THE HONG KONG POLYTECHNIC UNIVERSITY

Department of Civil and Structural Engineering

PRODUCTION OF HYDROXYALKANOATES FOR PHARMACEUTICAL AND MEDICAL APPLICATIONS

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A thesis submitted in partial fulfilment of the requirements for the degree of

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CERTIFICATION OF ORIGINALITY

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ABSTRACT

The replacement of traditional plastic by biodegradable plastic, polyhydroxyalkanoates (PHAs) is proposed to enhance the valuable plastic applications and to minimize its side-effects. However, the production cost of PHAs is too high at present, thus hampering the development of its manufacturing processes and widespread applications. The main aim of this research project is to increase the efficiency of PHAs production. Measures to reduce the cost of PHAs production were proposed. The potential application of PHAs in tissue engineering and drug delivery system was also assessed.

Two recombinant plasmids pBE2C1 and pBE2C1AB were constructed and then these two plasmids were transformed into *Bacillus subtilis* DB104 to generate *Bacillus subtilis*/pBE2C1 and *Bacillus subtilis*/pBE2C1AB, respectively. The two recombinants strains were subjected to fermentation and showed PHAs accumulation, which was the first report of production of mcl-PHAs in *Bacillus subtilis*. The result of Gas Chromatograpy (GC) analysis suggested that the product produced by *Bacillus subtilis*/ pBE2C1 was identified to be a hydroxydecanoate-co-hydroxydodecanoate (HD-co-HDD) polymer while the

product produced by *Bacillus subtilis*/pBE2C1AB was identified to be a hydroxybutyrate-co-hydroxydecanoate-co-hydroxydodecanoate (HB-HD-HDD) polymer.

The polyhydroxyalkanoate synthase gene pha, from *Bacillus thuringiensis* HD-81, was cloned using Polymerase Chain Reaction (PCR) cloning strategy based on the pha loci property. The complete open reading frames (ORFs) were identified from the PCR products. Using the sequence information, the complete PHAs synthase gene was PCR cloned directly from the genomic deoxyribonucleic acid (DNA) and expressed in *Escherichia coli* confirmed by Fourier Transform Infrared (FTIR) and GC.

It was also discovered that the recombinant *E. coli* yielded higher PHAs content (13.4 %) under the C: N ratios at 50:1. When the concentration of citric acid was 0.5 g/L, 16.72 % PHAs was obtained. With 0.5 g/L of tryptone, 11.03 % PHAs was recovered. A CDW of 8.62 g/L with 10.8 % PHAs was achieved when 2 g/L yeast extract was added. The PHAs content (14.91 %) was recorded when the recombinant *E. coli* was fed with sodium acetate.

Food wastes including tea waste, sugar cane waste, contaminated baked bread and contaminated candies were proposed to act as carbon source for recombinant

E. coli nutrient feeding to optimize the PHAs production. The contributions are not only to promote the use of biodegradable plastic but also effectively utilize of the wastes simultaneously. The achievement of this section of study was the optimization of PHAs production by using four proposed food wastes. The results showed that food wastes are the potential carbon source for PHAs production, and the pretreatment methods of waste materials could be further developed for PHAs production and commercial applications.

Protein drug delivery has been one of the very intensive studies of PHAs. In this study, PHAs produced by bacteria was proved that it can be used to produce nanoparticle and load with pyrene. It was found that PHAs nanoparticles had high loading capacity, high loading efficiency and low toxicity and they released in a stable manner which had very high potential for protein drug delivery.

Valvular heart disease is a significant cause of morbidity and mortality world-wide. Classical replacement surgery involves the implantation of mechanical valves or biological valves. Tissue engineering of heart valves has represented as a new experimental concept to improve current modes of therapy in valvular heart surgery. Preliminary investigation of the PHAs heart valves was also done in this study; it showed that PHAs could be an alternative material of heart valve for tissue engineering.

LIST OF PUBLICATIONS

Related Journal Papers:

Wang, **Y.J.**, Ruan, L.F., Chua, H. and Yu, H.F. (2006) Cloning and Expression the PHA Synthase Gene PhaCI into Bacilus. *World Journal of Microbiology and Biotechnology*. **22** (6): 559-563.

Wang, **Y.J.**, Ruan, L.F., Lo, W.H., Chua, H. and Yu, H.F. (2006) Construction of Recombinant *Bacillus subtilis* for Production of Polyhydroxyalkanoates. *Applied Biochemistry and Biotechnolog*. **132** (1-3): 1015-1022.

Wang, **Y.J.**, Hua, F.L., Tsang, Y.F., Chan, S.Y., Sin, S.N., Chua, H. and Yu, H.F. (2007) Synthesis of PHAs from Waster under Various C: N Ratios. *Bioresource Technology*. **98**: 1690-1693.

Hu, W.F., **Wang**, **Y.J.,** Hua, F.L., Chua, H., Sin, S.N. and Yu, H.F. (2006) Synthesis of Poly-hydroxyalkanoates from Activated Sludge under Various Oxidation-Reduction Potentials. *Annals of Microbiology*. **56**(3): 257-260.

Ruan, L.F., **Wang, Y.J.**, Yan, Q. and Yu, H.F. (2006) Microcalorimetric Studies on the Polyhydroxyalkanoates (PHAs) Production of Recombinant *Escherichia coli*. *Applied Biochemistry and Microbiology*. **42**(3): 270-273.

Ruan, L., **Wang, Y.J.**, Lam, W. and Yu, H.F. (2007) Microcalorimetric Research on Recombinant *Escherichia Coli* with High Production of Polyhydroxyalkanoates(PHAs). *Journal of Thermal Analysis and Calorimetry*. **89**(3): 953–956.

Feng, M., **Wang, Y. J.**, Peng, H. (2007) Transfect ion Efficiency of PEI2PLGA Cationic Nanoparticles Mediated Gene Transfer to Cervical Cancer. *Chinese Pharmaceutical Journal*. **42**(8): 585-589.

Yin, S., Cai, S., Zhang, X., **Wang, Y. J.**, Yu, H., Pan, S. (2006) Construction of tissue engineering heart valves. *Chinese Journal of Experimental Surgery*. **23**(8): 975-977.

Yin, S., Cai, S., **Wang, Y. J.**, Pan, S., Zhang, X., Yu, H. (2006) Promoting adhesion between tissue engineering heart valve and seed cells. *Chinese Journal of Experimental Surgery*. **23**(7): 847-848.

Cai, M.M., Chua, H., Sin, N., **Wang, Y. J.**, He, D. (2007) Proce ss regulation for monomer compo sition of polyhydroxyalkanoate s produced by activated sludge. *Journal of Chemical Industry and Engineering (China)*. **58**(10): 2427-2431.

Related Conference Papers:

Wang Y.J., Lam, W., Chua, H., Lo, W.H. and Yu, H.F. (2005) Integrated Process for Microbial Formation of Poly (β-hydroxyalkanoates) from Activated Sludges with Enhancement of Wastewater Treatment. *8th International Conference of the Aquatic Ecosystem Health and Management Society, Restoration and Remediation of Aquatic Ecosystems:Tools, Techniques and Mechanism*. Nanjing-Hangzhou, CHINA.

Wang, Y.J., Hua, F.L., Tsang, Y.F., Chan, S.Y., Chua, H. and Yu, H.F.(2006) Bio-plastics PHA Production by *B. subtilis*. *The International Association of Science and Technology for Development*. Spain.

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ABBREVIATIONS

^{13}C NMR	13 Carbon Nuclear Magnetic Resonance
Acetoacetyl-CoA	Acetoacetyl-coenzyme A
Acetyl-CoA	Acetyl-coenzyme A
B. cereus	*Bacillus cereus*
BOD	Biological Oxygen Demand
CDW	Cell Dry Weight
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
DSC	Differential Scanning Coulometry
E. coli	*Escherichia coli*
FTIR	Fourier Transform Infrared
GC	Gas Chromatograpy
GPC	Gel Permeation Chromatography
HB	3-hydroxybutyrate
HV	3-bydorxyvalerate or β- hydroxyvalerate
MCL-PHAs	Medium Chain Length Polyhydroxyalkanoates
NADH	Nicotinamide Adenine Dinucleotide

P(3HB-co-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PHAs	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate *or* Polyhydroxybutyric acid
PHB-co-PHV	Polyhydroxybutyrate-co-polyhydroxyvalerate
PHV	Polyhydroxyvalerate
SCL-PHAs	Short Chain Length Polyhydroxyalkanoates
TCA	Tricarboxylic Acid
TKN	Total Kjeldahl Nitrogen
TOC	Total Organic Carbon
HO	Hydroxyoctanoate
HD	Hydroxydecanoate
HDD	hydroxydodecanoate
DLC	Drug loading Capacity
DLE	Drug loading Efficiency

CHAPTER 1

INTRODUCTION

1.1 Plastics

Plastics are synthetic or semisynthetic polymers. They are mainly composed of carbon, hydrogen and some with other elements such as chlorine, oxygen and nitrogen. Small repeating units are bonded together and form a very long chainlike molecule. The repeating units are called monomers. Carbon makes up the backbone of the molecule while hydrogen and other atoms are bonded along it. Plastics are made by condensation or addition of monomers. The first man-made plastic was invented by Alexander Parkes in London in 1862. The first plastic he demonstrated was an organic material derived from cellulose.

Every polymer has very distinct characteristics, but most polymers have the general superior attributes. Firstly, polymers can be very resistant to chemicals. Secondly, they can be both thermal and electrical insulators. Thirdly, they are very light in weight with varying degrees of strength. Finally, the can be processed in various ways to produce thin fibers or very intricate parts. Elastomers and some plastics are very stretch and flexible. Polymers are materials with a seemingly limitless range of characteristics and color. They have many inherent properties that can be further enhanced by a wide range of additives to broaden their uses and applications.

Plastics today play an important part in cutting-edge technologies such as the space program, bullet-proof vests and prosthetic limbs, as well as in everyday products such as beverage containers, medical devices and automobiles.

1.2 Problems Caused by Plastics

Despite the advantages of plastics, there are also quite a number of problems caused by them. One of the most serious problems is environmental pollution. Since plastics degrade slowly, it causes deposal problems. A long period of time is required for plastics to degrade. As a result, burying plastics makes landfills take a long time to be recovered. Incineration of plastics also releases toxic gas such as hydrogen chloride gas and carbon monoxide gas, which causes air pollution. Beside pollutions, the raw material of plastics, petroleum, is non-renewable and limited. It is estimated that petroleum would be used up in a hundred years. Authorities are trying different methods to solve these problems, one of which is to recycle plastics, however, it is not under great success due to the difficulties in collecting plastics wastes and the high cost that make it unpopular in the market.

When such plastic materials are dumped into landfills, they can become “mummified” and persist for decades. Also, the supply of land is not inexhaustible and little land is available for new landfills in populated areas.

1.3 Environment-Friendly Plastics

Environment friendly plastics are biodegradable plastics, which from renewable sources may solve the problems cause by conventional plastics. Biodegradable plastics are plastics that can be degraded by microorganisms. Therefore, they cause less damage to the environment. They are usually produced from biological system such as plant and bacteria, or chemically synthesized by biological starting materials such as starch and cellulose. These are renewable sources which can be supplied in unlimited amount. A number of biodegradable plastics have been developed; examples are Polyhydroxyalkanoates (PHAs) from bacteria.

Polyhydroxyalkanoates (PHAs) are a family of optically active biopolymers that are fully biodegradable. PHAs are naturally produced in numerous genera of bacteria as intracellular carbon and energy storage compounds in the cytoplasm of the cell under restricted condition, and have been amplified through bacterial fermentation.

PHAs are biodegradable, biocompatible and thermoplastic polyesters produced by various microorganisms. In microorganisms, PHB serves as an intracellular energy and carbon storage product in much the same way as glycogen in mammalian tissues. The most extensively studied PHAs are the PHB and can be produced in high yield by fermentation of a variety of bacterial strains.

Its copolymers with varying ratios of hydroxyvalerate (HV) are the most widely used members. The copolymers of hydroxybutyrate (HB) with hydroxyvaleric (HV) acid are less crystalline, more flexible and more readily processable than PHB itself. Their various properties such as natural origin, biodegradability, biocompatibility, piezoelectricity, optical activity, and thermoplasticity make them suitable for a variety of applications in health industry.

The biocompatibility of PHB and PHBV has been studied by a number of different research groups. PHB has been found to have low toxicity, in part due to the fact that it degrades in vivo to hydroxybutyrate, a normal constituent of human blood.

PHBV emerge as a new generation of PHB-based materials with properties adjustable via changes in copolymer composition. Degradation rate of PHBV can be readily adjusted by changing the copolymer composition. The less crystalline

PHBV have a higher degradation rate in aqueous media. Biomaterials with optimum properties could be obtained by blending with suitable compounds or by selecting the appropriate PHAs. Thus, a wealth of options is available for cell carriers for specific engineering applications.

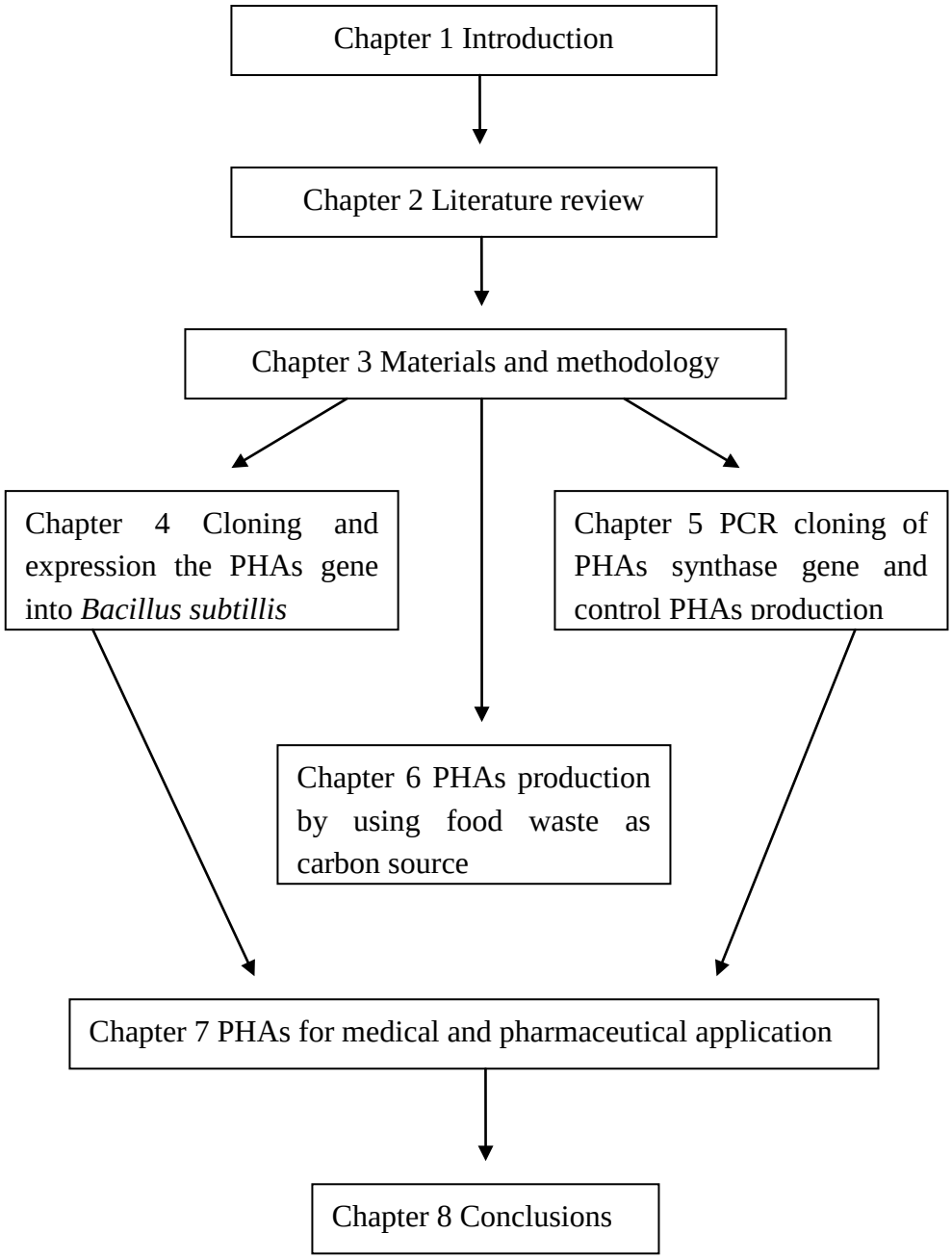
1.4 Objectives

The overall research aims to accumulate PHAs using the recombinant strain. The specific objectives of this study are:

- To evaluate factors affecting the productivity of PHAs.
- To reduce the cost of PHAs fermentation;
- To study the potential use of PHAs in tissue engineering and drug delivery system.

1.5 Organization of the Thesis

This thesis is divided into eight chapters. The present chapter covers the background, objectives, and scope of this research project. A detailed literature review is given in Chapter Two. Chapter Three focuses on the materials and methodology. Chapter Four concentrates on the results of cloning and expression the pha gene into *Bacillus subtilis*. Chapter Five presents the findings of the PCR cloning of PHAs synthase gene and control PHAs production. The results of PHAs production by using food waste as carbon source is evaluated in Chapter Six. Chapter Seven explores the applications of PHAs for medical and pharmaceutical uses. Finally, Chapter Eight gives a general summary of the major findings and overall conclusion in this study. The following flow chart briefly describes the overall organization of the thesis.


Chapter 1 Introduction
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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Polyhydroxyalkanoates (PHAs)

2.1.1 Storage materials

Polyhydroxyalkanoates (PHAs) is a class of polyesters which are synthesized by bacteria as an intracellular carbon and energy storage compound, especially under un-favourable conditions. So, PHAs can be fermented under carbon and nitrogen source limitation. There are approximately over 100 PHAs monomers, which include straight, branched, saturated, unsaturated and aromatic monomers,

with wide range of properties among the PHAs family (Zinn, 2001). It depends on the type of bacteria and growth conditions. Molecular weight of PHAs can be ranges from 2 x 10^5 to 3 x 10^6 Da (Lee, 1996). PHAs can be grown in both aerobic and anaerobic medium. PHAs are classified into short chain length PHAs (scl-PHAs, 3 carbons to 5 carbons), medium chain length PHAs (mcl-PHAs, 6 carbons to 14 carbons) and long chain length PHAs (lcl-PHAs, more than 14 carbons). Researches showed that the monomeric composition is usually dependent on the supply of particular fatty acid (Zinn, 2001). Researches even showed that low molecular weight PHB is present in eukaryotic such as yeast, peanut and sheep, in association with calcium ions (Reusch, 1995).

PHAs are thermal plastics which consist of important properties including biodegradability and biocompatibility. As a result, it is widely studied by researchers for its potential use in tissue engineering and medical uses.

The first discovered PHAs in the family was Polyhydroxybutyrate (PHB). It was found in *Bacillus megaterium* by a French scientist Lemoigne in 1926(Zinn, 2001). He found that the production of PHAs was often occurred during deficiency of nitrogen nutrients in the growing medium.

Table 2. 1 A selection of monomers found in poly (hydroxyalkanoic acids) (Zinn, 2001)

3-Hydroxy acids	3-Hydroxy acids (unsaturated)	3-Hydroxy acids (branched)	3-Hydroxy acids (substituted side-chain)	Other than 3-hydroxy acids
Propionic	2-Butenoic	2-Methylbutyric	Cyclohexylbutyric	4-Hydroxybutyric
Butyric	4-Pentenoic	2-Methylvaleric	5-Phenylvaleric	4-Hydroxyvaleric
Valeric	4-Hexenoic	2,6-Dimethyl-5-heptenoic	7-Fluoroheptanoic	4-Hydroxyhexanoic
Hexanoic	5-Hexenoic	4-Methylhexanoic	9-Fluorononanoic	4-Hydroxyheptanoic
Heptanoic	6-Heptenoic	5-Methylhexanoic	6-Chlorohexanoic	4-Hydroxyoctanoic
Octanoic	6-Octenoic	6-Methyloctanoic	8-Chlorooctanoic	5-Hydroxyvaleric
Nonanoic	7-Octenoic	7-Methyloctanoic	3-Bromohexanoic	5-Hydroxyhexanoic
Decanoic	8-Nonenoic	6-Methylnonanoic	8-Bromooctanoic	Malic
Undecanoic	9-Decanoic	7-Methylnonanoic	11-Bromoundecanoic	
Dodecanoic	10-Undecenoic	8-Methylnonanoic	7-Cyanoheptanoic	
Tetradecanoic	6-Dodecenoic	7-Mehtyldecanoic	9-Cyanononaic	

Polyhydroxyalkanoates (PHAs) are synthesized by numerous gram-positive and gram-negative bacteria. PHAs are accumulated as discrete granules to levels as high as 80 % of the cell dry weight and are generally believed to play an important role as sink for carbon. When nutrient supplies are imbalanced, it is advantageous for bacteria to store excess nutrients intracellular. By polymerizing soluble intermediates into insoluble molecules, the cell does not undergo alterations of its osmotic state and leakage of these valuable compounds out of the cell is prevented. Consequently, the nutrient stores will remain available at a relatively low maintenance cost and with a secured return on investment (Wang, 1998).

Once PHAs are extracted from the bacterial cell, however, these molecules show material properties that are similar to some common plastics such as polypropylene (Byrom, 1987). The bacterial origins of the PHAs make these polyesters a natural material and many microorganisms have evolved the ability to degrade these macromolecules. Besides being biodegradable, PHAs are recyclable like the petrochemical thermoplasts.

PHAs are accumulated and synthesized intracellularly in granular inclusion bodies as carbon and energy storage material by numerous bacteria (Table 2.2)

under conditions of metabolic stress, where nitrogen, phosphate, oxygen, or other nutrient is limiting in the presence of excess carbon (Doi, 1992; Poirier, 1995; Steinbuchel, 1996). Under these conditions, PHAs are accumulated as storage materials providing a reserve of carbon and energy. When the supply of the limiting nutrient is restored, the PHAs can be degraded by intracellular depolymerases and subsequently metabolized as carbon and energy source (Byrom, 1994; Doi, 1992).

Table 2. 2 Limiting compounds leading to PHAs formation (Poirier, 1995)

Compound	Key Organisms
Ammonium	*Alcaligenes eutrohus* *Alcaligenes latus* *Pseudomonas olevorans* *Rhodospirillum rubrum* *Rhodobacter sphaeroides* *Pseudomonas sp K* *Methylocystis parvus* *Rhizobium ORS571*
Iron	*Pseudomonas sp K*
Magnesium	*Pseudomonas sp K* *Pseudomonas olevorans* *Rhizobium ORS571*
Manganese	*Pseudomonas sp K*
Oxygen	*Azotobacter vinelandii* *Azotobacter beijerinckii* *Rhizobium ORS571*
Phosphate	*Rhodospirillum rubrum* *Rhodobacter sphaeroides* *Caulobacter crescentus* *Pseudomonas olevorans*
Potassium	*Bacillus thuringiensis*
Sulfate	*Pseudomonas sp K* *Pseudomonas olevorans* *Rhodospirillum rubrum* *Rhodobacter sphaeroides*

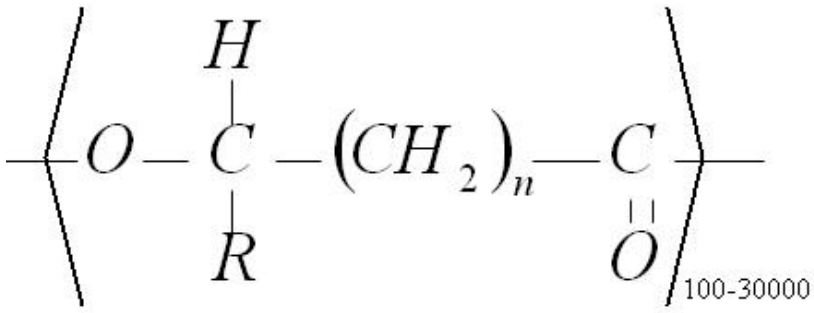
2.1.2 Chemical structure

A number of different PHAs that have been identified are primarily linear; head-tail polyesters composed of hydroxy fatty acid monomers. In the polymers, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer (Fig. 2.1).

In all PHAs that have been characterized so far, the hydroxyl-substituted carbon atom is of the *R* configuration, except in some special cases where there is no chirality. However, this alkyl side chain is not necessarily saturated: aromatic, unsaturated, halogenated, epoxidized, and branched monomers have been reported as well (Curley, 1996). Specialized, unnatural monomers such as 4-cyanophenylvalerate have been incorporated to obtain new polymers with special properties (Kim, 1995). As well as the variation in the alkyl substituent, the position of the hydroxyl group is somewhat variable and 4-, 5- and 6-hydroxy acids have been incorporated (Valentin, 1996). Substituents in the side chains of PHAs can be modified chemically, for instance by cross-linking of unsaturated bonds (Gagnon, 1994). This variation in the length and composition of the side chains and the ability to modify their reactive substituents is the basis for the

diversity of the PHAs polymer family and their vast array of potential application.

Polyhydroxybutyrate (PHB) has been studied most extensively and has triggered the commercial interest in these classes of polymers. PHB is the most common type of PHAs and the ability of bacteria to accumulate PHB is often used as a taxonomic characteristic.



R = n-alkyl pendant group of variable chain length

3HB, 3-hydroxybutrate where R = methyl

3HV, 3-hydroxyvalerate where R = ethyl

3HC, 3- hydroxycaproate where R = n-propyl

3HH, 3-hydroxyheptanoate where R = n-butyl

3HO, 3-hydroxyoctanoate where R = n-pentyl

3HN, 3-hydroxynonanoate where R = n-hexyl

3HD, 3-hydroxydecanoate where R = n-heptyl

3HUD, 3-hydroxyundecanoate where R = n-octyl

3HDD, 3-hydroxydodecanoate where R = n-nonyl

Fig. 2. 1 Chemical structure of PHAs (Kim, 1992)

2.1.3 Physical characteristics

The molecular mass of PHAs varies per PHAs producer but is generally on the order of 50,000 to 1,000,000 Da. This was primarily due to the use of relatively impure substrates at the time, which limited the molecular masses of these polymers to 20,000 to 30,000 Da (Marchessault, 1996). Bacterially produced PHB and other PHAs have a sufficiently high molecular mass to have polymer characteristics that are similar to conventional plastics such as polypropylene (Table 2.3).

Within the cell, PHB exists in a fluid, amorphous state. However, after extraction from the cell with organic solvents, PHB becomes highly crystalline and in this state is a stiff but brittle material (Dionisi, 2004). Because of its brittleness, PHB is not very stress resistant. Also, the relatively high melting temperature of PHB (around 170 °C) is close to the temperature where this polymer decomposes thermally and thus limits the ability to process the homopolymer. Initial biotechnological development was therefore aimed at making PHAs that were easier to process. The incorporation of HV into the PHB resulted in a poly (hydroxybutyrate-co-hydroxyvalerate) P (HB-co-HV) that is less stiff and brittle

than PHB, that can be used to prepare films with excellent water and gas barrier properties reminiscent of polypropylene, and that can be processed at a lower temperature while retaining most of the other excellent mechanical properties of PHB (Marchessault, 1996). In contrast to PHB and P (HB-co-HV), mcl-PHAs have a much lower level of crystallinity and are more elastic (Preusting, 1990). These mcl-PHAs have a potential different range of applications from the scl-PHAs.

Table 2. 3 Properties of PHAs and polypropylenea (Preusting, 1990)

Parameter	Value for[a]:			
	PHB	PHB-co-3HV	PHO-co-HH	PP
Tm (°C)[b]	177	145	61	176
Tg (°C)[c]	2	-1	-7	-10
Crystallinity (%)	70	56	30	60
Extension to break (%)	5	50	300	400

[a] PHB is polyhydroxybutyrate, PHB-co-HV is polyhydroxybutyrate-co-hydroxyvalerate containing 20 % HV, PHO-3HH is polyhydroxyoctanoate-co-hydroxyhexanoate containing 11 % HH, and PP is polypropylene.

[b] *Tm* is melting temperature.

[c] *Tg* is glass transition temperature.

PHAs mainly consist of 3-hydroxyalkanoic acid monomers, with saturated or unsaturated, straight or branched chains connecting aliphatic or aromatic side groups (De, 1983; Doi, 1992). PHAs are generally divided into two groups according to the number of carbon atoms in the monomer units. The short-chain-length PHAs consist of 3-5 carbon atoms, and the medium-chain-length PHAs consist of 6 or more carbon atoms. scl-PHAs are partially crystalline thermoplastic materials, while mcl-PHAs are sticky, elastic and amorphous materials (Park, 2001). Currently, about 150 hydroxyalkanoic acids other than HB have been identified as constituents of microbial polyesters (Jendrossek, 2002). Among different forms of PHAs, polyhydroxybutyrate (PHB) has the lowest molecular weight and is the most common type. Its molecular weight can be up to 2 million, or 20000 monomers per polymer molecule.

According to Huijberts (1992), PHB has some similar physical characteristics to polypropylene with three unique features: thermoplastic processibility, complete resistance to water and complete biodegradability. The following are some of the properties of PHB (Jendrossek, 1996):

(a) PHB is insoluble in water and relatively resistant to hydrolytic degradation but soluble in organic solvents such as chloroform and other chlorinated hydrocarbons.

(b) PHB shows good oxygen permeability.

(c) PHB has good ultraviolet resistance but has poor resistance to acids and bases.

(d) PHB is biocompatible and hence is suitable for medical applications.

(e) PHB has a melting point around 175 °C and a glass transition temperature around 15 °C.

(f) PHB has a tensile strength of 40 MPa, which is close to that of polypropylene.

(g) PHB sinks in water which facilitates its anaerobic biodegradation in sediments.

(h) PHB is non-toxic.

Table 2. 4 A comparison of physical properties of polypropylene and PHB (Park, 2001)

Physical Property	Polypropylene	PHB
Melting point (°C)	171-186	171-182
Glass transition point (°C)	-15	5-10
Crystallinity (%)	65-70	65-80
Density (g/cm^3)	0.905-0.94	1.23-1.25
Molecular weight (10^5)	2.2-7	1-8
Flexural modulus (GPa)	1.7	3.5-4
Tensile strength (MPa)	39	40
Extension to break (%)	400	6-8
Ultraviolet resistance	Poor	Good
Solvent resistance	Good	Poor
Oxygen permeability ($cm^3/m^2 \cdot atm \cdot d$)	1700	45
Biodegradability	None	Good

The high melting temperature of PHB around 170°C makes this material difficult to process. As a result, the poly (hydroxybutyrate-co-hydroxyvalerate) P (HB-co-HV) copolymer has been developed. The copolymer has much improved mechanical properties, such as greater flexibility and toughness than the HB homopolymer (Lee, 1991). Ojumu (2004) stated that the material properties of PHAs are controlled by adjusting the hydroxyvalerate (HV) fraction during fermentation. Depending on HV composition (0-25 mol %), P (HB-co-HV) copolymer showed higher elongation to break and reduction in melting points ranging from 100 °C to 160 °C. The brittleness of PHB decreases but strength increases by blending PHB with other polymers, such as polyhydroxyvalerate (PHV). The properties of some polymers are given in Table 2.5.

Ward (2005) discussed the significance of the molecular weight of PHAs. PHAs of high molecular weight and low polydispersity were desirable for making thermoplastics. A good illustrative example of this kind of plastic is a scl-PHAs copolymer P (HB-co-HV), with a molecular weight of 600,000 or above. Mcl-PHAs polymers have the potential to be specialty polymers. They can be applied as coatings, pressure-sensitive adhesives, polymer binding agents in organic-solvent-free paints and a range of medical applications.

Table 2. 5 Material properties of some PHAs (Ojumu, 2004)

	Glass transition point (°C)	Melting point (°C)	Tensile strength (MPa)	Crystallinity (%)	Elongation to break (%)
P(HB)	4	177	40	60	5
P(HB-co-10% HV)	6	162	36	69	10
P(HB-co-20% HV)	-1	145	32	53	-
P(HB-co-10l% HHX)	-1	127	21	34	400
P(HB-co-15% HHX)	0	115	23	26	760
P(HB-co-17% HHX)	-2	120	20	22	850
P(HB-co-6% HA)	-8	133, 146	17	-	680
PP	-30	130-161	29.3	40	400
LDPE	-30	120	15.2	-	620

PP: polypropylene LDPE: low density polyethylene - Data not available

2.1.4 Classification

The PHAs producing bacteria can be divided into two groups according to the number of carbon atoms in the monomer units of PHAs (Steinbuchel, 1991). It can be classified as short-chain-length PHAs and medium-chain-length PHAs. Scl-PHAs consist of 3-5 carbon atoms and mcl-PHAs consist of 6-14 carbon atoms (Lee, 1999).

One group of bacteria, including *Ralstonia eutrophus* produces a short chain length PHAs with 3 C to 5 C monomer units. While other group (*Pseudomonas oleovorans*) produces medium chain length PHAs with 6 C to 14 C monomer units (Steinbuchel, 2001). Although several bacteria can synthesize polyesters containing both short and medium chain length PHAs (Hiraishi, 2003).

Co-polymers of PHB can be formed by co-feeding of substrates and may result in the formation of polymers containing hydroxyvalerate (HV) or hydroxybutyrate (HB) monomers. Together, polymers containing such monomer form a class of PHAs typically referred to as short-chain-length PHAs (scl-PHAs).

In contrast, medium-chain-length PHAs (mcl-PHAs) are composed of 6 C to 14 C hydroxy fatty acids. These PHAs are synthesized from fatty acids or other aliphatic carbon sources, and, typically, the composition of the resulting PHAs depends on the growth substrate used (Huisman, 1989). Mcl-PHAs are also synthesized from carbohydrates, but the composition of these PHAs is not related to the carbon source (Huijberts, 1992). The vast majority of microbes synthesize either scl-PHAs containing primarily HB units or mcl-PHAs containing hydroxyoctanoate (HO) and hydroxydecanoate (HD) as the major monomers (Lee, 1994).

2.1.4.1 Short chain PHAs

Polyhydroxybutyrate (PHB) is one of the earliest discovered and widely studied one among the family due to its common occurrence. PHB is a relatively rigid and stiff material with tensile strength similar to polypropylene. The brittleness and stiffness limit the commercial use and application of PHB. To increase its flexibility, heteropolymers are introduced into PHB. Poly (hydroxybutyrate-co-hydroxyvalerate) (PHBV) contains both hydroxybutyrate (HB) and hydroxyvalerate (HV). By varying the ratio between HB and HV, the

mechanical properties can be designed. Beside increase in flexibility, it is also water resistant and impermeable to oxygen, which is advantage over pure PHB.

2.1.4.2 Medium chain PHAs

Medium chain length PHAs (mcl-PHAs) are PHAs with 6 carbons to 14 carbons. They are typically produced by fluorescent pseudomonads. PHAs properties depend on the bacterial strains and its environment. Mcl-PHAs is under great interest recently due to its rubbery and flexibility with low crystallinity. Since scl-PHAs have high crystallinity and thus rigid in nature, mcl-PHAs have potential in a wide range of industrial and medical uses that scl-PHAs be fulfilled (chan, 2006). One of the limitations of mcl-PHAs in wide commercial use is due to its high production cost. Finding alternate cheaper carbon source and increase efficiency by recombinant technologies are possible solutions.

2.2 PHAs Biosynthesis

The production of different types of PHAs significantly depends on the chosen microorganisms, fermentation conditions and different carbon sources. Some examples are shown in Table 2.6 (Steinbuchel, 1991). PHB can be produced when glucose as a carbon sources for bacteria, but growing the bacteria on valeric acid leads to production of PHV.

Zeneca was first to produce P (HB-co-HV) copolymer by manipulating the composition of media for bacteria. The copolymers are especially important because it is stronger and more flexible than regular PHB and making them more useful for applications (Pouton, 1996).

This is much interest in copolymers, in particular in copolymers of hydroxybutyric acid (HB) and hydroxyvaleric acid (HV) because they have melting points much lower, and are less crystalline, more ductile, easier to mould and tougher, than pure PHB (Loosdrecht, 1997). Variation in their HV content leads to a range of properties spanning a wide variety of thermo-mechanical

properties. The copolymer P (HB-co-HV) produced from *Alcaligenes eutrophus* and marketed as "*Biopol*" by Monsanto Company.

Table 2. 6 Synthesis of and accumulation of PHAs from unrelated substrates (Steinbuchel, 1991)

Microorganism	Carbon Source	PHA detected
Corynebacterium hydrocarboxydrans	fructose, glucose, succinate, acetate, lactate	Poly(HB-co-HV)
Haloferax medierranel	starch	Poly(HB-co-HV)
Rhodococcus Sp. ATCC 10970	fructose, glucose, sucrose, acetate, lactate	Poly(HB-co-HV)
Rhodococcus Sp. NCIMB 40126	fructose, glucose, molasses, succinate, acetate, lactate	Poly(HB-co-HV)
Nocardia strain 107-322	butane	Poly(HB-co-HBen)
Nocardia Incida	fructose, glucose, succinate, acetate	Poly(HB-co-HV)
Pseudomonas aeruginosa	gluconate, fructose, glucose, ethanol	Poly(HO-co-HD-HDD)
Rhodoboacter sphaeroides	acetate	Poly(HB-co-HV)

2.2.1 The three-step scl-PHAs biosynthetic pathway

2.2.1.1 β-Ketoacyl-CoA thiolase

β-Ketoacyl-CoA thiolase catalyzes the first step in PHB synthesis. The PHB biosynthetic thiolase is a member of a family of enzymes involved in the thiolytic cleavage of substrate into acyl-CoA plus acetyl-CoA. The β-ketoacyl-CoA thiolase is found throughout nature from higher eukaryotes to prokaryotes and is divided into two groups according to their substrate specificity. The first group consists of thiolase with a broad specificity for β-ketoacyl-CoAs ranging from 4 C to 16 C. This class of enzyme is involved in the degradation of fatty acids and is located in the mitochondria of mammalian, peroxisomes of plant cells and cytoplasm of prokaryotes. The second group of β-ketoacyl-CoA thiolase is considered biosynthetic and has a narrow range of chain length specificity, from C 3 to C 5. Throughout nature, these biosynthetic thiolase is specialized for a variety of roles such as ketone body formation, steroid and isoprenoid biosynthesis, and PHB synthesis. The thiolase involved in PHB formation is a biosynthetic thiolase with specificity primarily for acetoacetyl-CoA (Masamune, 1989).

Ralstonia eutropha contains two b-ketothiolases that can act in the biosynthetic pathway to PHB synthesis. The major difference between these two enzymes is their substrate specificity.

The enzyme A converts acetoacetyl-CoA and 3-ketopentanoyl-CoA. In contrast, the enzyme B has broader substrate specificity and cleaves acetoacetyl-CoA as well as 3-ketopentanoyl-CoA, 3-ketohexanoyl-CoA, 3-ketoheptanoyl-CoA, 3-ketooctanoyl-CoA, and 3-ketodecanoyl-CoA. Originally it was believed that the major role of the enzyme B is in fatty acid degradation while the primary role of the enzyme A is in the biosynthesis of PHB (Haywood, 1988). Recently, it has been shown that the enzyme B is the primary source of the 3HV monomer for PHB-co-HV formation (Slater, 1998).

The enzymatic mechanism of the enzyme A consists of two half-reactions that result in the condensation of two acetyl-CoA molecules into acetoacetyl-CoA. In the first half-reaction, an acetyl-*S*-CoA molecule is attacked to form an acetyl-*S*-enzyme intermediate by an active-site cysteine. In the second half-reaction, another acetyl-CoA is deprotonated by a second cysteine, resulting in an activated acetyl-CoA intermediate that can attack the acetyl-*S*-enzyme intermediate and form acetoacetyl-CoA (Masamune, 1989). The involvement of

a cysteine in the active site of the PHB thiolase was the first hypothesis because the thiolase was inhibited by sulphydryl-blocking agents (Lynen, 1953).

In the late 1980s, the roles of cysteine in the active site of the PHB thiolase were definitively determined, after the thiolase gene from *Zoogloea ramigera* had been cloned and the enzyme had been overproduced and purified. The cysteine involved in the acetyl-*S*-enzyme intermediate was identified as Cys89 by peptide sequencing of the radioactive peptide after tryptic digestion of radiolabeled enzyme with ^{14}C iodoacetamide or ^{14}C acetyl-CoA (Thompson, 1989). A C89S thiolase mutant was also constructed and determined to be severely affected in catalysis but not substrate affinity (Thompson, 1989).

The second cysteine in the active site of PHB thiolase was determined by using affinity labeled inactivators such as bromoacetyl-*S*-pantethene-11-pivalate. By using this inhibitor, Cys378 was identified as a potential residue for the second active-site cysteine that deprotonates the second acetyl-CoA molecule (Palmer, 1991) and the C378G mutant was virtually inactive (Palmer, 1991). So far, all PHB thiolase contain these two active-site cysteines, and it is believed that all the PHB thiolases use the same enzymatic mechanism to condense acetyl-CoA with either acetyl-CoA or acyl-CoA.

2.2.1.2 Acetoacetyl-CoA reductase

Acetoacetyl-CoA reductase is an (*R*)-3-hydroxyacyl-CoA dehydrogenase and catalyzes the second step in the PHB biosynthetic pathway by converting acetoacetyl-CoA into 3-hydroxybutyryl-CoA. The acetoacetyl-CoA reductase from *Z. ramigera* is a homotetramer and has been classified as an NADPH-dependent reductase (Fukui, 1987). Although both NADPH- and NADH-dependent acetoacetyl-CoA reductase activities have been observed in cell extracted of *R. eutropha*, only the former is involved in PHB synthesis (Haywood, 1989). The only known NADH-dependent acetoacetyl-CoA reductase involved in PHB formation was found in *Chromatium vinosum* (Liebergesell, 1992). Although the *phbB* gene product from *Paracoccus denitrificans* was initially ascribed to be NADH dependent (Yabutani, 1995), subsequent overexpression of this enzyme and characterization proved this reductase to be active only with NADPH (Cammas, 1999).

The enzymatic reaction involved in PHB synthesis has been analyzed by biochemical technique and provide clues about the regulation of this pathway. The preferred reaction of the thiolase is thiolytic cleavage, which occurs in the direction opposite to the PHB biosynthetic pathway. However, under the PHB accumulating condition, the enzyme acts against its thermodynamically favored

direction when the activities of acetoacetyl-CoA reductase and PHB polymerase pull the condensation reaction (Mergaert, 1993). The availability of reducing equivalents in the form of NADPH is considered to be the driving force for PHB synthesis.

In the PHB biosynthetic pathway, the monomer was provided for PHAs polymerization by the reaction catalyzed of thiolase and reductase. The kinetic characteristic and substrate specificity of these two enzymes are crucial in determining the range of product that can be expected to be synthesized in a thiolase, reductase, polymerase pathway, as showed in Fig. 2.2.

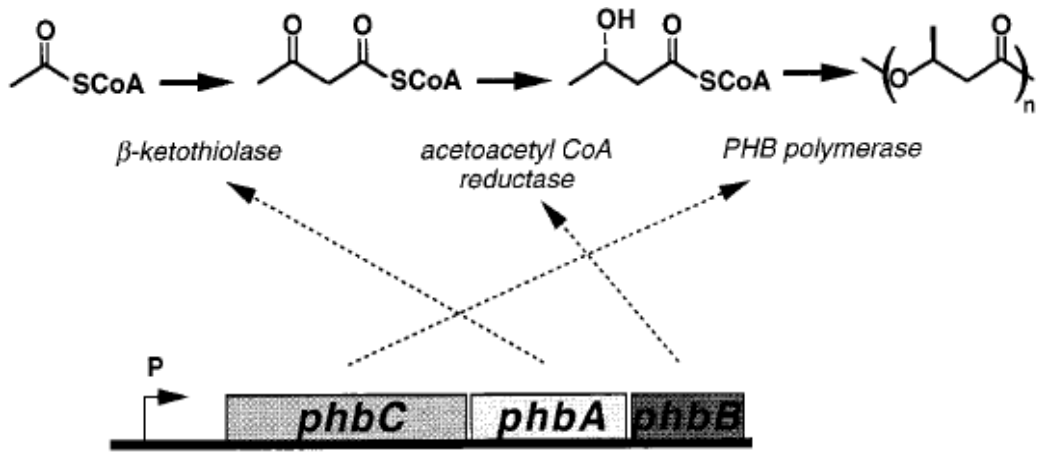


Fig. 2. 2 Biosynthetic pathway for PHB (Mergaert, 1993)

PHB is synthesized in a three-step pathway by the three enzymes, which are encoded by the genes of the *phbCAB* operon. A promoter upstream of *phbC* transcribes the complete operon.

2.2.1.3 PHB polymerase

PHB polymerase is the third enzyme in the biosynthetic pathway for PHB production. The first *phbC* nucleotide sequence to be reported was from *R. eutropha*. This gene was isolated by complementation of *R. eutropha* PHB negative mutants (Peoples, 1989a), and the promoter that drives the expression of *phbC* (Schubert, 1991) and the other genes in the *phb* operon (Peoples, 1989b) was mapped. Expression of these three genes in *Escherichia coli* resulted in the accumulation of PHB up to levels exceeding 50 % of the cell dry weight (Schubert, 1988).

PHB polymerase is one member of the PHAs polymerase family. All of the polymerases have molecular masses of around 63,000 Da, except for the polymerases from *C. vinosum* (Liebergesell, 1994), *Thiocystis violacea* (Liebergesell, 1993), and *Synechococcus* spp. (Kaneko, 1996), which are all

composed of two subunits with molecular masses of 40 and 45 kDa. PHAs polymerase is found in both soluble and granule-bound. A number of PHAs-producing microorganisms would represent a much spectrum of intracellular conditions to which these enzymes would have to be adapted.

2.2.2 Pathways for mcl-PHAs formation

2.2.2.1 mcl-PHAs from fatty acids

Mcl-PHAs were discovered in 1983, when Witholt and coworkers found that *P. oleovorans* grown on 50 % octane formed a material that was pliable under conditions where samples are prepared for freeze fracture electron microscopy. By using chemically synthesized standard, the inclusion formed from *n*-octane was determined to be made of copolyester consisting of 89 % hydroxyoctanoate and 11 % hydroxyhexanoate (Lageveen, 1988).

Subsequent studies showed that the composition of the PHAs formed by *pseudomonads* of the rRNA homology group I were directly related to the structure of the alkane, alkene, or fatty acid carbon source (Brandl, 1988). The monomer in the PHAs is the same length as the carbon source or has been

shortened by 2, 4, or 6 carbon atoms when the carbon source consists of 6 to 12 carbon atoms. When the carbon source is straight-chain 13 C to 18 C fatty acids, the composition of the polymer resembles that of the 11 C and 12 C grown bacteria (Huisman, 1991).

For instance, when *P. oleovorans* was supplied with mixtures of octane and 1-octene, the ratio of the monomer with an unsaturated bond ranged from 0 to 50 % depending on the fraction of 1-octene in the substrate (Lageveen, 1988). By analogy, substituted 3-hydroxyalkanoates were introduced to different levels by supplying 7-methyloctanoate, 8-bromooctanoate, phenylundecanoate, or cyanophenoxyhexanoate as the co-substrate (Fritzsche, 1990; Hazer, 1994). Incorporation of the last of these substrates results in PHAs with monomer constituents that are hyperpolarizable and may confer nonlinear optical properties to the polymer (Kim, 1995).

From Fig. 2.3, we knew that the mcl-PHAs biosynthetic pathway was a direct branch of the fatty acid oxidation pathway. The substrate specificity of the mcl-PHAs polymerase ranges from C 6 to C 14 hydroxy-alkanoyl-CoAs, with a preference for the C 8, C 9, and C 10 monomers (Huisman, 1992).

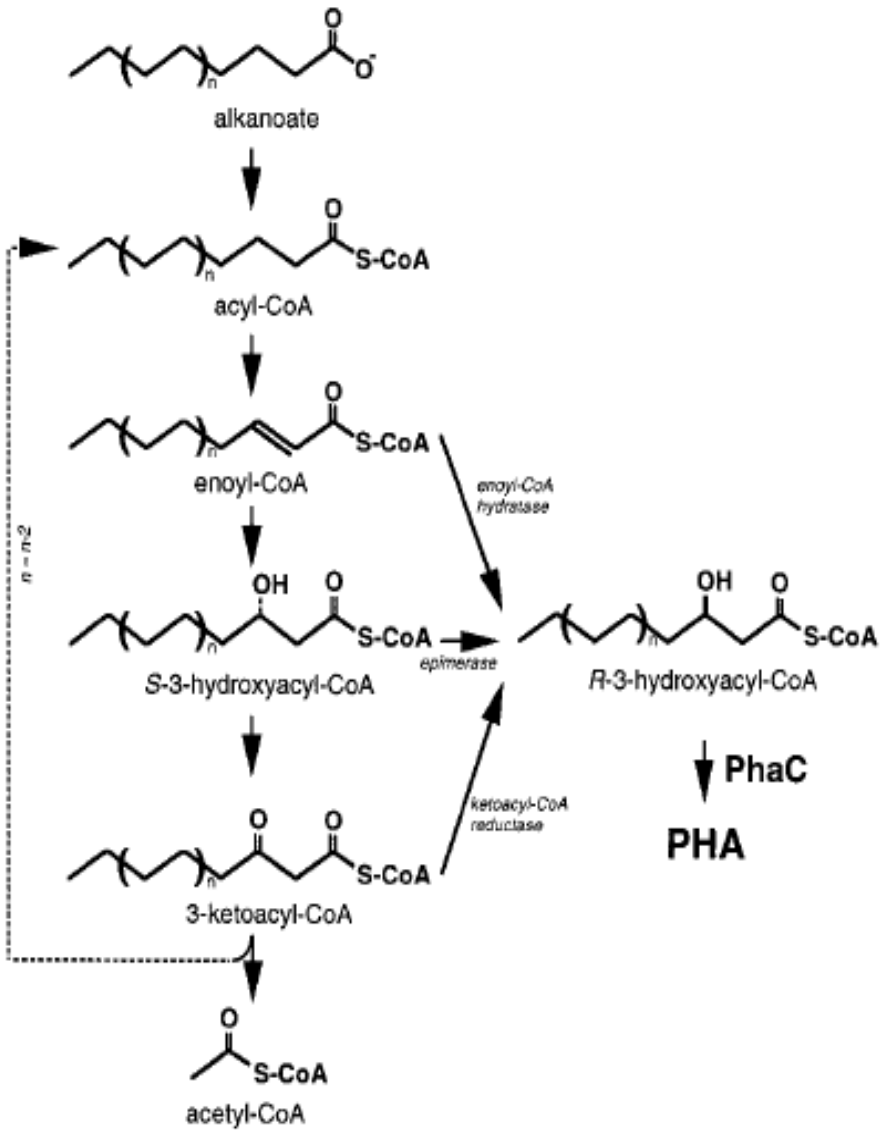


Fig. 2. 3 Biosynthetic pathway for mcl-PHAs from hydrocarbons (Brandl, 1988)

Fluorescent pseudomonads of rRNA homology group I can derive monomer for PHAs from fatty acid degradation. Intermediates from the β-oxidation cycle can be converted to hydroxyacyl-CoA by a hydratase, epimerase, or reductase activity. Any or all of these three enzymes and PHAs polymerase determine the limits to the substrate specificity, which is from C 6 to C 16 hydroxy fatty acids.

There are different biosynthetic pathways, so it is not surprising that the *pha* gene in the mcl-PHAs is very different from the *pha* gene in the scl-PHAs. Genes involved in mcl-PHAs have been characterized from *P. oleovorans* (Huisman, 1991) and *P. aeruginosa* (Timm, 1992). In the both species, two PHAs polymerases were identified, and PHAs polymerase genes are separated by one open reading frame. The two polymerases are approximately 50 % identical in their primary structure and appear equally active in PHAs synthesis from fatty acids (Huisman, 1991) or glucose (Huijberts, 1992). The open reading frame between *phaC1* and *phaC2* complements a mutation that prevents the utilization of accumulated PHAs.

Either of the two PHAs polymerase genes (*phaC1* or *phaC2*) was introduced to a multicopy plasmid, the molecular weight of the PHAs decreased. The reduction was not caused by an increase in PHAs depolymerase activity, since the

molecular weight of PHAs from a depolymerase mutant was not higher than that of PHAs from the wild type (Huisman, 1992). We suppose that the molecular weight of PHAs is determined by the activity of the PHAs polymerase. From the in vitro analysis of the PHAs polymerase, scientist suggested that the substrate is the limiting factor for PHAs production. These in vivo and in vitro experiments suggest that the substrate concentration and enzyme levels, determines the molecular weight of the resulting PHAs (Kraak, 1997).

2.2.2.2 mcl-PHAs from carbohydrates

When fluorescent pseudomonads of rRNA homology group I are grown on sugars, a PHAs that consists primarily of C 10 and C 8 monomers is obtained (Timm, 1990). Evidence suggests that the monomer is derived from intermediates of fatty acid biosynthesis and that the composition of the PHAs is maybe a reflection of the pool of fatty acid biosynthetic intermediates.

Temperature affects the fatty acid composition of bacterial membranes. Since this effect is due to enzyme activities in fatty acid biosynthesis, the PHAs composition was studied in relation to the growth temperature. Since the ratio of unsaturated to saturated monomers increases at lower temperature for both

membrane lipids and PHAs, a metabolic relationship between fatty acid biosynthesis and PHAs formation from glucose was suggested (Huijberts, 1992).

When carbon sources are in excess, *P. putida* are able to synthesize PHAs from glucose or fatty acids. But when a fatty acid synthesis inhibitor is added to the cell suspensions, no PHAs are formed from glucose whereas PHAs is still synthesized from fatty acids. Similarly, acrylic acid, a β-oxidation blocker, prevents the formation of PHAs from octanoate but not from glucose.

The experiment confirmed that PHAs accumulation from glucose is linked to fatty acid biosynthesis showed in Fig. 2.4. Recently, peoples determined that the gene product of *phaG* is responsible for this conversion (Rehm, 1998).

Some *Pseudomonas* spp. can incorporate both scl- and mcl-PHAs monomer into the same polymer chain. Typically, these PHAs are produced when these bacteria are grown on unrelated carbon source such as carbohydrates or 1, 3-butanediol (Abe, 1994). The PHAs polymerases synthesizing these scl- and mcl-PHAs must have a broad substrate range. This was concluded from experiment where a recombinant *P. putida* strain containing both the chromosomal *phaC* and *R. eutropha phbC* on one plasmid was shown to accumulate individual granules composed of either PHB or PHA (Preusting,

1993). The isolation of PHAs polymerase genes from Pseudomonas accumulates PHB and PHB-co-PHAs granules from glucose (Kassab, 1997), that should provide further insights into the simultaneous metabolism of the two types of PHAs.

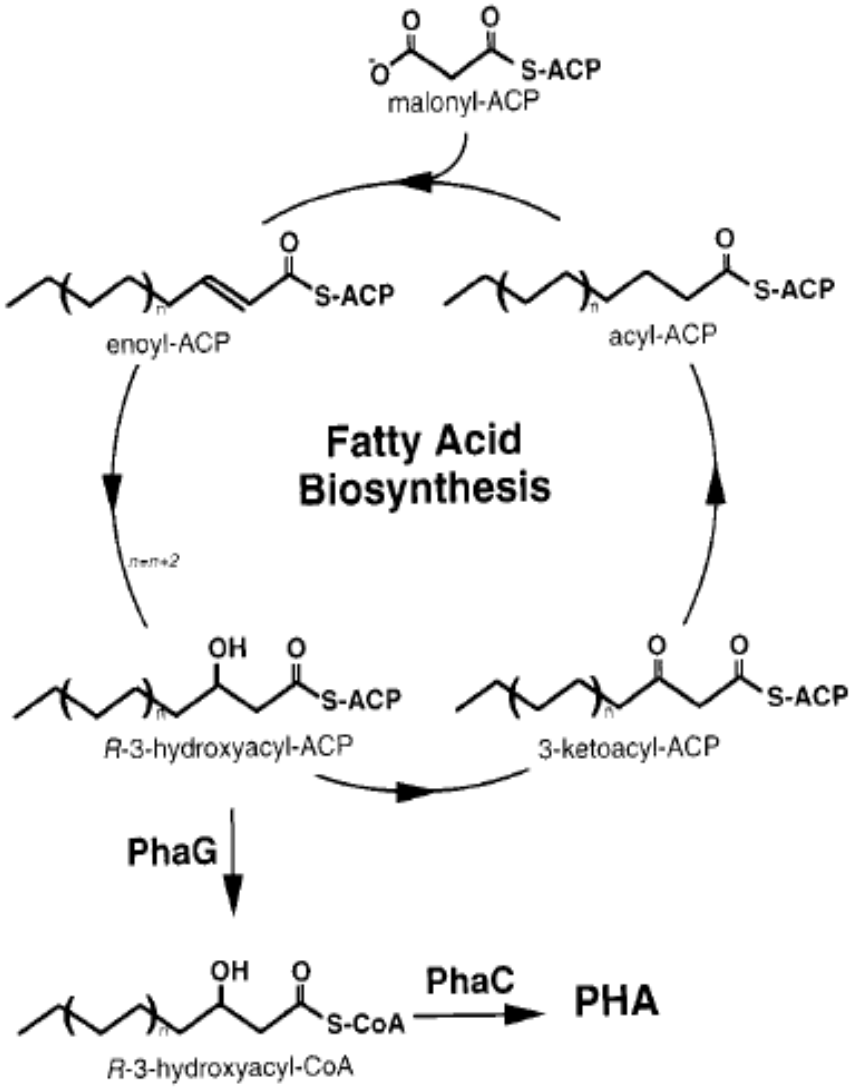


Fig. 2. 4 Biosynthetic pathway for mcl-PHAs from carbohydrates (Rehm, 1998)

2.2.3 Factors affecting synthesis and composition of PHAs

2.2.3.1 Substrate and growth conditions

Doi (1992) reported that the composition of PHAs is influenced by the substrate used. When an even chain length of alkanoic acid is employed as a substrate, a PHB homopolymer is produced by *Alcaligenes eutrophus*, while a P (HB-co-HV) copolymer is produced when an odd chain length of substrate is used. The amount of polymer formed is dependent on the concentration of the substrate supplied.

2.2.3.2 Bacterial strain

Though more than 300 microorganisms are known to synthesize PHAs, only several of them, such as *Alcaligenes eutrophus, Alcaligenes latus, Azotobacter vinelandii* and recombinant *Escherichia coli* are suitable for the production of highly concentrated PHAs and at a high productivity (Lee, 2001).

Jogdand listed some advantages of using recombinant *E. coli* for production of PHAs:

(a) Recombinant *E. coli* can use a wide range of substrates, such as lactose, xylose and sucrose. Agricultural wastes including whey and molasses as well as industrial food waste like soy, malt and ice cream wastes are good choices of the substrates.

(b) The genetics and biochemistry of *E. coli* are understood best among any microorganisms worldwide, so further metabolic engineering and production of new polymer composition are feasible.

(c) The sources of *E. coli* are unlimited and easily accessed.

(d) Recombinant *E. coli* can produce PHB within 24 hours, while non-engineered species take 3 days or more.

(e) It is easier and cheaper to purify polymers from recombinant *E. coli* because *E. coli* becomes fragile when the biopolymer accumulates.

(f) Recombinant *E. coli* does not possess PHAs depolymerase like other

natural PHAs producers. Hence synthesized PHAs are not degraded by recombinant *E. coli* itself.

(g) The molecular weight and polydispersity index of PHB produced by recombinant *E. coli* can be controlled by modulating the activity of PHAs synthase.

(h) High level of polymer production of up to 90 % of cell dry weight has been recorded, thus less carbon source is required to generate extraneous biomass.

The production of PHAs by different microorganisms under different growth limiting conditions has been summarized by Suzuki (1986) and Punrattanasin (2001).

2.2.3.3 Method of isolation

The extraction with neutral solvents yields a polymer of higher molecular weight than sodium hypochlorite (Dawes, 1973). Even the digestion condition is optimized, the molecular weight of the recovered PHB is merely half of that of

the polymer existing in the original biomass (6×10^5 versus 1.2×10^6) (Berger, 1989). Nonetheless, if the biomass is pretreated with a surfactant such as sodium dodecyl sulfate or Triton X-100 before digestion, a polymer of higher purity and higher molecular weight (7.3×10^5 to 7.9×10^5) was acquired (Ramsay, 1994).

Table 2. 7 Composition of PHAs accumulated by *Alcaligenes eutrophus* ATCC 17699 from various carbon sources (Punrattanasin, 2001)

Carbon source	Concn (g/L)	PHA (%, wt/wt)	Monomer composition (mol %)	
			HB	HV
$CH_3(CH_2)_3COOH$	20	36	10	90
$Cl(CH_2)_3COOH$	18	27	89	
$HO(CH_2)_3COOH$	16.5	30	67	
$HO(CH_2)_3COOH$	9.6	43	82	
$HO(CH_2)_4OH$	20	8	75	
$Cl(CH_2)_4COOH$	15	29	63	
$O(CH_2)_3CO$	20	21	83	
$O(CH_2)_3CO$	10	65	76	
$Cl(CH_2)_4COOH$	20	1	24	24
$Cl(CH_2)_4COOH$	5	19	26	65
$HO(CH_2)_3COOH$	17.5	18	32	23

2.3 Environment-Friendly Biopolymer

2.3.1 Biodegradation

The biodegradability of PHAs, which is rapid and requires no special environmental conditions, is their most attractive feature. Various environments have isolated numbers of aerobic and anaerobic PHAs degrading bacteria and fungi, says, species of *Aspergillus*. Biodegradation starts when microorganisms begin to grow on the surface of the plastic and excrete extracellular PHAs depolymerase enzymes that break down the polymer into its molecular building blocks, called hydroxyacids. The microorganisms then take up these water-soluble monomers and oligomonomers which are used as carbon source for growth. So in aerobic condition, CO_2 and H_2O are the end products of PHAs but it turns into methane under anaerobic condition. Figure 2.5 shows the biocycle of PHAs. At the biochemical and molecular level, the extracellular depolymerase have been studied. It was found that the enzymes have a hydrophobic domain as a binding site that adheres to the surface of PHAs and a

catalytic domain containing the lipase consensus sequence (Jan, 1995).

The rates of biodegradation of P (HB-co-HV) have been studied in various environments such as soil, sludge, seawater, etc (Doi, 1992). The rate of biodegradation of PHB is higher than that of the P (HB-co-HV).

However the rate of biodegradation of PHAs is dependent upon a number of factors such as microbial activity of the environment, exposed surface area, temperature, pH, and properties of the plastic material to be degraded (Jendrossek, 1996).

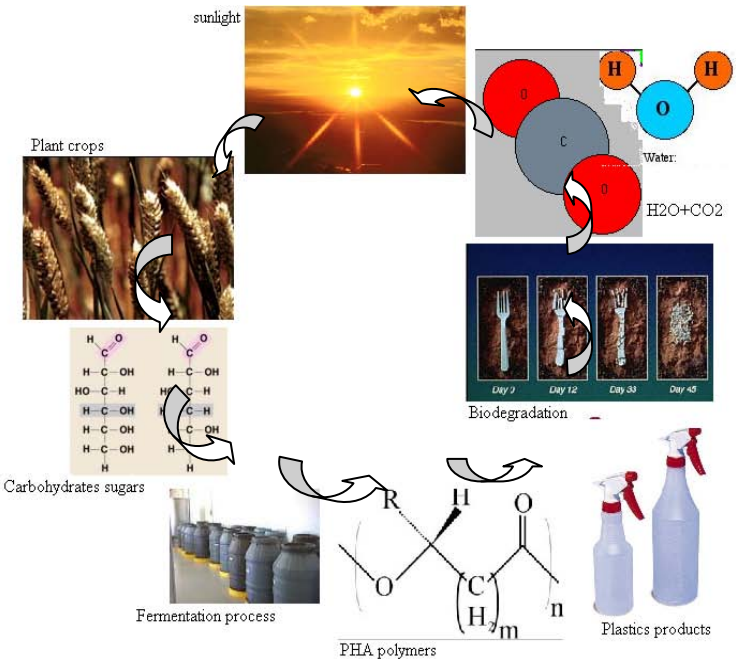


Fig. 2. 5 Biocycle of Polyhydroxyalkonate (Jendrossek, 1996)

2.3.1.1 Microbial degradation

The potential use of PHAs as commodity plastic relies on its biodegradation in the natural environment. Extracellular enzyme of a great deal of microorganisms has been reported to degrade PHAs (Dawes, 1973). Many soils bacteria are known to degrade PHAs and utilize the polymers as the carbon source (Laffferty, 1988). In a recent research, about 300 dominant strains of bacteria were identified which were capable of degrading PHAs (Mergaert, 1993). There were 36 *Bacillus* 105 gram-negative bacteria 86 mold strains and 68 *Streptomyces*. It was showed that extracellular degradation of PHAs by *Pseudomonas* sp. produced the dimeric hydroxybutyrate (Merrick, 1964) which was reported to being actively taken into the bacterial cell and hydrolyse to the monomer by the intracellular hydrolase (Lusty, 1966). The monomer could take part in the intracellular biochemical pathways providing the energy for the bacteria.

2.3.1.2 Hydrolytic and in vivo degradation of PHAs

Hydrolytic degradation of PHAs in vitro proceeds to the hydroxybutyric acid. This acid is a normal constituent of blood with acetoacetate and acetone in

common is one of the three ketone bodies which are produced endogenously by the process known as ketogenesis. It is thought that PHAs will be well tolerated in vivo.

2.3.2 Biocompatibility

PHAs for inclusion in drug delivery or other biomedical applications will not only depend on the biodegradation but also on their biocompatibility. Biocompatibility refers to the mutual acceptability of the polymer and its surrounding physiological environment. Biocompatibility has been described as a two component phenomenon by the previous research: the effects of the physiological environment on the polymer and the effects of the polymer on the same environment (Williams, 1999). The former will influence polymer degradation and the implanted tissue.

2.4 Low Costs of Production

In PHAs production, about 50 % of the total production cost is for the carbon source (Choi, 1999, Beom, 2000). In order to reduce the cost of production, the use of a cheaper carbon source is required. Recently, it was reported that some recombinant *E. coli* strains could produce PHB from whey (Lee, 1997; Wong, 2000), molasses (Li, 1990; Liu, 1998), soy waste and malt waste (Yu, 1999). Summary of PHB production from inexpensive substrates by various microorganisms is given in table 2.8 (Beom, 2000).

The price of P (HB-co-HV) exceeds 10 US dollars per kilogram, which is much higher than the cost of conventional oil-derived plastic. Substrate cost is one of the major factors in the economical production of PHAs. The carbon source should be less expensive since it is the major contributor to the total substrate cost (up to 50 % of the total operating cost) and thus an expensive carbon source is not practical in large scale industrial production. There are generally two approached that can be taken in the development of bacterial strains in order to produce PHAs from an inexpensive carbon substrate: (i) Substrate utilization

genes can be introduced into PHAs production. (ii) PHAs biosynthesis genes can be introduced into the non-PHAs production, which can utilize cheap substrates.

The utilization of waste materials as the substrate is good alternatives to reduce the cost of the production. Several studies had investigated the use of low cost substrates for PHAs production, such as xylose which is a significant component of hemicellulose of hardwoods and crop residues, molasses, malt waste and soya waste.

Malt waste contains wide variety of sugars is generated in a very large amount during the beer brewery production in Carsberg Brewery Hong Kong Ltd. The ratio of the carbon and nitrogen contents is 7:1 (Yu, 1997). The malt waste was also used as a carbon source for the production of PHB (Wong, 2000).

Table 2. 8 Production of PHB from cheaper carbons substrates (Beom, 2000)

Organism	Carbon Source	Culture time (h)	Cell conc. (g/l)	PHB conc. (g/l)	PHB content (%)
Azotobacter chroococcum	Starch	70	54	25	46
Azotobacter chroococcum	Starch	58	1.17	0.864	73.9
Azotobacter chroococcum H23	Starch	72	5.19	3.85	74.2
Ralstonia eutropha	Tapioca Hydrolysate	59	106	61	58
Recombinant E.coli	Whey	52	31	25	80
Methylobacterium sp. ZP24	Whey	48	9.9	5.9	59.6
Pseudomonas cepacia	Lactose	120	6.57	2	56
Pseudomonas cepacia	Xylose	60	2.59	1.55	60
*Aztobacter vinelandii*UWD	Molasses	36	33	22	66
Alcaligenes latus DSM1124	Malt waste	69		32.4	22.7
Alcaligenes latus DSM1124	Soy waste		69	18.4	6

2.5 PHAs Production by Recombinant Strains

PHAs yield is another major factor of the economical production of PHAs. Bacterial *E. coli* and *B. subtilis* have proven to be powerful tools in the microbial synthesis of bio-products, which is using molecular biology techniques. Recombinant DNA techniques can be used to modify or to introduce new metabolic pathways to broaden the utilizable substrate range, so as to enhance PHAs synthetic capacity and to produce novel PHAs.

To increase the efficiency of production of PHAs, recombinant method is used to modify gene in bacteria to give a higher production of PHAs. Vector pKS containing PHB operon from *Alcaligenes eutrophus* was introduced into *E. coli* XL-1 Blue to produce scl-PHAs and it was greatly successful (Kichise, 2002). Recent research also cloned PHAs synthase gene (phaC1) from indigenous *Pseudomonas sp.* LDC-5 in *E. coli* to increase production of mcl-PHAs (Sudesh, 2000).

2.5.1 Use of recombinant *E. coli*

There are several advantages of employing recombinant *E. coli* for the production of PHAs such as short generation time, the best understanding in both genetics and biochemistry and broad substrate utilization ability.

PHAs biosynthesis genes of *Alcaligenes eutrophus* had been sequenced and characterized in detail, and they were found to form an operon, which could be expressed from its own promoter in *E. coli*. The use of recombinant *E. coli* harboring the PHAs biosynthesis genes was attractive for the production of PHAs.

Although acetate can be utilized by wild type *E. coli* strains, the saturated short chain fatty acids such as butyrate, propionate and valerate can not directly serve as substrates for the enzymes. For these short chain fatty acids to be metabolized, the enzyme are required as well as the fatty acid degradation enzymes encoded by fad genes. Since the expression of the fad structural genes is not induced by saturated shot chain fatty acids, two mutations causing constitutive expression of the fad structural genes are necessary for the metabolism.

E. coli was supposed lacking an efficient system for the conversion of propionate

to propionyl-CoA, and it is consistent with the fact that when *E. coli* is grown in a rich medium, the pathways of fatty acid uptake and fatty acid utilization are not expressed because of regulation by the fad gene. The mechanism is complex and involves at least two pathways.

For the successful application of commercial PHAs production systems, it is an essential to optimize all factors of the fermentation condition. The price of the PHAs product will depend on the parameters such as substrate cost, PHAs yield on the substrate, the efficiency of the production in the downstream processing. This indicates that high PHAs percentage of the cell dry weight (CDW) is desirable (De, 1997). On the other hand, natural PHAs producers accumulate PHAs during evolution, they often have a long generation time and low optimal growth temperature, they are also hard to lyse, and contain pathways for PHAs degradation. Bacteria such as *E. coli* do not have the capacity to synthesize or degrade PHAs, nevertheless *E. coli* grows fast and at a higher temperature and it is easy to lyse. The faster growth enables a shorter cycle time for the production process, while the higher growth temperature can decrease the cost associated with fermentor cooling. The easier lysis of the strains provides a low cost during the purification of the PHAs.

The availability of a great deal of PHAs biosynthetic genes facilitates the

construction of recombinant for the production of PHB. Although *R. eutropha* is a perfect producer of PHB, the strain has certain limitations that prevent it from being useful for the commercial application of PHB. For example, it grows slowly and is difficult to lyse. In addition, it does not have a good genetically character. PHB production with recombinant strain may be able to overcome these obstructions.

Recombinant *E. coli* could potentially be used to address these problems, since it has a very good genetically characterized. PHB accumulation in *E. coli* must be engineered, because this microorganism does not have the PHAs synthesis gene. Since the first *phb* genes were expressed in *E. coli* (Slater, 1988), a variety of other polymers, such as P (HB-co-HV) and P (HO-co-HH), have been synthesized by *E. coli* following genetic engineering.

2.5.2 Use of recombinant *B. subtilis*

Bacillus subtilis is also used as a host because it offers advantages such as short generation time, absence of endotoxin, and secretion of amylases and proteinases that utilize food wastes for nutrients, reducing the cost of production of PHAs. Moreover, secretion of proteins may circumvent the formation of inactive

inclusion bodies, which occurs during the overexpression of foreign genes in *E. coli*. Furthermore, *B. subtilis* is not a human pathogen and can be considered biologically safe. Therefore, *B. subtilis* has become an attractive alternative to *E. coli* as a host for the expression of foreign genes.

2.6 PHAs Recovery Process

Because PHAs are produced intracellular as discrete granules, it is necessary to extract them from the cells for purification. Three methods are adopted for PHAs recovery process. They are as follows: (i) solvent extraction, (ii) sodium hypochlorite digestion, and (iii) enzymatic digestion.

2.6.1 Solvent extraction

Solvent extraction is very commonly used because it is applicable to many PHAs producing bacteria. This process requires a large amount of solvent because PHAs solution is highly viscous (Punrattanasin, 2001). Solvents such as chloroform, methylene chloride, propylene carbonate and dichloroethane are commonly used to extract PHAs from the biomass (Baptist, 1962; Lafferty, 1988). Doi (1992) reported that PHAs is first extracted with a hot solvent in an apparatus for over 1 hour. The PHAs extracted is then separated from lipids by precipitating it with another solvent, such as diethyl ether, hexane, methanol or

ethanol.

Finally PHAs is redissolved in chloroform and is further purified by precipitation with hexane. The maximum purity of PHAs retrieved by chloroform can reach over 90 %, but further digestion results in a reduction in the molecular weight of PHAs (Ramsay, 1990).

2.6.2 Sodium hypochlorite digestion

The principle of sodium hypochlorite digestion is to solubilize non-PHAs cellular materials and leave PHAs intact (Punrattanasin, 2001). PHAs can then be separated from the solution by centrifugation. However it is often revealed that PHAs has degraded severely during the process because of the strong oxidizing property of sodium hydroxide. A 50 % reduction in the molecular weight of the polymers was observed by Berger (1989) when the biomass was digested with sodium hypochlorite. This method has been improved by surfactant pretreatment that requires a shorter recovery time to give a high molecular weight of extracted PHAs.

2.6.3 Enzymatic digestion

This process includes thermal treatment to lyse the cells and denature nucleic acids, enzymatic digestion and washing with anionic surfactant to dissolve non-PHAs cellular materials (Punrattanasin, 2001). Concentrated PHAs from centrifugation is then bleached with hydrogen peroxide. Enzymes like lysozyme, phospholipase, lecithinase, proteinase and others are used in the digestion stage (Steinbuchel, 1996). These enzymes hydrolyze most of the non-PHAs cellular materials but PHAs remains unaffected. The major drawback of this method is the efficiency to release PHB is only 50 to 70 %, which is significantly lower than that of chloroform extraction.

Table 2. 9 Evaluation of different extraction methods from *Rhodospirillum rubrum* (Lafferty, 1988)

	Chloroform extraction	Hypochlorite extraction	Lysozyme digestion
PHAs content (%)	13.6	13.6	13.6
Isolated PHAs (%)	12.5	12.1	9.7
Recovery %	92	89	71
M_w (10^{-6})	1.5	0.94	0.92
M_n (10^{-6})	1.1	0.37	0.36
Polydispersity (M_w/M_n)	1.4	2.5	2.6

2.7 PHAs Applications

Being a potential replacement for conventional plastics, PHAs have several applications in the following areas:

2.7.1 Medical applications of PHAs

A high biocompatibility is essential for the acceptance of a transplant by humans and mammals. Several factors determine whether a polymer is biocompatible: shape, surface porosity, chemistry component, and the tissue where it is incorporated. New materials are needed now because some of these traditional materials, such as silicone, are suspected to have malign effect and to cause cancer (Cammas, 1999). PHAs have the potential to become the important compound for medical applications. Tests have shown that PHB is biocompatible, which is not surprising when considering the fact that *R*-3-hydroxybutyric acid is a normal constituent of blood at concentrations between 0.3 and 1.3 mM (Anderson, 1990) and is also found in the cell envelope of eukaryotes (Reis,

1991). PHB has a low degradation rate in the body comparison with heteropolymers (Pouton, 1996). Thus, medical studies focused on the copolymer.

2.7.1.1 PHAs as drug carrier

PHAs could become candidate as drug carriers due to their biodegradability and biocompatibility in the early 1990s (Pouton, 1996). Microsphere of PHB loaded with rifampicin was investigated for their use as a chemoembolizing agent (Kassab, 1997). The microsphere was prepared by the solvent evaporation technique with distilled water as the dispersion medium, polyvinyl alcohol as an emulsifier and methylene chloride as the solvent. The granule size varied between 5 and 100 mm. These granules could be separated into classes of different size using a gravity field-flow fractionation technique (Kassab, 1997). The drug releases of all the microspheres were rapid within 24 hours. It was possible to be control the drug release rate by the drug loading and the particle size. Recently, similar behaviors were described for P (HB-co-HV) supplemented with tetra-cycline in the acidic and neutral form (Sendil, 1999). The neutral forms were more efficient for encapsulation (52–65 %, w/w) than the acidic forms, however both forms of the drug were rapidly released without any signs of particle degradations.

Also the usage of PHAs as an antibiotic-loaded carrier to treat transplant related and chronic osteomyelitis was investigated (Yagmurlu, 1999). Sulbactam-cefoperazone, the antibiotic used in the study, was integrated into rods (1x 0.3 x 0.3 cm^3, 100 mg) made of poly (hydroxybutyrate-co–22 mol %-hydroxyvalerate) and implanted into a rabbit tibia that was artificially infected by *Staphylococcus aureus*. The infection subsided after 15 days and almost complete healing was found on 30 days.

All these examples indicated that the release of drugs from microspheres made of scl-PHAs occurred at excessive rates. Whether this effect could be related to the manufacturing process was not clear. However, it can be speculated that PHAs with a lower crystallinity such as mcl-PHAs, may be used for granule with lower drug release rates.

2.7.1.2 Problems of protein drug delivery

A lot of proteins and peptides, such as hormone and enzymes can act as drugs. Examples like insulin and thyroid hormone has been used as commercial drug since the beginning of 20th century. Most protein drugs in the market are

delivered in parenteral route because it avoids biological barriers that is difficult for protein to pass, easy administration and easy to achieve effective dose in a short period of time. In addition to parenteral administration, local deliveries of proteins orally or by inhalation are in great interest. However, protein drug delivery through these systems is not very successful due to proteolysis and low rate of absorption. The use of biodegradable polymer as drug vector for controlled release of protein drug could be one of the solutions.

2.7.1.3 PHAs nanoparticles as drug delivery system

Biodegradable polymeric nanoparticles for drug delivery vehicles have attracted increasing attention through the past decade due to its biocompatibility, biodegradability and increase efficiency in drug releasing and entering cells (Williams, 2002). There are several advantages of biodegradable polymer as drug delivery systems over conventional drug therapies. First, delivery of the drug can be localized to a particular tissue or cell type, so that systemic drug level can be lowered. Second, the polymer coat protects drugs from degradation in the body and improves drug efficacy. Besides, there is advantage of biodegradable polymers over non-degradable systems that they are not required to be removed from the patient. The rate of drug release could also be controlled. They are

commonly prepared by block of copolymers consisting of aliphatic polyesters and polyethers (Chen, 2006), such as poly (L-lactic acid) (PLA) – poly (ethylene glycol) (PEG), and poly (lactide-co-glycolidel) (PLGA)-PEG. PEG is most frequently used in associate to form nanoparticle due to its water solubility so that the nanoparticles can be effectively stabilized in the blood and reduce the uptake at the reticuloendothelial sites, such as liver. The core is usually constructed with biodegradable polyesters, since they can be completely degraded by enzymes inside body.

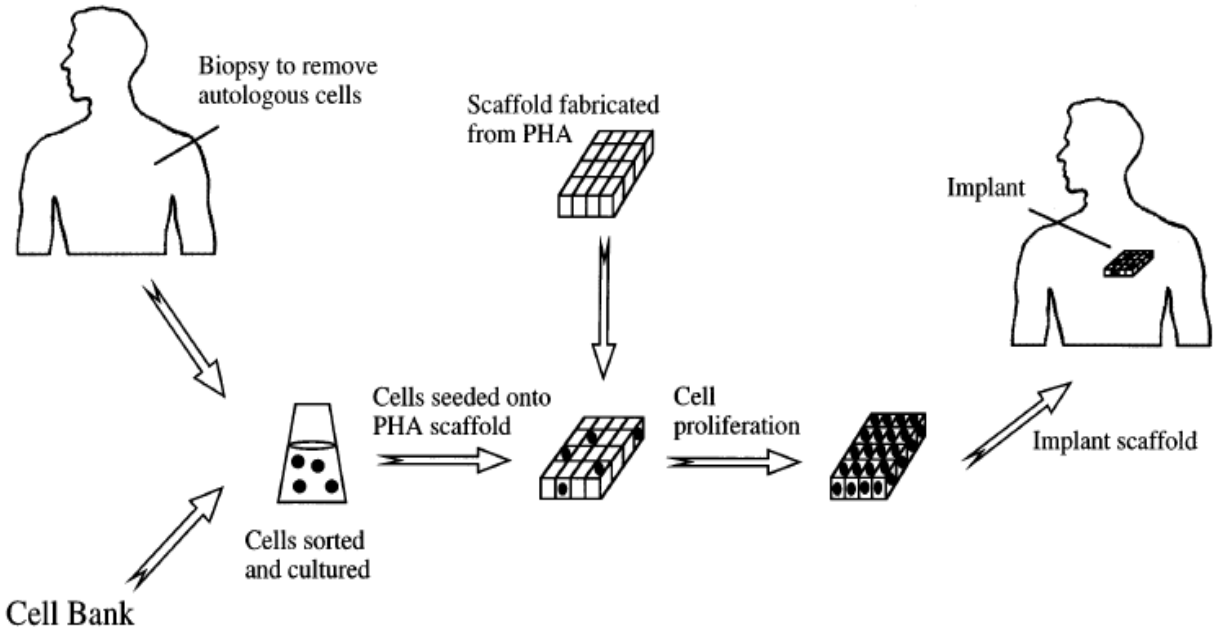
2.7.1.4 PHAs as scaffold material in tissue engineering

Williams (1999) had defined five key parameters that scaffolds need to fulfill for successful tissue engineering: (1) biocompatibility, (2) support of cell growth and cell adhesion, (3) guide and organize the cells, (4) allow ingrowth of cells and passage of nutrients and waste products, (5) biodegradable without formation of toxic compounds. In addition, tissue engineers had determined that the surface structure was also an important factor. Porous surfaces could be produced by the leaching technique, which was done by blending of PHAs with a salt that can be washed out by water. The surface of PHAs materials could be rendered more hydrophilic as was shown by the treatment of P (HB-co-HV) with

allyl alcohol gas plasma that led to an increase of wettability (Mas, 1997).

An interesting aspect of PHAs scaffolds was the fact that the tissue engineered cells could be transplanted together with the supporting scaffold (Williams, 1999). This approach was exemplified (Sodian, 2000) which used scl-PHAs for the fabrication of a tri-leaflet heart valve scaffold. A porous surface was reached with the salt leaching technique resulting in pore sizes between 80 and 200 mm. The scaffolds were seeded with vascular cells from ovine carotid artery and subsequently tested in a pulsatile flow bio reactor. The cells formed a confluent layer on the tir-leaflets.

It is believed that this degradation product is biocompatible with animal tissues, thus implantation of PHB in animal tissues imposes no toxic effects. There are some pharmaceutical applications of PHAs, which include biodegradable carriers for long term drug dosages inside human body, surgical pins, wound dressings, bone and blood vessel replacements. Surgical removal of the bio-tissues is not required after their implantation. Apart from this, PHAs have the potential as constituents for anti-human immunodeficiency virus drugs, anticancer drugs, antibiotics and vitamins (Ward, 2005).



Stages

1. PHA scaffold fabricated for application
2. Tissue specific cells obtained from biopsy or cell bank
3. Cells sorted, cultured and seeded into scaffold
4. Cells proliferate on scaffold and are implanted at tissue engineering site

Fig. 2. 6 Role of PHAs in tissue engineering (Williams, 1999)

2.7.1.5 Advantage of PHAs in medical use

One of the most extensively investigating fields is in medical area since PHAs polymers are biocompatible. The monomeric component of PHB, for example, R-hydroxybutanoic acid, is a normal metabolite found in human blood and present as a ketone body at 3-10 mg per 100 ml in normal human blood. (Williams, 2002) Thus, it has very high potential in medical application. The advantage of PHAs over other heteropolymers, like poly (lactate-co-glycolate) is that it has a lower degradation rate (Zinn, 2001). Hydroxy acids that PHAs releases after breakdown are also less toxic and less inflammatory. For example, hydroxybutanoic acids from PHB are significantly less acidic than 2-hydroxy acids from PGA and poly-lactic acid (PLA) (Williams, 2002). Besides, as the production cost of PHAs is quite high, which is around US$15-30 (Witholt, 1999), it is more profitable to develop PHAs in medical field. As a result, the application of PHAs in a wide range of scope in medical field is studied, one of the most popular were drug delivery and tissue engineering.

2.7.2 Commodity packaging and agricultural containers

Having some mechanical properties similar to polyethylene and polystyrene, such as tensile strength and flexibility, PHAs can be used in extrusion and molding processes, as well as blending with synthetic polymers like chlorinated polyethylene. With a high PHV content, the biopolymer is more suitable for extrusion processes, while a low PHV content favors injection molding process (Tsuchikura, 1994). Because PHB has low oxygen permeability, it is satisfactory for use in food packaging. For example, The P (HB-co-HV) copolymer can be used for films and blow-molded bottles (Ojumu, 2004).

Punrattanasin (2001) suggested that PHAs are favorably used as biodegradable carriers for long-term dosage of insecticides, herbicides, seedling containers or tubing for crop irrigation. At the end of the harvesting season, it is not essential to remove the biodegradable items.

Fig. 2. 7 PHA as biodegradable plastic bags (Ojumu, 2004)

Fig. 2. 8 PHA as agricultural containers (Ojumu, 2004)

2.7.3 Water and wastewater treatment

A promising application of PHAs is in the use as a solid substrate for denitrification in water and wastewater treatment (Hiraishi, 2003). The established nitrogen removal system in wastewater treatment confronts the threat of overdosing, which results in deterioration of effluent quality. However, the use of some conventional liquid substrates does not guarantee a high and stable denitrification rate. It was found through thorough investigations that PHAs are the most suitable solid substrates among the biopolymers because they are microbial storage materials, and are anticipated to be readily mobilized by bacteria under denitrifying and aerobic conditions. Solid substrates such as straw, bark, wood and other biopolymers have been tested for finding a good alternative.

This solid-phase denitrification method has some advantages over the traditional treatment system. Firstly, PHAs are insoluble in water but easily biodegradable, thus they are constant sources of reducing agents for denitrification (Muller, 1992). Secondly, PHAs do not cause deterioration in effluent quality because they release dissolved organic carbon. Thirdly, PHAs serve as sites for the

development of microbial films for water treatment. The bio-films can keep forming while PHAs are in excess in the system. With these characteristics, PHAs are good candidates to assist in water treatment process.

Fig. 2. 9 PHAs as a substrate in water denitrification process (Hiraishi, 2003)

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Strains

Bacillus subtilis DB104

Escherichia coli HB101

E. coli XL-1/pKSCAB

Bacillus thuringiensis HD-81

3.1.2 Plasmids

pBE2 (Fig.3.1) was an *E. coli- B. subtilis* shuttle vector.

pBHR71 (Fig.3.2) contains *phaC1* gene from *Pseudomonas aeruginosa* (Langenbach, 1997).

pJM9131 (Fig.3.3) contains *phaAB* gene from *Ralstonia eutropha* (Valentin, 1994).

pGEMT (Fig3.4) was used for ligation of PCR mixture.

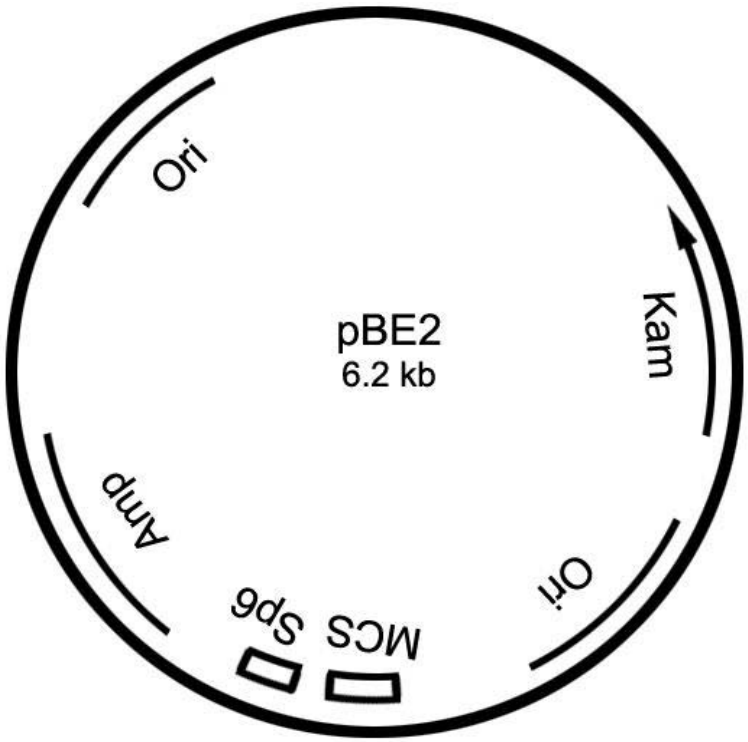


Fig. 3. 1 Gene map of plasmid pBE2 (Guo, 1991)

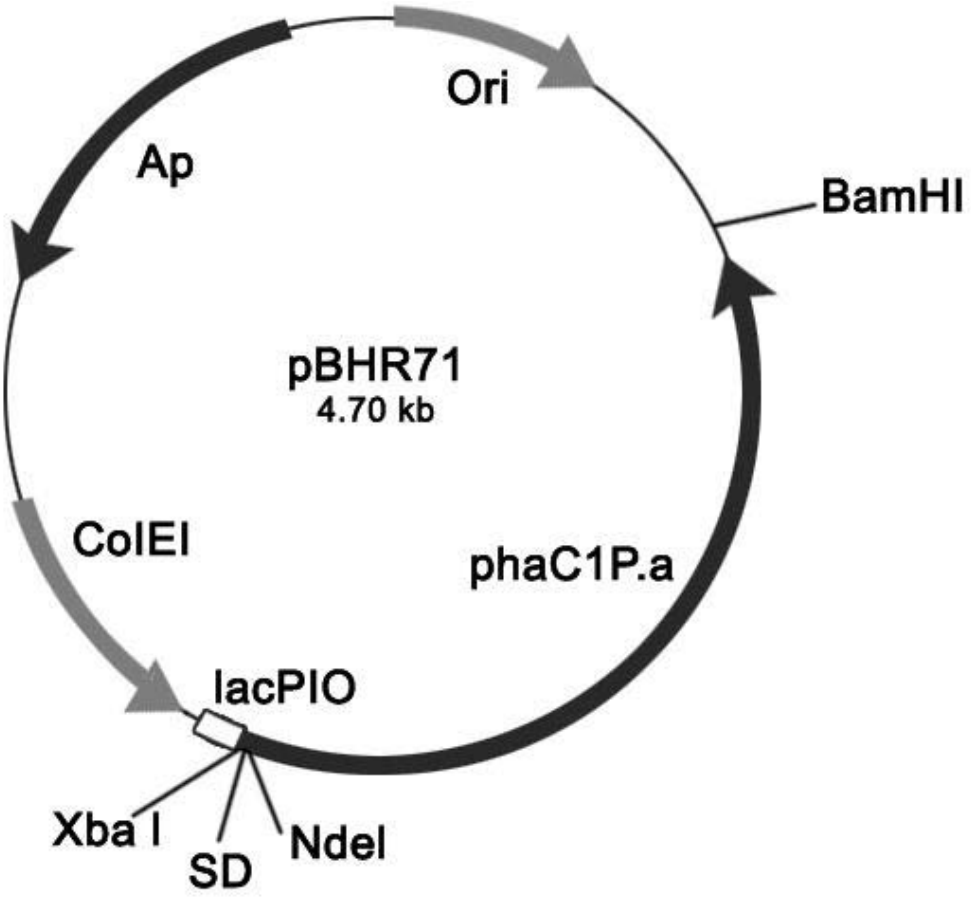


Fig. 3. 2 Gene map of plasmid pBHR71 (Langenbach, 1997)

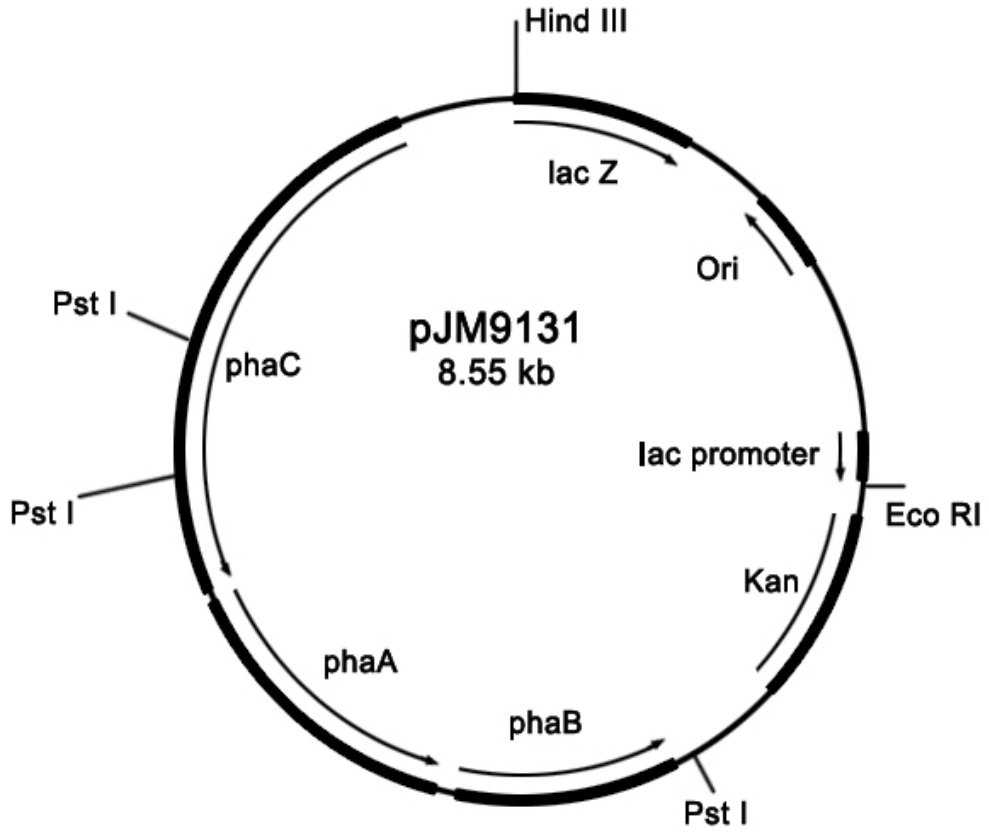


Fig. 3. 3 Gene map of plasmid pJM9131 (Valentin, 1994)

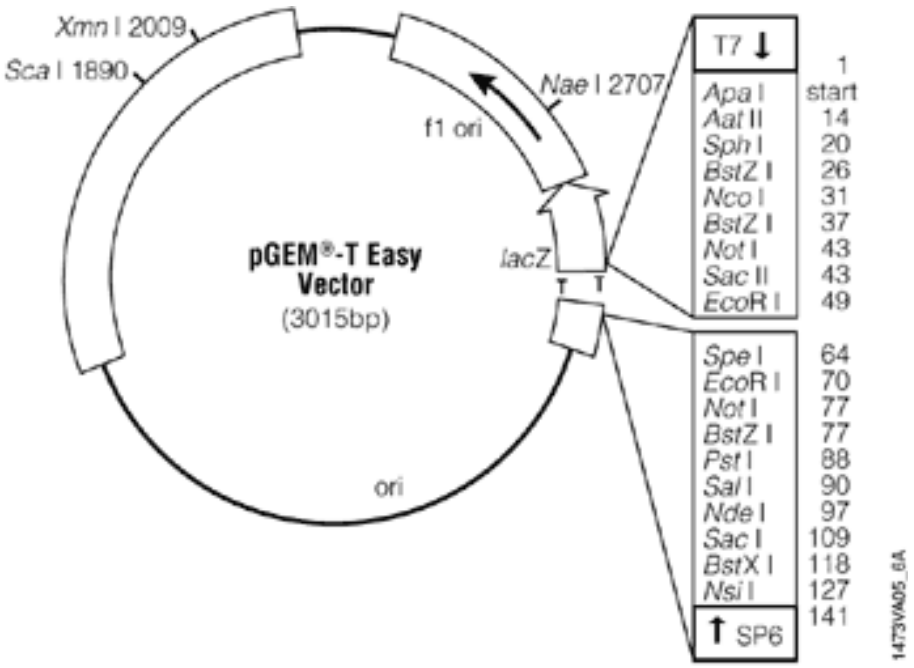


Fig. 3. 4 Gene map of plasmid pGEMT (Gaurivaud, 2000)

3.1.3 Elementary media

PHAs fermentation medium (per L):

10 g glucose, 7 g KH_2PO_4, 1 g $MgSO_4$, 1.5 g citrate, 6 g tryptone, 2 g yeast extract and the pH was adjusted to 7.0.

Nutrient broth (per L):

10 g tryptone, 5 g yeast extract, 10 g NaCl.

Cell Lines Culture Media:

DMEM (Dubecco's Modified Eagle Medium, Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen)

3.1.4 Proposed waste for culture media

3.1.4.1 Tea waste

The tea waste was dumped in large amount from The Maxim's Chinese

Restaurant. Tea waste contained certain amount polyphenols/tannic acid ($C_{76}H_{52}O_{46}$) (20-30 %) which were organic compounds having 1 to 4 hydroxyl group, -OH, attached to benzene ring. They react like alcohols but also show weak acidic properties.

3.1.4.2 Contaminated candies

The contaminated candies came from the candy production line and packing machine in The Garden Company Limited. The effluent was the first washing water for the candy machine. It contained a large amount glucose (Monosaccharide) and sucrose (Disaccharide) after rinsing the internal cavity.

3.1.4.3 Sugar cane waste

The sugar cane waste was generated by the Hung Fook Tong Shop which mainly sells herbal tea. After the cane juice was squeezed from sugar cane, the cane dregs still contained a high proportion of sucrose (Disaccharide).

3.1.4.4 Contaminated baked bread

In The Garden Company Limited, the contaminated baked bread was the defective products which could not pass the quality control and the bread which stocked up over its expiry date. The major ingredients were flour, milk, oil and egg. It consisted with a high quantity of starch (Polysaccharide), protein (polypeptide) and amino acid.

3.1.4.5 Malt waste

Malt waste was obtained from Carlsberg Company and San Miguel Ltd., both local beer breweries, which were mostly semisolids of spent barley and millet refuse.

3.1.5 Preparation of waste medium

3.1.5.1 Tea waste hydrolysate

The obtained wet tea waste was dried in oven for 3 hours and then grinded into

powder and stored in air tight bag for future use. 50 g of tea waste powder was hydrolyzed by adding 400 ml 5 N sodium hydroxide (NaOH) in bottle. Then, the bottle was put into autoclave for 60 minutes at 121 °C. The hydrolyzed mixture was centrifuged for 10 minutes at 3000 rpm and the supernatant was separated from the mixture and neutralized by certain amount of concentrated hydrochloric acid (HCl). Medium was re-centrifuged for 10 minutes at 3000 rpm to separate the suspended residue due to the neutralization process. Finally, the clear tea waste hydrolysate was obtained and stored at low temperature for future use.

3.1.5.2 Contaminated candies hydrolysate

The effluent was centrifuged for 10 minutes at 3000 rpm to separate the suspended residue. The supernatant was neutralized by small amount of NaOH. After neutralization, media was re-centrifuged for 10 minutes at 3000 rpm. Finally, the clear candies hydrolysate was obtained and kept at low temperature.

The 50 g crushed candies were melted by adding 500 ml deionized distilled water (ddH_2O) in bottle. Then, the mixture was put in autoclave for 30 minutes at 121 °C and treated as the same as the fist washing effluent above.

3.1.5.3 Sugar cane waste hydrolyses

The obtained cane waste was grinded with some lubricating ddH_2O. The cane waste paste was stored in air tight bag for future medium digestion. 50 g of cane waste paste was hydrolyzed by adding 500 ml of 0.3M HCl in bottle. Then, the bottle was put into autoclave for 60 minutes at 121 °C. The hydrolyzed mixture was centrifuged for 10 minutes at 3000 rpm and the supernatant was separated from the mixture and neutralized by certain amount of concentrated NaOH. Media was re-centrifuged for 10 minutes at 3000 rpm to separate the suspended residue. Finally, the clear cane waste hydrolysate was obtained and was stored at low temperature.

3.1.5.4 Contaminated baked bread hydrolyses

30 g of baked bread was hydrolyzed by adding 200 ml of 5 N NaOH in bottle. Then, the bottle was put into autoclave for 60 minutes at 121°C. The hydrolyzed mixture was centrifuged for 10 minutes at 3000 rpm and the supernatant was separated from the mixture and neutralized by certain amount of concentrated NaOH. Media was re-centrifuged for 10 minutes at 3000 rpm to separate the suspended residue. Finally, the clear baked bread hydrolysate was obtained and

was stored at low temperature.

3.1.5.5 Malt waste hydrolyses

150 g of the waste was hydrolyzed with 1 L of 0.5 M HCL. The mixture was incubated at 121 °C for 30 min at evaluated pressure (1 Kgf/cm^2). The resultant mixture was centrifuged at 14, 333 g for 20 min. The supernatant was adjusted to pH 7 by NaOH and filtered to remove debris. The medium was autoclaved and used as substrate for the growth of the bacteria.

3.2 Methodology

3.2.1 Culture condition of the elementary culture

B. subtilis was grown at 30 °C and *E. coli* was grown at 37 °C. They were first inoculated into 5 ml nutrient broth for 16 hours, and then 1 % inoculum was used in fermentation medium or in waste medium with shaking 280 rpm. Antibiotic concentrations for bacterial selection were as follows: 100 μg ampicillin /ml for *E. coli* and 80 μg ampicillin /ml for *B. s*ubtilis. The medium volume was 100 ml in 500 ml flask or 200 ml in 1000 ml flask.

3.2.2 Determination of carbon concentration

3.2.2.1 Determination of TOC by using TOC analyzer

After fermentation, the supernatants were diluted by adding ddH_2O. Then, 5 ml of the diluted supernatants were put into 2 TOC tubes for each. 2 blank ddH_2O

were prepared for correction. Finally, the sample tubes were put into the Auto sampler of TOC Analyzer. All the steps were repeated other 3 times for increasing the accuracy.

3.2.2.2 Chemical oxygen demand (COD)

20 ml of water sample and 20 ml deionized water were transferred to two separate flasks, with the latter being as a blank. A spoonful of mercury sulfate powder and 5 ml of concentrated sulfuric acid was added to each flask. 10 ml of standardized potassium dichromate solution and 25 ml of sulfuric acid containing dissolved silver sulfate were then added. The flasks were refluxed for 2 hours. Finally, the mixtures were titrated with standardized FAS solution with Ferrion indicator. The color change from bluish green to reddish brown marked the endpoint of titration. By deducting the blank, COD was obtained.

3.2.3 Determination of TKN by Kjeldahl method

The supernatant of the fermentation culture were diluted by adding ddH_2O. 50ml diluted sample were put into kjeldahl tube. Two K_2SO_4 tablets with mercury oxide and 10 ml H_2SO_4 was then added in the tube. The tube was heated at

250 °C for 120-150 minutes until the mixture turned black and then got clear greenish blue in color. After cool down, 70 ml of ddH_2O was added to the tube to carry out crystallization. The nitrogen in different samples was distillated by using 50ml 40 % NaOH. The nitrogen was dissolved in distillate and the distillate was received in 10ml receiving solution. The nitrogen was titrated against the 0.0023 N HCl. The TKN was calculated after the deduction of blank.

3.2.4 Measurement of cell dry weight (CDW)

The fermentation culture samples were centrifuged at 4,000 rpm for 20 minutes and the cells were washed by ddH_2O. Then, it was filtered through the dried and pre-weighted 0.45 μm pore member filter and dried at 100 °C for 2-3 hours. The filter paper together with the biomass was weighted to obtain the CDW.

3.2.5 Gas chromatography (GC) analysis

PHA content in the cells was determined by GC with benzoic acid as an internal standard. 15 mg of dried cells were placed in small GC tubes with screw cap and the cells were dissolved in 1 ml of esterification solution and 1 ml of chloroform. The screw cap was closed tightly before put in the oven. The tubes were heated

at 100 °C for 4 hours to convert the polymer into their methyl esters. After esterification, tubes were cool down and 1 ml of ddH_2O was added to the tubes and vortexed well. At the same time 10 mg of standard PHAs also was prepared as same as above method for standard purpose.

After settle down, three layers were observed such as upper aqueous layer, middle cell layer and lower chloroform with esterified PHAs.

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0.2 µl of lower organic phase was drawn for GC analysis. GC was performed on a Hewlett Packard 5890A Series II Gas chromatograph, using an Ultra 2 (Crosslinked 5 % Ph Me Silicone) Capillary column with 0.2 mm diameter and 25 m in length.

The column temperature was started at 70 °C for 3 minutes and increased at the rate of 10 °C / min, and then it was raised to 250 °C for 20 minutes to remove all nonvolatile components. The fractionated products were detected by flame ionization with condition of 30 ml of hydrogen min^{-1}, 20 ml of air min^{-1} and 1 ml of auxiliary nitrogen min^{-1}. The temperature of the GC was set up as follows, oven temperature at 135 °C, injector A at 260 °C and detector A at 300 °C.

3.2.6 Calculation of the PHAs

The percentage of PHAs content was calculated as follows,

$$standardized \quad area \quad of \quad PHAs = \frac{peak \quad area \quad of \quad standard}{weight \quad of \quad standard} \frac{PHAs}{PHAs}$$

$$PHAs\% = \frac{peak \quad area \quad of \quad PHAs}{standardized \quad area \; of \; PHAs \times weight \; of \; sample} \times \frac{peak \; area \; of \; standard \; benzoic \; acid}{peak \; area \; of \; sample \; benzoic \; acid}$$

3.2.7 Fourier transform infrared (FTIR) spectroscopy

Two milliliter of the cell culture was centrifuged at 2,610 g for 15 min. The cells were transferred onto IR window (ZnSe Disc, Spectratech) and dried on it. A mirror was used to give the reflected infrared signal to the horizontally laid window. With a scan number of 32, resolution of 16 and auto gain, spectra were recorded at wavenumbers (cm^{-1}) from 400 to 4000 using an Avatar 360 FT-IR (Nicolet) (Hong, 2000). The PHA peak was observed at wavenumbers about 1726–1740 cm^{-1} (Yoshiharu, 1989).

3.2.8 Extraction of PHAs

An appropriate amount of freeze dried cell powder was treated with 20 ml chloroform per gram cell powder. The mixture was oven at 50 ℃ for 24 hours then was filtered by using a glass fiber filter paper GC 50 two times. The liquid was put into the fume cupboard for air dry. The PHAs was precipitated by adding the methanol with the chloroform (methanol: chloroform = 9:1) in the cold room overnight. Pour away the upper layer and air dry in the fume cupboard, so obtain the pure PHAs.

3.2.9 Mini-preparation of plasmid DNA

Wizard Plus SV Minipreps DNA Purification System (Promega) was used for plasmid DNA isolation from the bacterial cells. Bacterial culture was prepared in the way similar to previous minipreps system.

The culture was pelleted in eppendorf for 3 minutes at 10,000 rpm; 4.5 ml culture was used for high copy number plasmid. The cell pellet was resuspended in 250 µl Cell Resuspended Solution (50 mM Tris, pH 7.5; 10 mM EDTA; 100 µl / ml Rnase A). 250 µl of cell Lysis Solution (0.2 M NaOH and 1 % SDS) was added

and mixed by inverting the eppendorf several times and the cell suspension became clear. 10 µl Alkaline Protease Solution was added into the suspension and mixed by inverting the eppendorf. The mixture was incubated at room temperature less than 5 min; otherwise the nicked plasmid may occur. The mixture was neutralized by 350 µl Neutralization Solution (4.09 M guanidine hydrochloride; 0.759 M potassium acetate; 2.12 M glacial acetic acid; pH 4.2) followed by inverting several times and centrifugation at 14,000 rpm in micro-centrifuge for 10 min.

The cleared lysate was transferred to the Spin Column with 2 ml Collection Tube. After centrifugation at 14,000 rpm for 1 min, the flow-through was discarded. 750µl of the Column Wash Solution (60 mM potassium acetate, 10 mM Tris-HCL, pH 7.5 and 60 % ethanol) was added and the column was centrifuged at 14,000 rpm for 1 min and the flow-through was also discarded. 250 µl Column Wash Solution was added again and the column was centrifuged at 14,000 rpm for 5 min. This spin column was then transferred to an autoclaved 1.5 ml eppendorf, 100 µl Nuclease-free Water was added into spin column and centrifuged at 14,000 rpm for 1 min to elute the DNA.

3.2.10 Midi preparation of plasmid DNA

QIAGEN Midipreps System was used for large scale plasmid preparation. Glycerol store of the bacterial strain was inoculated into 5 ml of the LB medium with appropriate antibiotic and incubated 8 hours at 37 °C with 280 rpm shaking. 1 % of culture was inoculated into 100 ml LB medium and incubated at 37 °C 280 rpm shaking overnight. 100 ml culture was centrifuged at 6,000 g for 15 min at 4 °C. The cell pellets was resuspended in 4 ml Buffer P 1, 4 ml of Buffer P 2 was then added and was incubated for 5 min at room temperature. 4 ml of chilled Buffer P 3 was added and incubated in ice for 15 min. It was centrifuged at 20,000 g for 45 min at 4 °C and the supernatant containing the plasmid DNA was transferred to QIAGEN tip 500 column that was pre-equilibrated by 4 ml Buffer QBT. Twice of 10 ml Buffer QC was then applied to the column and the DNA was eluted by 5 ml of Buffer QF. 3.5 ml of isopropanol was added to the eluted plasmid DNA and was centrifuged at 15,000 g for 30 min at 4 °C. The supernatant was removed and 1.5 ml 70 % ethanol was added with centrifuged at 15,000 g for 10 min. The ethanol was carefully removed and air dried the pellet. Finally, 100 µl deionized distilled water was added to re-dissolve the plasmid DNA.

3.2.11 Agarose gel electrophoresis

Different sizes of DNA were separated by electrophoresis in horizontal slab agarose gel containing 0.8 % (w/v) agarose and 0.5 µg/ml ethidium bromide in 1 x TBE buffer. The DNA sample was mixed with appropriate amount of 6 x agarose gel loading buffer to give 1 x final concentration. The samples were loaded onto slots of the gel placed in the electrophoresis tank submersed by 1 x TBE buffer. 100 volts was applied in electrophoresis and the gel was examined under UV after the bromophenol blue with sample was migrated for an appropriate distance through the gel. The DNA marker used was 1 kb ladder (Promega).

3.2.12 Restriction digestion of DNA

Restriction digestion of DNA was carried out in a volume of 10 µl to 50 µl in an appropriate buffer as suggested by the supplier. The reaction was incubated at the optimal temperature of the restriction enzyme for 2 hours or longer. After digestion, the reaction mixture was analyzed by agarose gel electrophoresis.

3.2.13 Dephosphorylation of plasmid vector

After plasmid vector was completely digested by the suitable restriction enzyme, 1 U of Shrimp Alkaline Phophatase (SAP) was added and incubated at 37 °C for 1 hour. Dephosphorylated plasmid was recovered and purified by agarose gel extraction system.

3.2.14 Chromosomal DNA isolation

5 ml culture was centrifuged down and the cell pellet was resuspended by TE buffer, pH 8 and the suspension was freezed by liquid nitrogen for 2 min and thawed at 50 °C for 2 min. The procedures were repeated for 6 times. Finally, the suspension was incubated at 85 °C for 5 min to remove undesirable protease and centrifuged at 12,000 rpm for 30 s and the supernatant was collected. In order to obtain higher purity of the genomic DNA, the supernatant was undergone phenol-chloroform extraction and ethanol preoccupation to remove contaminants.

3.2.15 Primer design

There were a few considerations for primer design. The primers should be between 18 and 30 bases in length, short primer would give rise to higher risk of annealing at more than one complementary site, leading to amplification of non-specific temperature cycling products. Besides, the melting temperature (Tm) of the primer should not be too low that will lead to higher chance of non-specific binding. In addition, the primers optimally should have GC content 40-60 % and should terminate in 1 or more C or G bases. The primer sequences should also be check for self-complementarily that could introduce secondary structures like hairpin loops.

3.2.16 Polymerase chain reaction (PCR)

The Expand High Fidelity PCR System or Taq DNA Polymerase was used to select and amplify the *pha* gene cluster of *Bacillus thuringiensis* by using a pair of oligonucleotide primer each complementary to the end of the target DNA sequence. Two primers were extended toward each other by thermostable DNA sequence in a reaction cycle of denaturation, primer annealing and polymerization.

Final concentration of 1 x Expand HF Buffer or Taq Buffer, 200 µM dNTP, 300 nM each of forward and reverse primer and 0.1 µg – 0.75 µg of template DNA were added and mixed in a 0.2 ml PCR eppendorf tube. Sterile water was added to make a final volume of 50 µl. 2.6 units of Expend HF enzyme mix or 2.5 units of Taq polymerase was added and subjected to the thermocycle in a thermocycler.

General amplification was carried out under the following conditions:

Step 1: 94 °C first denaturation for 5 minutes

Step 2: 94 °C cycle denaturation for 1 minute

Step 3: 50 °C primer annealing for 1 minute

Step 4: 72 °C extension for 1 minute

Step 5: repeat step 2 to 4 for 30 times

Step 6: 4 °C for infinity

3.2.17 Phenol /chloroform extraction

Equal volume of Phenol /chloroform mixture (Phenol: chloroform: isoamyl

alcohol in ratio of 25: 24: 1) was added to reaction mixture after the CIAP hydrolyzation, the mixture was gently mixed by hand agitation until the emulsion was formed. The eppendorf was then centrifuged at 14,000 rpm for 30 seconds in microcentrifuge.

The upper aqueous phase was transferred to an autoclaved eppendorf and added with an equal volume of the chloroform mixture (chloroform: isoamyl alcohol in ratio of 24: 1). The eppendorf was then centrifuged again for 30 seconds in after gently mixing. The upper aqueous was then transferred to an eppendorf, which was ready for DNA purification by ethanol precipitation.

3.2.18 Ethanol precipitation

Equal volume of 0.3 M sodium acetate (NaOAc, pH 5.5) was added to DNA solution prepared and well mixed by hand agitation. 2 volume of the cold absolute ethanol was added and mixed by hand agitation, and then the eppendorf was quick spun for a few seconds.

The eppendorf was deep frozen at -80 °C for 30 min. In order to precipitate the DNA, and was subjected to centrifuge at 14,000 rpm for 10 min. The supernatant

was discarded and the pellet was washed by cold 70 % ethanol (without disturb the pellet) and spun for 2 min. Supernatant was discarded and the pellet of the eppendorf was dried by DNA SpeedVac (Savant Model SC 100) for 20 min. 20 µl sterile water was added to dissolve the dried pellet and incubated at 37 °C for 5 min to enhance the detached of DNA pellet from the wall of eppendorf.

3.2.19 Purification of DNA fragments from agarose gel

The DNA sample was electrophoresed on a 1 x TBE agarose gel. The desired DNA was purified by QIAEX II Gel Extraction Kit (Qiagen). Desired DNA band in gel was excised and dissolved in 3 volume of buffer QX1 and 10 µl of resuspended QIAEX II was added. It was incubated in 50 °C for 10 min with vortex mixing at every 2 min in order to keep the QIAEX II in suspension. After the gel slice was completely dissolved, the mixture was then centrifuged at 14,000 rpm for 30 seconds and resin was pellet with the discarded supernatant. 500 µl of buffer QX1 was added to remove any residual agarose contaminants and the pellet was suspended by vortex, and repeated the above centrifugation steps. Above wash steps were repeated twice by using another buffer, buffer PE, to remove any residual salt contaminants.

The pellet was air dried and 20 µl of the sterile TE buffer was added for elution of DNA from the resin. The pellet was then suspended and incubated at 50 °C for 5 min. The eppendorf was finally centrifuged at 14,000 rpm for 30 seconds; the supernatant containing desired DNA was transferred to a clean autoclaved eppendorf.

3.2.20 Preparation of competent cell

Appropriate *E. coli* from glycerol stock was inoculated in 5 ml sterilized 2XYT and incubated at 37 °C overnight with 250 rpm shaking. 200 µl portion of the culture was added into 20 ml sterilized 2XYT and then incubated at 37 °C until the A600 was reached the range of 0.3-0.4. The cells were collected by centrifugation at 2,500 rpm for 15 min at 4 °C. The supernatant was discarded and 10 ml cold 100 mM sterilized $CaCl_2$ was added to suspend the pellet by slightly vortex. The tube was incubated in ice for 25 min and the cells were pellet again by 2,500 rpm for 15 min in 4 °C and suspended in 2 ml cold 100 mM sterilized $CaCl_2$ solution.

The resulting competent cells were incubated in 4 °C overnight. 50 % glycerol stock was added in the tube to a final concentration of 15 % (v/v) and slightly

vortex mixed. The competent cells were aliquoted (200 µl each) in eppendorf tubes and the eppendorf tubes were frozen in liquid nitrogen and finally stored in -80 °C.

3.2.21 Ligation reaction

An appropriate amount of the vector and insert (about 1: 5 in concentration) were mixed with the addition of 5 x ligation buffer to reaction mixture to a final concentration 1 x. The mixture was mixed well followed by quick spin, and then incubated at 42 °C for 5 min and in ice for 3 min to terminate the reaction. One unit of T4 DNA ligase (Life Technologies) was then added and the mixture was incubated at 4 °C overnight.

3.2.22 Transformation of ligation reaction mixture into *E. coli*

Frozen competent cells were incubated on ice until the cell suspension was just thawed. Ligation reaction mixture and 50 µl competent cells were mixed together in an ice cold eppendorf, which was incubated in ice for 25 min and heat shocked at 42 °C for 2 min and immediately chilled in ice for 2 min. 200 µl 2 x YT or LB medium was added and incubated at 37 °C for 1.5 hours. Portion of 50 µl

transformed cells were spread on nutrient agar plates with appropriate concentration of antibiotics and the plates were incubated at 37 °C overnight.

3.2.23 Transformation of *B. subtilis*

B. subtilis strain was firstly streaked on nutrient agar plate with appropriate antibiotic and incubated overnight at 37 °C. Cell from the overnight incubated plate heavily incubated (about 0.9 at A600) into 100 ml flask containing 5 ml pre-transformation medium (PTM). The culture was incubated at 37 °C 280 rpm shaking. After the A600 was reached 3.0, the stationary phase of the culture was reached and the cells were most competent in this state. 100 µl of the competent culture was transferred into 1 ml pre-warmed transformation medium TM in a universal bottle and at least 2 µg of plasmid DNA was added into TM. The universal bottle was incubated at 37 °C with 280 rpm shaking for 1.5 hours, then 600 µl and 100 µl of transformed cultures were plated on nutrient agar plate with antibiotic chloramphenicol (5µg/ml). The plates were incubated at 37 °C overnight or 2 days and the colonies on agar plates were counted.

3.2.24 Construction of recombinants

Plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). The isolated plasmids and digested DNA fragments were analyzed by electrophoresis in horizontal slab gels containing 0.7 % (w/v) agarose, and a 1 kb DNA ladder (promega, Madison, WI) was used as standard marker. DNA restriction fragments were isolated from the agarose gel using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA). Restriction enzymes and T4 DNA ligase (Promega) were used according to the instructions provided by the supplier. A 1.8 kb *Bam* HI/*Xba* I restriction fragment containing *phaC1* gene obtained from plasmid pBHR71 was used as an insert, and plasmid pBE2 was employed as vector. A 2.5 kb *Pst* I restriction fragment containing *phaAB* gene obtained from plasmid pJM9131 was used as an insert, and plasmid pBE2C1 was employed as vector. Ligation products were mixed with competent cells of *B. subtilis* DB104, and the resulting transformants were selected on nutrient agar plates containing 100µg ampicillin /ml. The positive transformants were proved by restriction digestion followed by agarose gel electrophoresis.

3.2.25 Cultivation of recombinant *B. subtilis* in hydrolyzed malt waste

Malt waste, mostly semisolids of spent barley and millet refuse was obtained from Carlsberg Company, a Hong Kong beer brewery. Eighty grams waste was hydrolyzed with 1 L 0.6 M HCl. The mixture was incubated at 121 °C for 30 min at evaluated pressure (1 kgf/cm^2). The resultant mixture was neutralized, centrifuged and filtered. The recombinant *B. subtilis* was cultivated by acid hydrolyzed malt waste and compared to the fermentation medium, in order to observe the ability of the strain in utilization of malt waste.

3.2.26 Cultivation of recombinant *E. coli* in food waste

The recombinant *E. coli* was grown at 37 °C. It was first inoculated into 5 ml nutrient broth for 16 hours, and then 1 % inoculum was used in food waste medium with shaking 280 rpm for 48 hours. The medium volume was 100 ml in 500 ml flask or 200 ml in 1000 ml flask.

3.2.27 Preparation of nanoparticles using PHAs produced by bacteria

0.03 g sample PHAs was dissolved in 4 ml chloroform in a reflux system for 30 minutes. 0.4 g Poloxamer 188 which dissolved in 20 ml ddH_2O (2 % Poloxamer 188) was added to the sample. 0.02 ml oleic acid followed by 0.04 ml SPAN 80 was then added. The mixture was subjected to homogenization at 20, 000/min for 5 minutes. Chloroform is evaporated by rotatory evaporator. The mixture was stirred overnight by magnetic stir bar.

3.2.28 Preparation of pyrene loaded nanoparticles

Pyrene was chosen to imitate protein drug due to its hydrophobicity and fluorescence nature. Nanoparticles were prepared in dispersion of pyrene in ddH_2O by method the same as above. Unloaded pyrene was removed by dialysis in ddH_2O for 2 days.

3.2.29 Measurement of size

The particle size was measured using a Malvern Zetasizer 3000HSA (Malvern, Worcs., UK). Size of nanoparticles prepared were measured and compared.

3.2.30 Drug loading capacity and drug loading efficiency

Nanoparticles loaded with pyrene prepared above were centrifuged so that pyrene in nanoparticles was released. The amount of pyrene loaded was determined by measuring the absorbance of supernatant by Luminescence Spectrometer LS 50B (Perkin Elmer).

3.2.31 Drug in vitro release

Extracellular PHAs depolymerase solution (0.01 ml, 0.05 ml and 0.1 ml) was added into nanoparticles loaded with pyrene (0.5 mg/ml, 1 mg/ml and 2 mg/ml). Fluorescence intensity was measured and recorded which indicated amount of drug release.

3.2.32 Biocompatibility test

Human breast adenocarcinoma cell line (MCF7) was used to investigate the biocompatibility of the nanoparticles. MCF7 cells were placed in 96 well tissue-culture plates and incubated in a 5 % CO_2 incubator for 4 hours. The amount was 2 x 10^5 cells /ml. 8µl (12 µg, 6 µg, 4 µg, 2 µg, 1 µg, 0.5 µg respectively) of nanoparticle dispersions was added to 200 µl cells with media and incubated for 1 day. The viability of cells was evaluated by 3-(4, 5-dimethylthiazolyl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2G-tetrazolium. Viable cell number was determined by measuring absorbance at 490 nm.

CHAPTER 4

CLONING AND EXPRESSION THE PHAs GENE INTO *BACILLUS SUBTILIS*

4.1 Introduction

Polyhydroxyalkanoates (PHAs) function as carbon and energy reserves in prokaryotic cells (Anderson, 1990): They are accumulated by a wide range of bacteria when a carbon resource is provided in excess and one essential growth nutrient is limited (Ramsay, 1990; Steinbuchel, 1991). Since their physical characteristics are similar to those of petrochemical polymers such as polypropylene, PHAs have been studied intensively by academia and industry and are considered good candidates for biodegradable plastics and elastomers

(Anderson, 1990; Poirier, 1995). The synthesis of PHAs requires the enzyme PHAs synthase (*phaC*), which uses ß-hydroxyacyl-coenzyme as a substrate for polymerization. The production of such substrates can occur by a variety of pathways (Madison, 1999), including the simplest using the enzymes ß-ketothiolase (encoded by *phaA*) and acetoacetyl-CoA reductase (encoded by *phaB*), ß-oxidation (Page, 1995), and a fatty acid *de novo* synthesis pathway (Rehm, 1998).

In this research, *B. subtilis* was used as host because it offered advantages such as short generation time, absence of endotoxin, and secretion of amylases and proteinases that utilize food wastes for nutrients, reducing the cost of production of polyhydroxyalkanoates (PHAs). Moreover, secretion of proteins may circumvent the formation of inactive inclusion bodies, which occurs during the overexpression of foreign genes in *E. coli*. Furthermore, *B. subtilis* is not a human pathogen and can be considered biologically safe. Therefore, *B. subtilis* has become an attractive alternative to *E. coli* as a host for the expression of foreign genes.

Recently, there had been growing interest in P (HB-co-mcl HA) because it had more excellent physical chemical character. In this study, we first reported the expression of the *phaC1* gene from *Ps. aeruginosa* responsible for mcl PHAs

synthesis in *B. subtilis* DB104. The resulting recombinant strain *B. subtilis*/pBE2C1 showed P (HD-co-HDD) copolymer accumulation in *B. subtilis* DB104.

In order to synthesize the P (HB-co-mcl HA) polymer in *B. subtilis*, the *phaC1* gene was further coexpressed with *phaAB* gene from *R. eutropha* responsible for PHB synthesis in *B. subtilis* DB104. Our research result suggested that the resulting recombinant strain *B. subtilis*/pBE2C1AB could produce P (HB-HD-HDD) polymer. Our research results showed promise of significant improvement in PHAs production.

4.2 Construction of the Recombinant Strain

4.2.1 Construction of recombinant plasmids

Plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). The isolated plasmids and digested DNA fragments were analyzed by electrophoresis in horizontal slab gels containing 0.7 % (w/v) agarose, and a 1 kb DNA ladder (promega, Madison, WI) was used as standard marker. DNA restriction fragments were isolated from the agarose gel using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA). Restriction enzymes and T4 DNA ligase (Promega) were used according to the instructions provided by the supplier.

A 1.8 kb *Bam* HI/*Xba* I restriction fragment containing *phaC1* gene obtained from plasmid pBHR71 was used as an insert, and plasmid pBE2 was employed as vector (Fig. 4.1).

A 2.5 kb *Pst* I restriction fragment containing *phaAB* gene obtained from plasmid pJM9131 was used as an insert, and plasmid pBE2C1 was employed as vector (Fig. 4.2).

Ligation products were mixed with competent cells to obtain the recombinant plasmids. The positive plasmids were proved by restriction digestion followed by agarose gel electrophoresis (Fig. 4.3). The size of the vector pBE2 is around 6.2 kb and the inserts around 1.8 kb (*phaC1* gene) and 4.3 kb (*phaC1AB* gene).

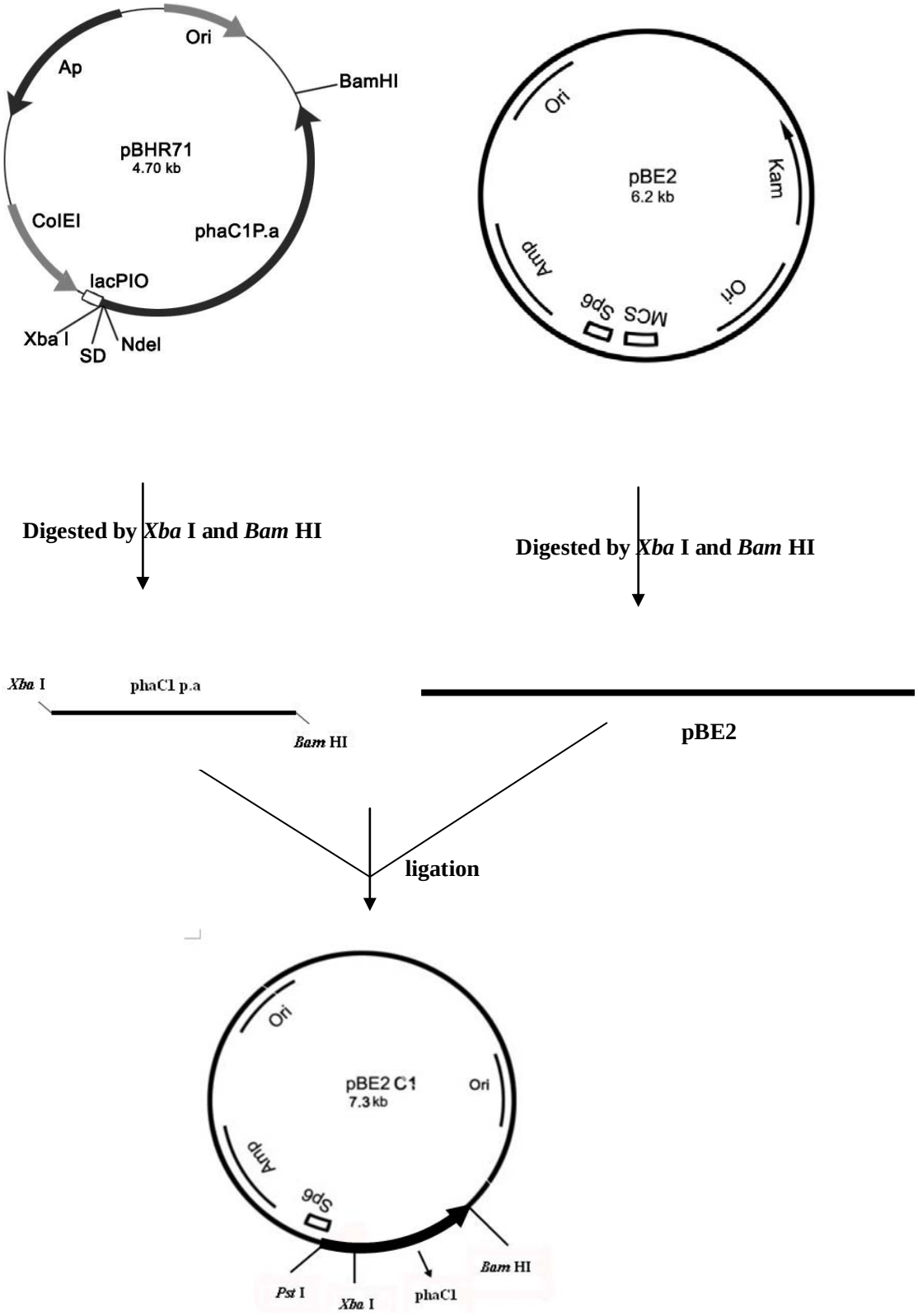


Fig. 4. 1 Construction of plasmid pBE2C1

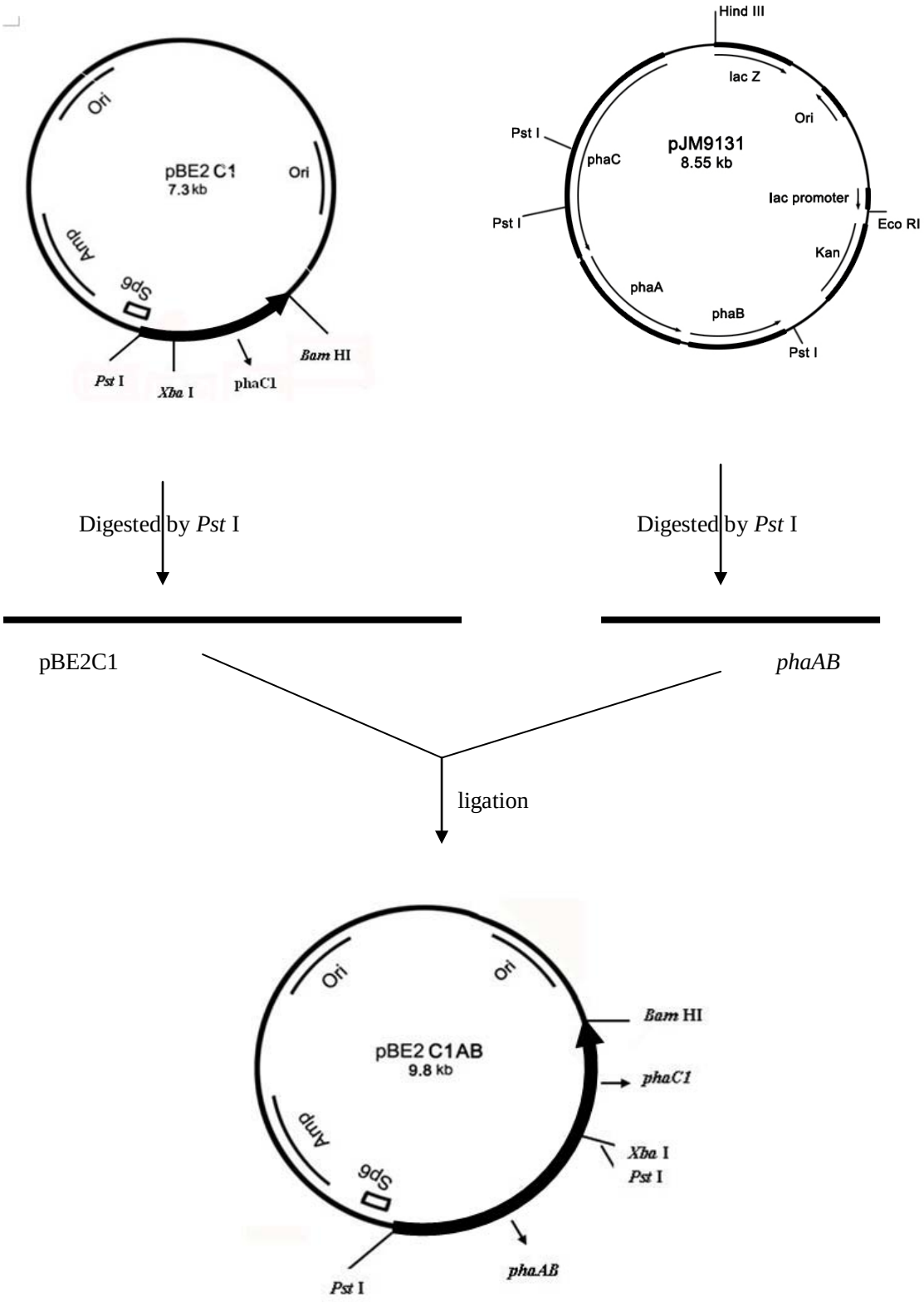


Fig. 4. 2 Construction of plasmid pBE2C1AB

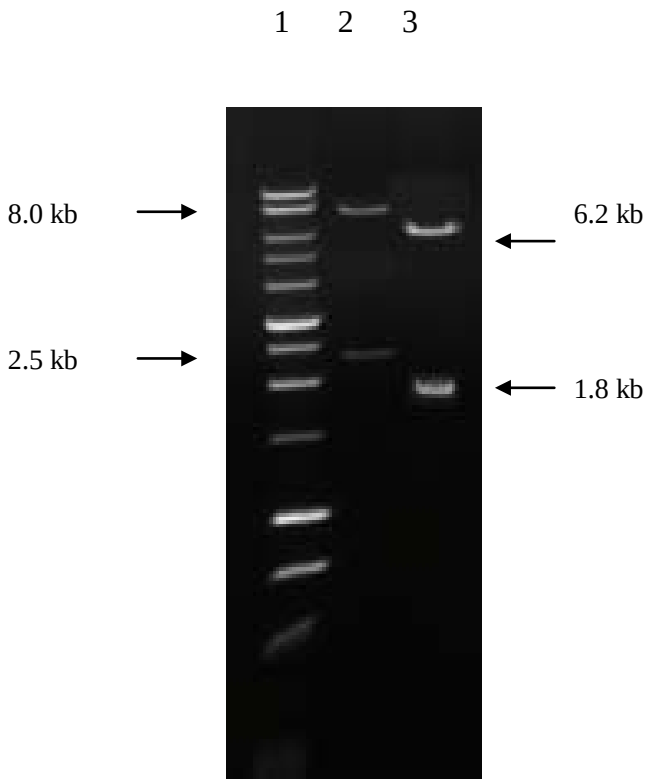


Fig. 4. 3 Agarose gel electrophoresis

1. DNA ladder 2. pBE2C1AB/*Pst* I 3. pBE2C1/ *Xba* I and *Bam* HI

4.2.2 Construction of recombinant strains

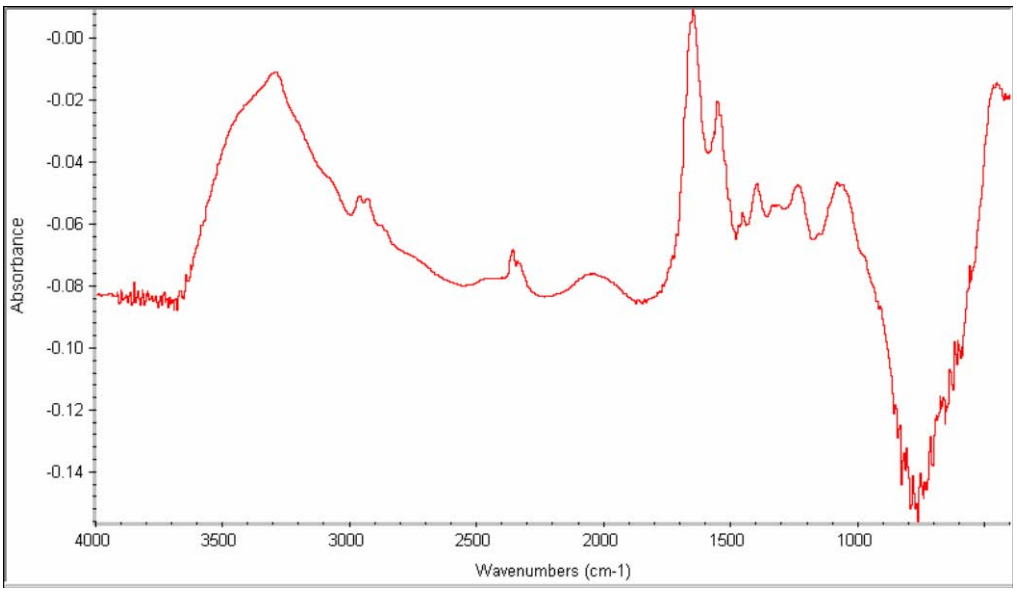
The recombinant plasmid pBE2C1 was obtained by using a 1.8 kb *Bam* HI/*Xba* I restriction fragment *phaC1* gene from *Ps. aeruginosa* obtained from plasmid pBHR71 as an insert, and plasmid pBE2 as vector. The recombinant plasmid pBE2C1AB was obtained by using a 2.5 kb *Pst* I restriction fragment containing the *phaAB* genes obtained from plasmid pJM9131 as an insert, and plasmid pBE2C1 as vector. The constructed plasmids were confirmed by restriction digestions and agarose gel electrophoresis. The newly cloned plasmids were amplified in *E. coli*. The size of the pBE2C1 is around 8.0 kb and the size of the pBE2C1AB is around 10.5 kb. These two newly constructed plasmids were transformed into *B. subtilis.* The positive transformants were checked by restriction digestions followed by agarose gel electrophoresis. These result showed that the foreign fragment has been successfully inserted into the plasmids.

4.3 Gene Expression in the Recombinant Strains

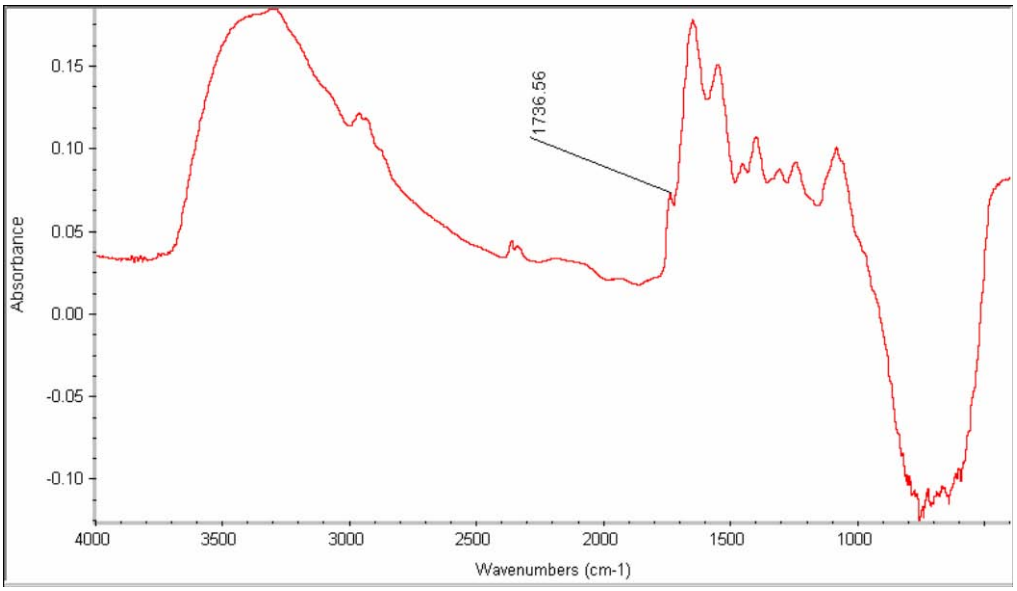
4.3.1 FTIR analysis of the recombinant strains

The culture was incubated at 28 °C and shaken at 280 rpm. Two milliliter of the cell culture was centrifuged at 2,610 *g* for 15 min. The cells were transferred onto IR window (ZnSe Disc, Spectratech) and dried on it. A mirror was used to give the reflected infrared signal to the horizontally laid window. The PHAs peak was observed at wavenumbers about 1726–1740 cm^{-1}.

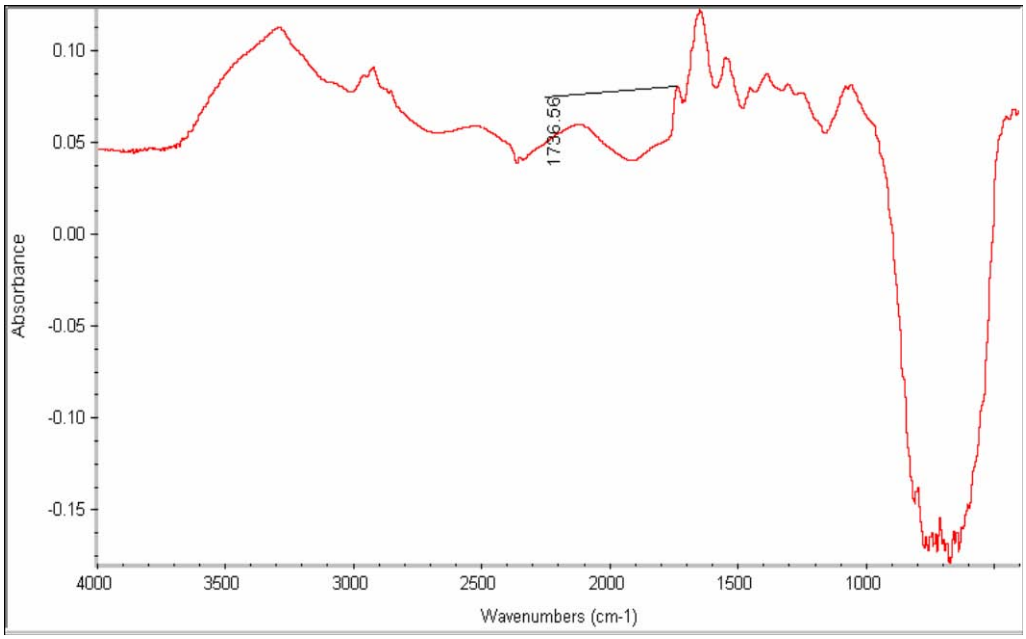
From Fig. 4.4, we knew that there was no 1726–1740 cm^{-1} peak in Fig. (a), so there was no PHAs accumulation in the host strain *B. subtilis*. But there were obvious 1726–1740 cm^{-1} peak in Fig (b) and Fig (c), which showed that there were PHAs accumulations in these two recombinants---*B. subtilis*/pBE2C1 and *B. subtilis*/pBE2C1AB. The result suggested that the recombinant strains *B. subtilis*/pBE2C1 and *B. subtilis*/pBE2C1AB could produce PHAs. Therefore the *pha* gene was expressed in the host strain *B. subtilis*.



(a) The negative control of the host strain-*B. subtilis*



(b) The FTIR result of recombinant *B. subtilis*/pBE2C1



(c) The FTIR result of recombinant *B. subtilis*/pBE2C1AB

Fig. 4. 4 The FTIR results of the recombinants

4.3.2 GC analysis of the recombinant strains

After fermentation, the cells were freeze-dried. One milliliter esterification solution, 15 mg freeze-dried cells and 1 ml chloroform were mixed and heated at 100 °C for 4 hours, 1 ml ddH_2O was added to the cooled mixture, which was then vortexed for phase separation. One microliter portion of the lower organic phase was subjected to GC analysis, which was performed on a Hewlett Packard 5890 Series II Gas Chromatograph, using a 6 ft Supelco (10 % Carbowax 20 M with 80/100 in mesh size Chromosorb WAW) Packed Column. Nitrogen was used as the carrier gas at the flow rate of 20 ml/min. The analysis was started at 135 °C and the temperature was kept stable for 10 min to determine both the content and composition of the polymer.

The result was showed in Fig.4.5. The data was analyzed by two-sample t-test. There was no significant difference at the beginning, but there was a significant difference at 0.01 level of significance after 25 hours. From Fig. 4.5, we knew that the PHAs was accumulated PHAs from the 8th hour and the content of the PHAs of the recombinant strain *B. subtilis*/pBE2C1AB was the same as that of

the recombinant strain *B. subtilis*/pBE2C1 at the beginning. The PHAs produced by the recombinant strain *B. subtilis*/pBE2C1AB was much higher than that by the recombinant strain *B. subtilis*/pBE2C1 after 25 hours. These showed that the foreign genes were expressed in the host. The expression in the recombinant strains is controlled by the promoter SP6 of pBE2.

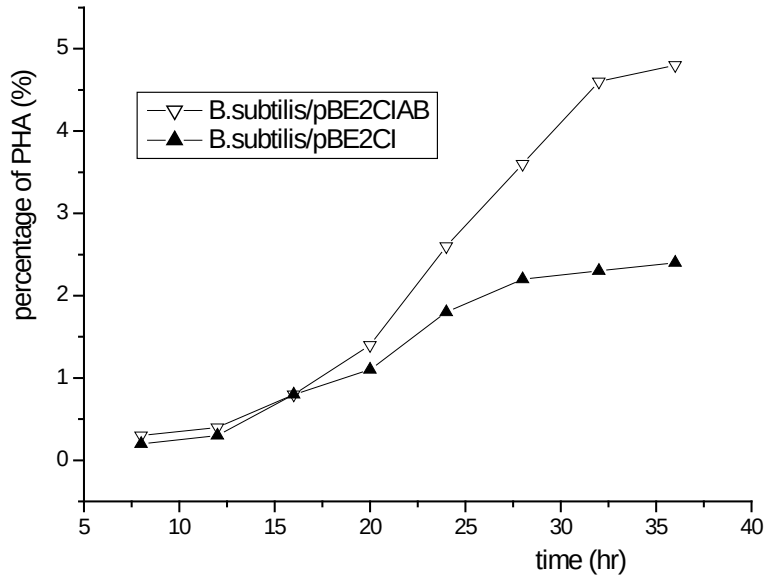


Fig. 4. 5 PHAs production in recombinants after cultivated for different time

We had known that there was no PHAs accumulation in the host strain *B. subtilis* DB104. However, *B. subtilis*/pBE2C1 and *B. subtilis*/pBE2C1AB, recombinants containing *pha* genes, showed PHAs accumulation---- the first reported expression of *pha* genes from *Ps .aeruginosa* and *R. eutropha* into *B. subtilis*. As

shown in Fig.4.5, PHAs production by the recombinant *B. subtilis*/pBE2C1AB is higher than that by *B. subtilis*/pBE2C1.

The PHAs produced by these two recombinant strains reached maximum at the 36th hours, which were 4.8 % and 2.4 %, respectively. GC analysis further identified the product synthesized by *B. subtilis*/pBE2C1 to be a HD-HDD polymer which was a mcl-PHAs and contain 10 C and 12 C polymers while the product synthesised by *B. subtilis*/pBE2C1AB was identified to be a HB-HD-HDD polymer which was scl- co-mcl-PHAs containing 4 C, 10 C and 12 C polymers (Table 4.1).

Table 4. 1 Monomer composition of the PHAs accumulated by recombinant strains

strains	HB mol%	HD mol%	HDD mol%
B. subtilis/pBE2C1	0	58±3	42±3
B. subtilis/pBE2C1AB	16±2	62±3	22±3

Values are means ± S.D. (n=4)

The ratio of the HD and HDD monomer in the PHAs produced by the

recombinant strain *B. subtilis*/pBE2C1 was about to 1:1. While the ratio of the HD and HDD monomer in the PHAs produced by the recombinant strain *B. subtilis*/pBE2C1AB was about to 3:1. And the ratio of the HB, HD and HDD monomer in the PHAs produced by the recombinant strain *B. subtilis*/pBE2C1AB was about to 1:3:1.

The *B. subtilis*/pBE2C1 and *B. subtilis*/pBE2C1AB carrying *pha* gene were subjected to fermentation and showed PHAs accumulation, providing the first evidence that the *pha* gene from *Ps. aeruginosa* and *R. eutropha* can be expressed in *B. subtilis*.

4.4 Cell Dry Weight (CDW) and C: N Ratio in the Fermentation

4.4.1 CDW in the fermentation

These two recombinant strains were cultured in the fermentation medium for 36 hours. The fermentation culture samples were centrifuged at 4000 rpm for 20 minutes and the cells were washed by ddH_2O twice. Then, they were filtered through the dried and pre-weighted 0.45 μm pore member filter and dried at 100 °C for 2-3 hours. The filter paper together with the biomass was weighted to obtain the CDW. The CDW of these two recombinant strains were showed in Fig. 4.6. The data was analyzed by two-sample t-test. There was no significant difference.

From Fig. 4.6, we knew that the CDW of these two recombinants reached the peak at the 32nd hours, and it was about to 3 g/L.

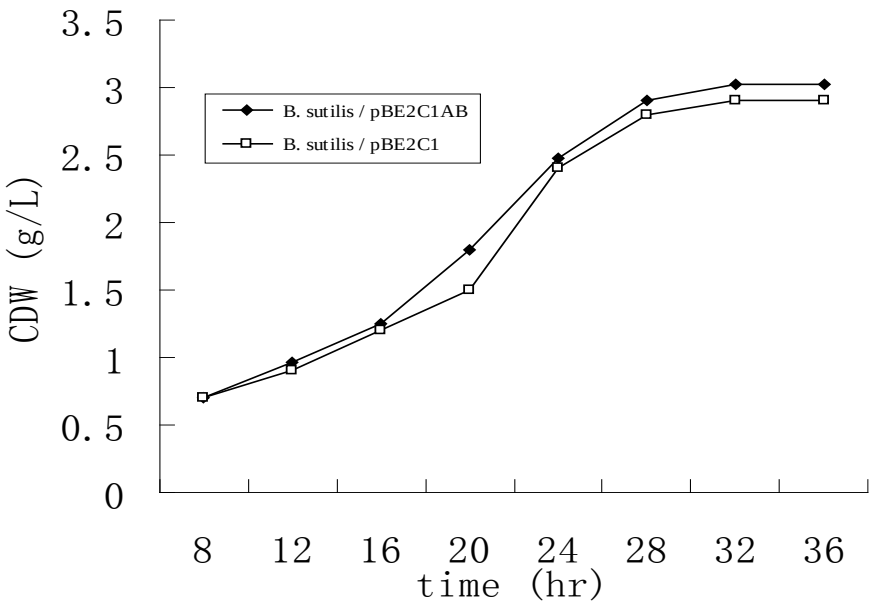


Fig. 4. 6 CDW of the recombinants after cultivated for different time

4.4.2 C: N ratio in the fermentation

After the fermentation, the mixture was centrifuged at the 4,000 rpm for 20 min. The deposit was subject to freezing dry, the supernatants were subject to measure the concentration of the C and N.

For TOC, the supernatants were diluted by adding ddH_2O. Then, 5 ml of the

diluted supernatants were put into 2 TOC tubes for each. 2 blank ddH_2O were prepared for correction. Finally, the sample tubes were put into the Auto sampler of TOC Analyzer.

For TKN, the supernatants of the fermentation culture were diluted by adding ddH_2O. 50 ml diluted sample were put into kjeldahl tube. Two K_2SO_4 tablets with mercury oxide and 10 ml H_2SO_4 was then added in the tube. The tube was heated at 250 °C for 120-150 minutes until the mixture turned black and then got clear greenish blue in color. After cool down, 70 ml of ddH_2O was added to the tube to carry out crystallization. The nitrogen in different samples was distillated by using 50 ml 40 % NaOH. The nitrogen was dissolved in distillate and the distillate was received in 10 ml receiving solution. The nitrogen was titrated against the 0.0023 N HCl. The TKN was calculated after the deduction of blank.

The results were showed in Fig. 4.7. The concentrations of the carbon and nitrogen were decreased during the fermentation. The concentration of the carbon was decreased calmer than that of the nitrogen. The concentration of the nitrogen was decreased acutely at the 28th hours. So the maximum of the PHAs accumulation was at the end of the fermentation, which was according to the condition of the PHAs production.

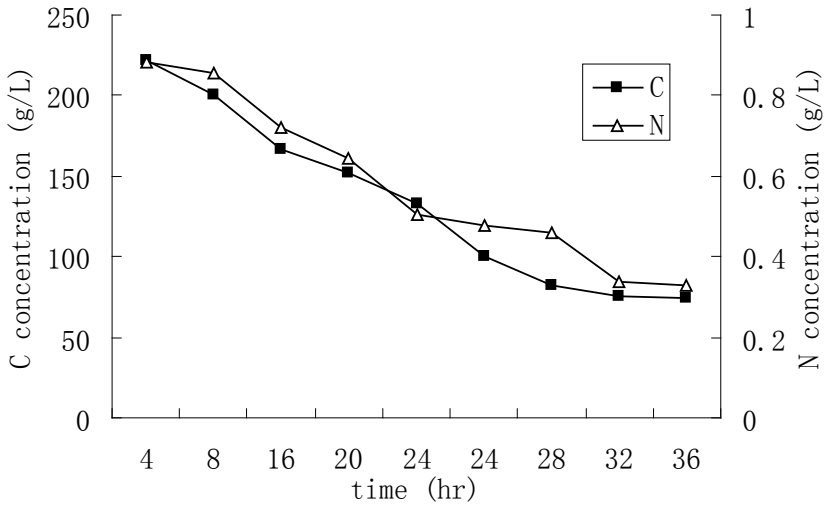


Fig. 4. 7 C: N concentration of the recombinants after cultivated for different time

4.5 Cultivation of Recombinant *B. subtilis* in Hydrolyzed Malt Waste

Malt waste, mostly semisolids of spent barley and millet refuse was obtained from Carlsberg Company, a Hong Kong beer brewery. Eighty grams waste was hydrolyzed with 1 L 0.6 M HCl. The mixture was incubated at 121 °C for 30 min at evaluated pressure (1 kgf/cm^2). The resultant mixture was neutralized, centrifuged and filtered. The recombinant *B. subtilis* was cultivated by acid hydrolyzed malt waste and compared to the fermentation medium, in order to observe the ability of the strain in utilization of malt waste.

The hydrolyzed malt waste was diluted with fourth volume of ddH_2O; the growth of the recombinant *B. subtilis*/pBE2C1AB in hydrolyzed malt waste was compared with the fermentation medium.

The fermentation mixture was centrifuged at 4,000 rpm for 20 min. The deposit was subject to freezing dry, and we got the dry cell to GC. The CDW of the recombinant in the malt waste medium was 2.1 g/L, while the CDW of the

recombinant in the fermentation medium was 1.9 g/L. The data was analyzed by two-sample t-test; there is a statistical significance at the 0.01 level. The results of CDW showed that the recombinant *B. subtilis/*pBE2C1AB was better growth in malt waste medium than that in the fermentation medium (Fig. 4.8).

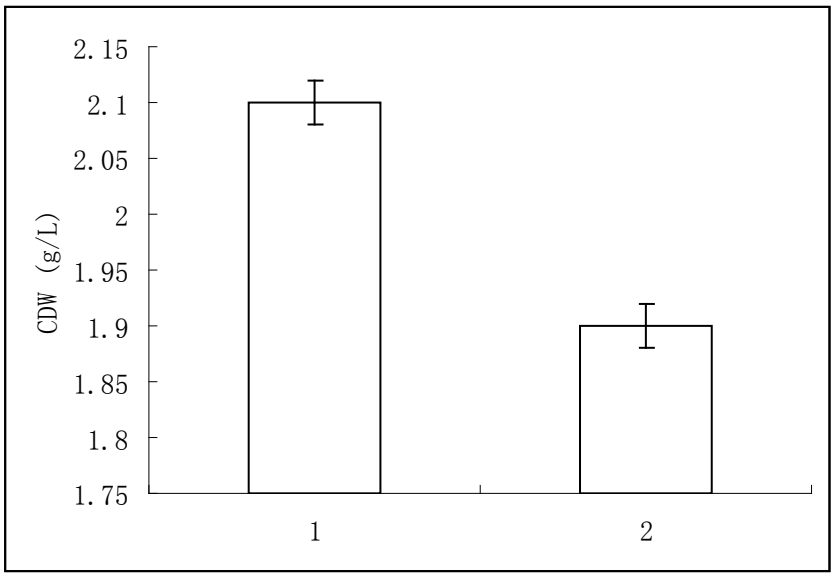


Fig. 4. 8 The CDW of the recombinant in the different medium

1. Malt waste medium 2. Fermentation medium

The data was analyzed by two-sample t-test. As P-Value < 0.01, there is a significant difference.

Carbon source is the major contributor to the total substrate cost (up to 50 % of the total operating cost). The well utilization of the malt waste medium can greatly reduce the cost for PHAs production. The PHAs accumulation of the recombinant *B. subtilis/* pBE2C1AB in malt waste medium was 6.7 % of the

CDW, the PHAs accumulation of the recombinant in fermentation medium was 4.8 %. The GC result showed that the recombinant *B. subtilis/*pBE2C1AB could produce more PHAs in malt waste medium than that in the fermentation medium (Fig. 4.9). The data was analyzed by two-sample t-test; there is a statistical significance at the 0.01 level. GC analysis further identified the product synthesized by *B. subtilis*/pBE2C1AB was to be a HB-HD-HDD polymer, which was also a scl-co-mcl PHAs.

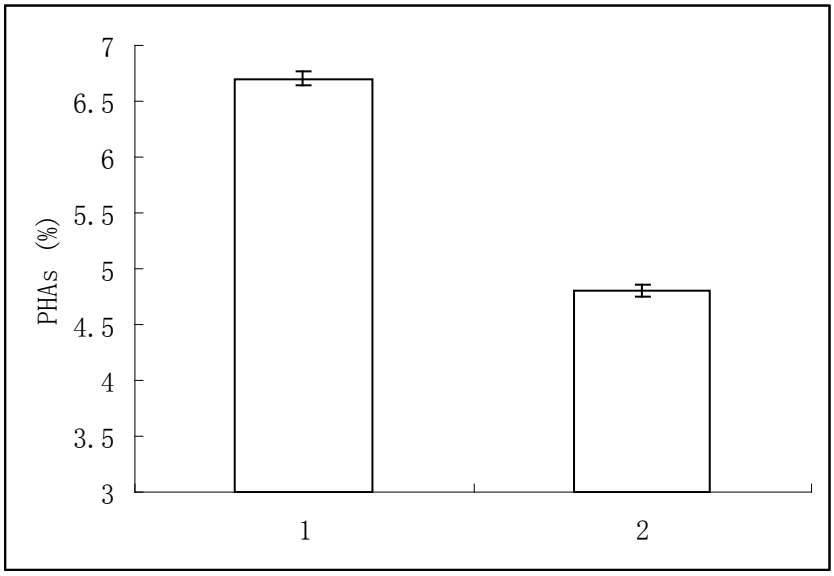


Fig. 4. 9 The PHAs of the CDW in the different medium

1. Malt waste medium 2. Fermentation medium

The data was analyzed by two-sample t-test. As P-Value < 0.01, there is a significant difference.

4.6 Discussion

This work was the first report to demonstrate the expression of the mcl-PHA synthase gene obtained from *Ps. aeruginosa* into *B. subtilis*. And the recombinant strains successfully produced mcl-PHA. *Ps. aeruginosa* can synthesize mcl-PHA by ß-fatty acid oxidation which uses fatty acid as substrate, and by the de novo fatty acid synthesis pathway which uses simple molecules such as glucose as substrate, but production of the latter is lower. In this work, the medium incorporated glucose as a substrate, so the production of the PHAs is low. Some mcl fatty acids can be added in future experiments to improve the production of the PHAs.

As the results show, the recombinant *B. subtilis*/pBE2C1 and *B. subtilis*/pBE2C1AB can synthesize monomer mcl-PHA, which accords with the character of PHAs synthase gene *phaC1*. In order to synthesize P (HB-co-mcl HA), whose physical-co-chemical character was more excellent than PHB or mcl PHA in *B. subtilis*, the ß-ketothiolase gene (*phbA*) and acetoacetyl-CoA reductase gene (*phbB)* from *R. eutropha* were subcloned into plasmid pBE2C1 to obtain

plasmid pBE2C1AB. The recombinant *B. subtilis*/pBE2C1AB was found to produce PHB (Kichise, 2002).

Bacillus strain was used as industrial production organisms. *B. subtilis* was a Gram-positive non PHAs accumulation but is easy to grow at very high cell density when utilizing inexpensive carbon and nitrogen sources. It was the best studied member of the genus Bacillus and well characterized in both genetics and physiology. It was generally recognized as nonpathogenic and did not produce endotoxins.

B. subtilis expression system was shown to be good for protein overproduction. The expression in the recombinant *B. subtilis* without heat shock was controlled by the native promoter of SP6 in vector pBE2. The recombinant *B. subtilis* had the PHAs accumulation after fermentation. The use of recombinant *B. subtilis* together with inexpensive carbon source such as malt waste as culture media in our research would make PHAs production economical and allow easier commercialization of PHAs.

CHAPTER 5

PCR CLONING OF PHAs SYNTHASE GENE AND CONTROL PHAs PRODUCTION

5.1 Introduction

Polyhydroxyalkanoic acids (PHAs) represent a rather complex class of polyesters that are synthesized by most genera of bacterial. Most of these prokaryotes synthesize PHB and other PHAs as storage compounds and deposit these polyesters as insoluble inclusions in the cytoplasm. Since they are biodegradable, they are considered for several applications in the packaging industry or as raw materials for the synthesis of pure chemicals and the production of paints. Many prokaryotic and eukaryotic organisms are able to produce low molecular weight

PHB molecules that are complex with other biomolecules and that occur at concentrations which are three to four orders of magnitude less than storage PHAs in the cells.

The synthesis of PHAs required the enzyme PHAs synthase, which are the key enzymes of PHAs biosynthesis. The synthase used hydroxyacyl-coenzyme A substrates for polymerization. The production of such substrates can occur by a variety of pathways, including the simplest using the enzymes ketothiolase and acetoacetyl-CoA reductase, oxidation, and a fatty acid synthesis pathway. The synthase use coenzyme A thioesters of HA as substrates and catalyze the polymerization of HAs into PHAs with the concomitant release of CoA.

So far, after the cloning of the PHAs synthase operon of *R. eutropha*, as much as 42 different kinds of PHAs synthesis operons had been cloned and analyzed from a variety of bacteria, the results reveal that the proteins required for PHAs biosynthesis pathways had diverged considerably. Nevertheless, PHAs synthase is crucial enzyme in all PHAs synthesis pathways.

In this section, the polyhydroxyalkanoate synthase gene pha, from *Bacillus thuringiensis* HD-81, was cloned using PCR cloning strategy based on the pha loci property. The complete open reading frames (ORFs) were identified from the

PCR products. Using the sequence information, the complete PHAs synthase gene was PCR cloned directly from the genomic DNA and expressed in *E. coli* confirmed by FTIR and GC. The control operating conditions of CDW and PHAs production were also studied.

5.2 Construction Recombinant Strain by Genetic Engineering

5.2.1 PCR reaction for new PHAs synthase gene

5.2.1.1 PCR primer design

Primer was designed based on multiple sequence alignment result. In this section, the primers in the cloning strategy were designed according to the homologous region of ORF, the PHAs biosynthesis gene loci.

The primers were as follows,

5' TGGGGATCCTTAATTAGAACGCTCTTCAA 3'

5' GGTGAGCTCTTACTTGATGGAAGTAAATA 3'

5.2.1.2 PCR reaction

Purified genomic DNA was used as template and PCR technology was applied to obtain the preferred product and minimize the artifact products and primer. After the 5 minutes of initial denaturation at 94 °C, the general denaturation was 1 minute, the annealing elongation temperature was 50 °C for 1 minute and the elongation temperature was 72 °C for 2 minutes, there were 30 cycles and the last elongation step was 10 minutes at 72 °C. The expected fragments were around 3 kb for the pha gene product.

5.2.1.3 Gene sequence

The cloning vector pGEMT was used for the ligation of PCR products and the obtained plasmid was sequenced.

The gene and amino acid sequences were list at appendix.

5.2.2 Construction of the recombinant plasmid and strain

The PHAs synthase gene in the *B. thuringiensis* HD-81 isolated by using PCR

was ligation into the plasmid pGEMT. Ligation products were mixed with competent cells to obtain the recombinant plasmids. The positive plasmids were proved by restriction digestion followed by agarose gel electrophoresis. The size of the vector pGEMT is around 3 kb and the inserts around 3 kb (*pha* gene).

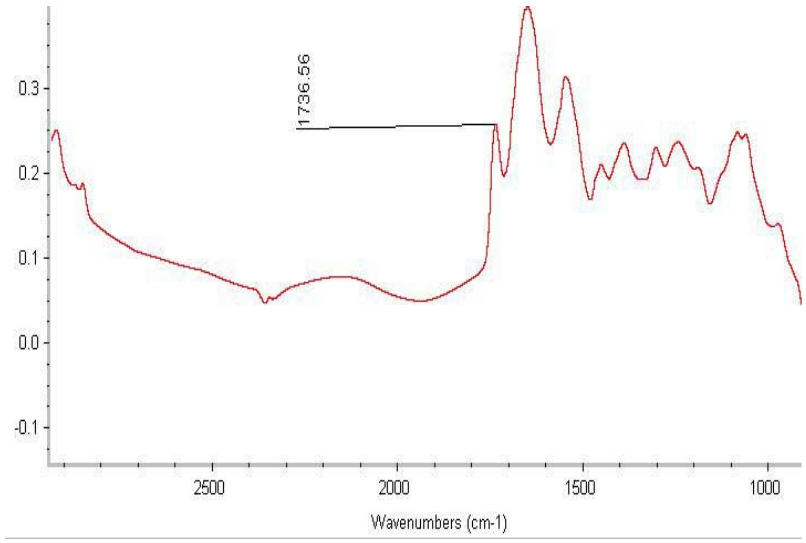
The constructed plasmid pGEMT-pha was confirmed by restriction digestions and agarose gel electrophoresis. The newly cloned plasmids were transformed in *E. coli* for further research. The positive transformants were checked by restriction digestions followed by agarose gel electrophoresis. The recombinant strain was named *E. coli/ pGEMT-pha.*

5.3 Gene Expression in the Recombinant Strain

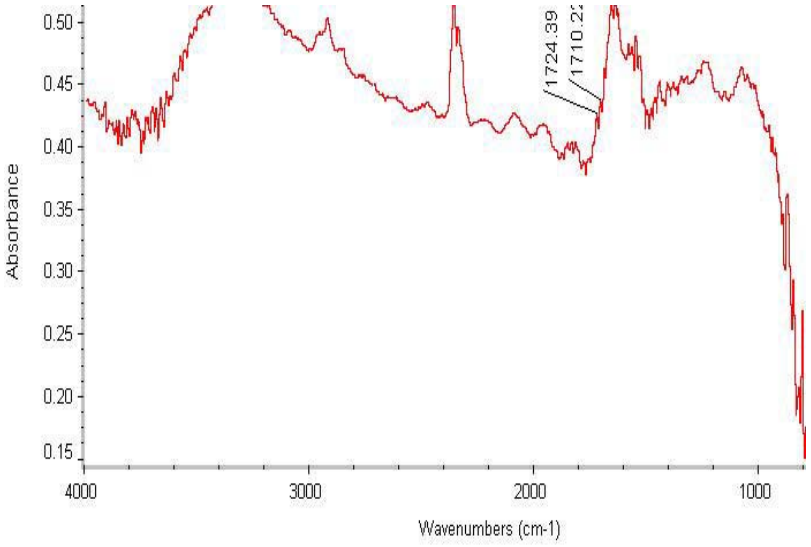
5.3.1 FTIR analysis of the recombinant strain

The culture was incubated at 37 °C and shaken at 280 rpm. Two milliliter of the cell culture was centrifuged. The cells were transferred onto IR window (ZnSe Disc, Spectratech) and dried on it. A mirror was used to give the reflected infrared signal to the horizontally laid window. The PHAs peak was observed at wavenumbers about 1726–1740 cm^{-1}.

From Fig. 5.1, we knew that there was 1726–1740 cm^{-1} peak in Fig. (a), so there was PHAs accumulation in the donor strain *B. thuringiensis*. That was to say, the donor strain *B. thuringiensis* had the PHAs synthase gene. There was also obvious 1726–1740 cm^{-1} peak in Fig (b), which showed that there were PHAs accumulations in the recombinants *E. coli/ pGEMT-pha*. The result suggested that the recombinant strains *E. coli/ pGEMT-pha* could produce PHAs. Therefore the *pha* gene was cloned and expressed in the host strain *E. coli*.



(a) *B. thuringiensis* showed PHAs accumulation by the FTIR



(b) *E. coli pGEMT-pha* showed PHAs accumulation by the FTIR

Fig. 5. 1 The FTIR results of the recombinants

5.3.2 GC analysis of the recombinant strain

After fermentation, the cells were freeze-dried. One milliliter esterification solution, 15 mg freeze-dried cells and 1 ml chloroform were mixed and heated at 100 °C for 4 hours, 1 ml ddH_2O was added to the cooled mixture, which was then vortexed for phase separation. One microliter portion of the lower organic phase was subjected to GC analysis, which was performed on a Hewlett Packard 5890 Series II Gas Chromatograph, using a 6 ft Supelco (10 % Carbowax 20 M with 80/100 in mesh size Chromosorb WAW) Packed Column. Nitrogen was used as the carrier gas at the flow rate of 20 ml/min. The analysis was started at 135 °C and the temperature was kept stable for 10 min to determine both the content and composition of the polymer.

The PHAs percentage was calculated by comparison between the peak area of PHAs standard and the peak of the extracted biopolymers. The peak of benzoic acid was the reference.

Table 5. 1 Monomer composition of the PHAs accumulated

	HB %	HD %	HDD %	PHAs %
E. coli/ pGEMT-pha	9.14±1.28	1.77±0.23	1.43±0.15	12.34±1.51

Values are means ± S.D. (n=4)

We had known that there was no PHAs accumulation in the host strain *E. coli*. However, *E. coli/ pGEMT-pha*, recombinants containing *pha* genes, showed PHAs accumulation.

GC analysis further identified the product synthesized by *E. coli/pGEMT-pha* to be a HB-HD-HDD polymer which was scl-co-mcl-PHAs containing 4 C, 10 C and 12 C polymers. PHAs accumulated by the recombinant strain *E. coli pGEMT-pha* reached 12.34 %, the HB, HD and HDD monomer percentage of the PHAs was 9.14 %, 1.77 % and 1.43 %, respectively.

The *E. coli/pGEMT-pha* carrying *pha* gene was subjected to fermentation and showed PHAs accumulation, providing the first evidence that the *pha* gene from *B. thuringiensis* can be expressed in *E. coli*.

5.4 Factors Affecting of CDW and PHAs Production

5.4.1 Preparation of the fermentation medium

5.4.1.1 Effect of carbon-nitrogen ratio

Four C: N ratios, 20:1, 50:1, 80:1 and 100:1 were tried to investigate the effect of carbon content on the productivity of PHAs using recombinant *E. coli/pGEMT-pha*.

Table 5. 2 PHAs production by *E. coli/pGEMT-pha* using different C: N ratios

Proposed C:N	Required C substrate (g/L)
20:1	2.72
50:1	6.99
80:1	11.27
100:1	14.12

5.4.1.2 Effect of carbon substrates

Many carbon substrates, sodium acetate, glucose, fructose, sucrose, citrate, propionate, octanoic acid, nonanoic acid, decanoic acid, lauric acid and benzoic acid were tried to analyze its effect on PHAs production. C: N ratio was kept at 50:1 for all samples. Recombinant *E. coli/ pGEMT-pha* was the producer.

5.4.1.3 Effect of citric acid

Two concentrations of citric acid (0.5 g/L, 2 g/L) were tried to analyze its effect on PHAs production. C: N ratio was kept at 50:1 for all samples and glucose was used as the carbon source for cultivating recombinant *E. coli/ pGEMT-pha*.

5.4.1.4 Effect of yeast extract

Two concentrations of yeast (0.5 g/L, 2 g/L) were tried to analyze its effect on PHAs production. C: N ratio was kept at 50:1 for all samples and glucose was

used as the carbon source for cultivating recombinant *E. coli/pGEMT-pha*.

5.4.1.5 Effect of tryptone

Two concentrations of tryptone (0.5 g/L, 2 g/L) were tried to analyze its effect on PHAs production. C: N ratio was kept at 50:1 for all samples and glucose was used as the carbon source for cultivating recombinant *E. coli/ pGEMT-pha*.

5.4.2 Effect of carbon-nitrogen ratio

Recombinant *E. coli/pGEMT-pha* was observed to yield higher cell dry weight (CDW) and PHAs contents.13.4 % of PHAs was the highest recording among the results when C: N reached 50:1. The highest CDW observed was 5.156 g/L when the recombinant *E. coli/pGEMT-pha* was supplied with glucose at C: N ratio 100:1.

Table 5. 3 CDW and PHAs produced by the recombinant *E. coli/ pGEMT-pha* under different C: N ratios

C:N	CDW (g/L)	PHAs (%)
20:1	3.957±0.15	9.12±0.31
50:1	4.668±0.11	13.4±0.56
80:1	4.511±0.17	9.03±0.22
100:1	5.156±0.13	7.15±0.32

Values are means ± S.D. (n=4)

From the recombinant *E. coli/pGEMT-pha*, 1 L of water sample could produce 4.668 g of cell dry weight (CDW) which contained 13.4 % of PHAs. In other words, 4.668(0.134) = 0.626 g PHAs /L was recovered.

5.4.3 Effect of carbon substrates

The cell dry weight and PHAs of the samples using sodium acetate and glucose were higher than that of others. These could be explained by the fact that the

simpler molecular structure of the substrate eases its consumption by bacteria. Hence the use of complex aromatic benzoic acid prohibits the growth of polymer. The CDW of 5.802 g/L and 14.91 % PHAs, which was the highest yield in this research, were obtained when sodium acetate was the substrate.

From the recombinant *E. coli*, 1 L of water sample could produce 5.802 g of cell dry weight (CDW) which contained 14.91 % of PHAs. In other words, 5.802(0.1491) = 0.865 g PHAs /L was recovered.

Table 5. 4 CDW and PHAs produced under different carbon substrates

Substrate	CDW (g/L)	PHAs (%)
Sodium acetate	5.802±0.09	14.91±1.8
Glucose	4.266±0.07	14.82±1.67
Benzoic acid	1.398±0.02	3.68±0.53
Fructose	0.7±0.01	-
Sucrose	1.2±0.02	9.13±1.01
Propionate	0.6±0.01	-
Octanoic acid	0.67±0.01	2.08±0.33
Nonanoic acid	0.55±0.01	2.24±0.53
Decanoic acid	0.87±0.01	-
Lauric acid	0.45±0.01	2.10±0.48
Fructose	0.77±0.01	-

Values are means ± S.D. (n=4)

5.4.4 Effect of citric acid

Citric acid could be considered as a secondary carbon source for the growth of bacteria. When 0.5 g/L of citric acid was employed, a CDW of 6.98 g/L and 16.72 % PHAs were acquired. When 2 g/L of citric acid was employed, a CDW of 6.12 g/L and 14.98 % PHAs were acquired. The data was analyzed by two-sample t-test; there is a statistical significance at the 0.01 level. The CDW and PHAs of adding 0.5 g/L citric acid were much higher than that of adding 2 g/L citric acid.

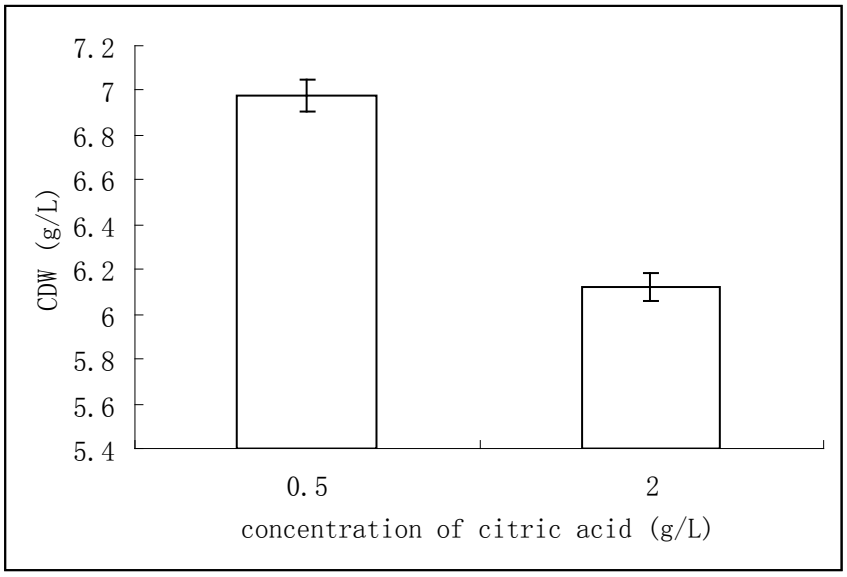


Fig. 5. 2 CDW produced under different citric acid contents

The data was analyzed by two-sample t-test. As P-Value < 0.01, there is a significant difference.

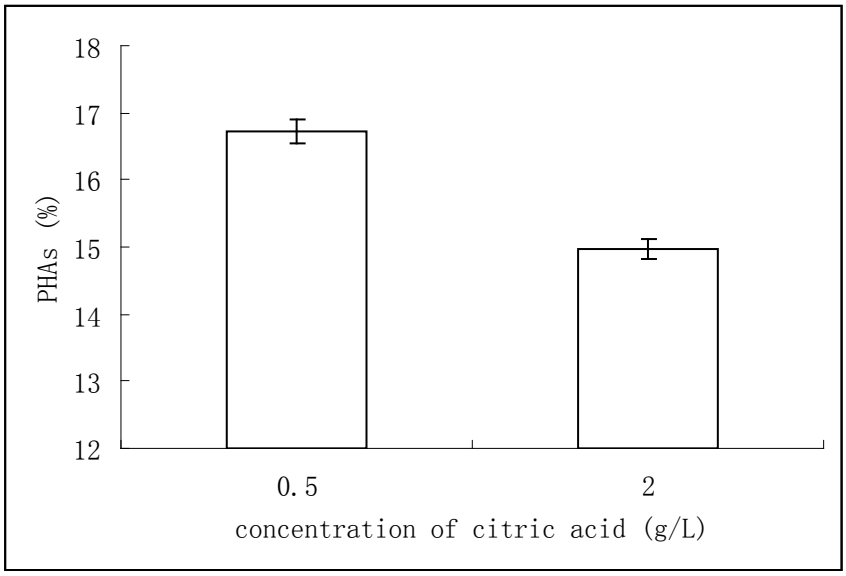


Fig. 5. 3 PHAs produced under different citric acid contents

The data was analyzed by two-sample t-test. As P-Value < 0.01, there is a significant difference.

From the recombinant *E. coli*, 1 L of water sample could produce 6.98 g of cell dry weight (CDW) which contained 16.72% of PHAs. In other words, 6.98(0.1672) = 1.17 g PHAs /L was recovered.

5.4.5 Effect of yeast extract

The function of yeast extract was to provide supplementary nutrition to the bacteria, such as proteins and vitamins. Larger amounts of yeast extract used enhanced the growth of bacteria and thus the PHAs production. 10.8 % and

9.5 % PHAs were retrieved when the content of yeast extract was 2 g/L and 0.5 g/L, respectively. The CDW recorded in this research was 8.62 g/L and 5.8 g/L, respectively. The data was analyzed by two-sample t-test; there is a statistical significance at the 0.01 level. The CDW and PHAs of adding 2 g/L yeast extract were higher than that of adding 0.5 g/L yeast extract.

From the recombinant *E. coli*, 1 L of water sample could produce 8.62 g of cell dry weight (CDW) which contained 10.8 % of PHAs. In other words, 8.62(0.108) = 0.931 g PHAs /L was recovered.

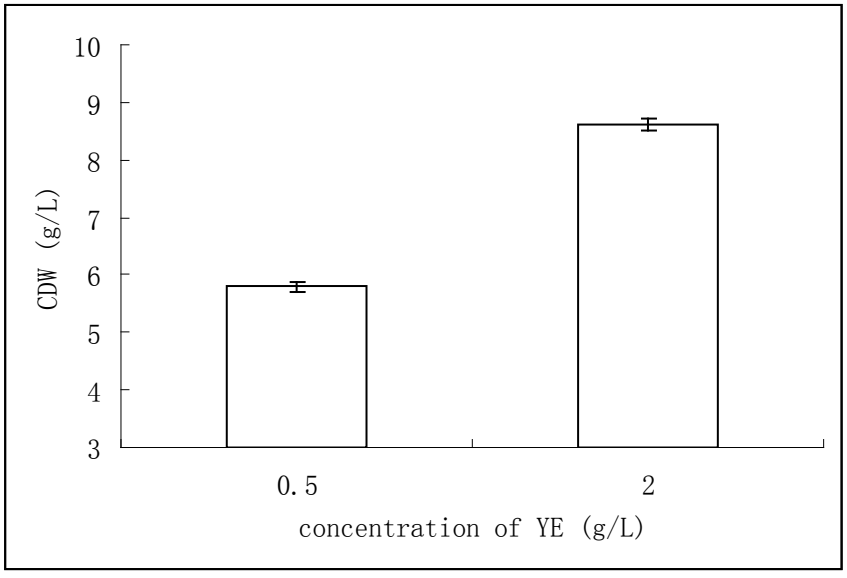


Fig. 5. 4 CDW produced under different yeast extract contents

The data was analyzed by two-sample t-test. As P-Value < 0.01, there is a significant difference.

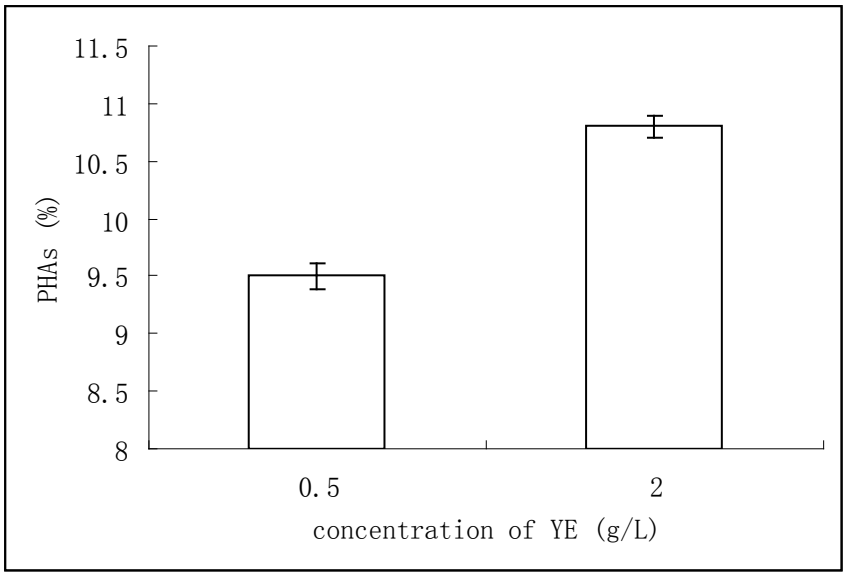


Fig. 5. 5 PHAs produced under different yeast extract contents

The data was analyzed by two-sample t-test. As P-Value < 0.01, there is a significant difference.

5.4.6 Effect of tryptone

Tryptone was principally served as a nutrition-rich medium for the growth of *E. coli*. It was a source of peptides and peptone, which contain nitrogen for bacterial cultivation. Hence the addition of tryptone decreased the carbon-nitrogen ratio. When 0.5 g/L of tryptone was added, a CDW of 7.3 g/L and 11.03 % PHAs were acquired. When 2 g/L of tryptone was added, a CDW of 6.4 g/L and 10.01 % PHAs were acquired. The data was analyzed by two-sample t-test; there is a

statistical significance at the 0.01 level. It could be seen that the sample with 0.5 g/L tryptone yields higher PHAs.

From the recombinant *E. coli*, 1 L of water sample could produce 7.3 g of cell dry weight (CDW) which contained 11.03 % of PHAs. In other words, 7.3(0.1103) = 0.81 g PHAs /L was recovered.

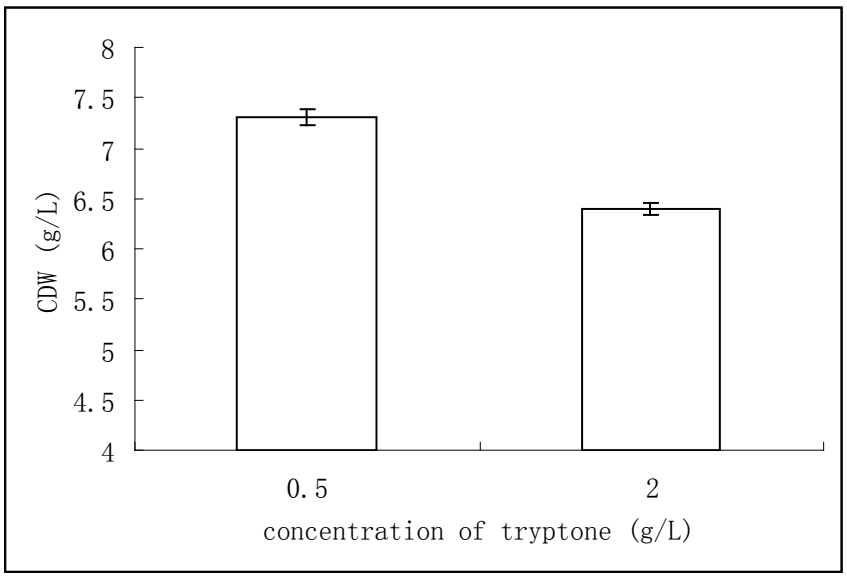


Fig. 5. 6 CDW produced under different tryptone contents

The data was analyzed by two-sample t-test. As P-Value < 0.01, there is a significant difference.

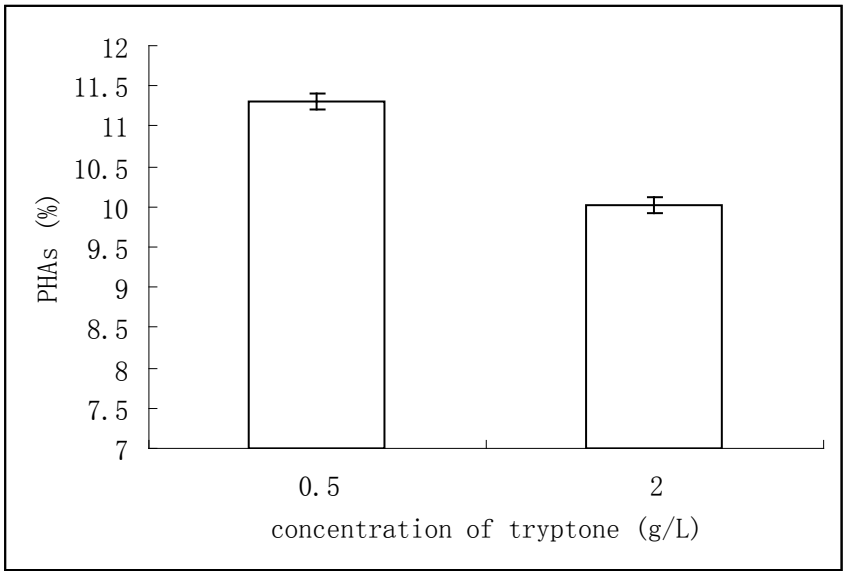


Fig. 5. 7 PHAs produced under different tryptone contents

The data was analyzed by two-sample t-test. As P-Value < 0.01, there is a significant difference.

5.5 Discussion

PHAs biosynthesis gene had been cloned and functionally expressed by various research group from a large number of different bacteria. In the section, the PHAs synthesis genes locus was cloned directly from *B. thuringiensis* genome using the PCR. This successful pha locus cloning from *B. thuringiensis* showed that the rapid PCR cloning strategy could be used for cloning Bacillus strain. The analysis of the PHAs synthase derived from the cloned PHAs synthase genes with the known PHAs synthase showed that the PHAs gene were highly homologous. The function of the PHAs synthase gene was identified by the expression the in the recombinant *E. coli/pGEMT-pha*.

It could be noticed that PHAs was the dominant polymer species produced by the recombinant strain *E. coli/pGEMT-pha*. Overall speaking, recombinant *E. coli/pGEMT-pha* was a better species to generate PHAs, which complied with the previous results that engineered organisms were better producers than primitive bacterial strains.

CHAPTER 6

PHAs PRODUCTION BY USING FOOD WASTE AS CARBON SOURCE

6.1 Introduction

The price of P (HB-co-HV) exceeds 10 US dollars per kilogram, which is much higher than the cost of conventional oil-derived plastic. Substrate cost is one of the major factors in the economical production of PHAs. The carbon source should be less expensive since it is the major contributor to the total substrate cost (up to 50 % of the total operating cost) and thus an expensive carbon source is not practical in large scale industrial production.

There are generally two approached that can be taken in the development of bacterial strains in order to produce PHAs from an inexpensive carbon substrate:

(a) Substrate utilization genes can be introduced into PHAs production.

(b) PHAs biosynthesis genes can be introduced into the non-PHAs production, which can utilize cheap substrates.

The utilization of waste materials as the substrate is good alternatives to reduce the cost of the production. Several studies had investigated the use of low cost substrates for PHAs production, such as xylose which was a significant component of hemicellulose of hardwoods and crop residues, malt waste and soya waste.

The tea waste contained certain amount polyphenols/tannic acid ($C_{76}H_{52}O_{46}$) (20-30 %) which were organic compounds having 1 to 4 hydroxyl group, -OH, attached to benzene ring. They react like alcohols but also show weak acidic properties. The effluent was the first washing water for the candy machine. It contained a large amount glucose (Monosaccharide) and sucrose (Disaccharide) after rinsing the internal cavity. The sugar cane dregs still contained a high

proportion of sucrose (Disaccharide) after the cane juice was squeezed from sugar cane. The contaminated baked bread was the defective products which could not pass the quality control and the bread which stocked up over its expiry date. The major ingredients were flour, milk, oil and egg. It consisted with a high quantity of starch (Polysaccharide), protein (polypeptide) and amino acid.

In this section, we used the above food wastes to produce PHAs. Also we used the recombinant strain *E. coli/pKSCAB* harboring the pha synthase gene as the producer.

6.2 Analysis of the Food Wastes

6.2.1 Pre-treatment of the food wastes

The obtained wet tea waste was dried in oven and then grinded into powder and stored in air tight bag for future use. The tea waste powder was hydrolyzed by sodium hydroxide (NaOH) in bottle. Then, the bottle was put into autoclave. The hydrolyzed mixture was centrifuged and the supernatant was separated from the mixture and neutralized by certain amount of concentrated hydrochloric acid (HCl). Medium was re-centrifuged to separate the suspended residue due to the neutralization process. Finally, the clear tea waste hydrolysate was obtained and stored at low temperature for future use.

The effluent was centrifuged to separate the suspended residue. The supernatant was neutralized by small amount of NaOH. After neutralization, media was re-centrifuged. Finally, the clear candies hydrolysate was obtained and kept at low temperature.

The crushed candies were melted by deionized distilled water (ddH_2O) in bottle. Then, the mixture was put in autoclave and treated as the same as the fist washing effluent above.

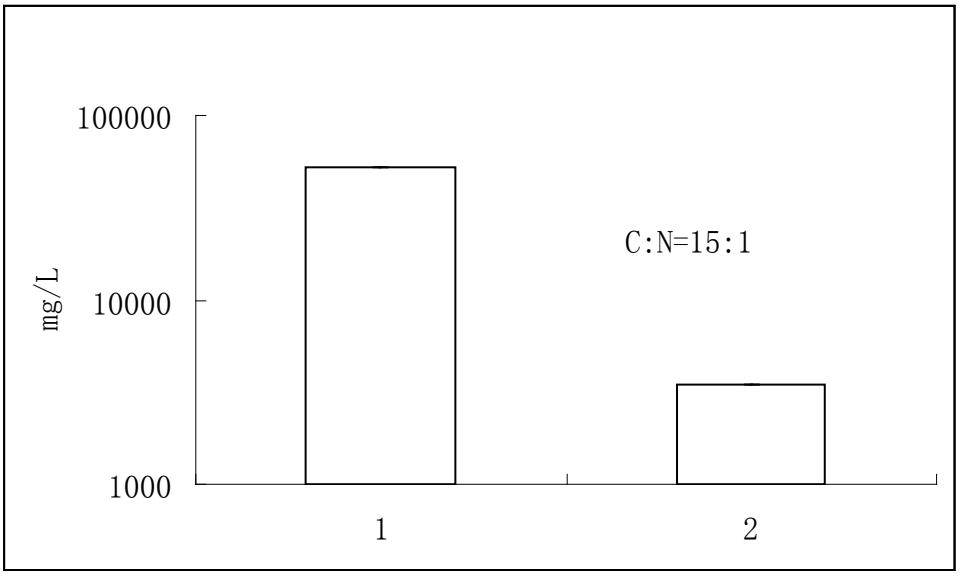
The obtained cane waste was grinded with some lubricating ddH_2O. The cane waste paste was stored in air tight bag for future medium digestion. The cane waste paste was hydrolyzed by adding HCl in bottle. Then, the bottle was put into autoclave. The hydrolyzed mixture was centrifuged and the supernatant was separated from the mixture and neutralized by certain amount of concentrated NaOH. Media was re-centrifuged to separate the suspended residue. Finally, the clear cane waste hydrolysate was obtained and was stored at low temperature.

The baked bread was hydrolyzed by NaOH in bottle. Then, the bottle was put into autoclave. The hydrolyzed mixture was centrifuged and the supernatant was separated from the mixture and neutralized by certain amount of concentrated NaOH. Media was re-centrifuged to separate the suspended residue. Finally, the clear baked bread hydrolysate was obtained and was stored at low temperature.

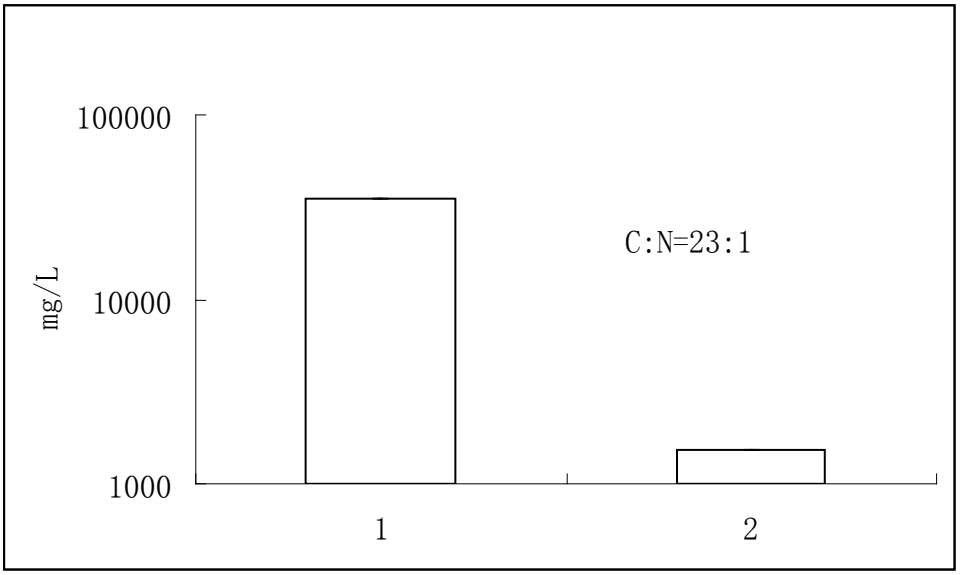
6.2.2 C: N ratio identification of the food wastes

The above pre-treated food wastes supernatant were subject to TOC and TKN. For TOC, the supernatants were diluted by adding ddH_2O. Then, the diluted supernatants were put into TOC tubes for each. Blank ddH_2O were prepared for correction. Finally, the sample tubes were put into the Auto sampler of TOC Analyzer. For TKN, the supernatants of the fermentation culture were diluted by adding ddH_2O. The samples were put into kjeldahl tube. Two K_2SO_4 tablets with mercury oxide and H_2SO_4 were then added in the tube. The tube was heated until the mixture turned black and then got clear greenish blue in color. After cool down, ddH_2O was added to the tube to carry out crystallization. The nitrogen in different samples was distillated by NaOH. The nitrogen was dissolved in distillate and the distillate was received in 10 ml receiving solution. The nitrogen was titrated against the HCl. The TKN was calculated after the deduction of blank.

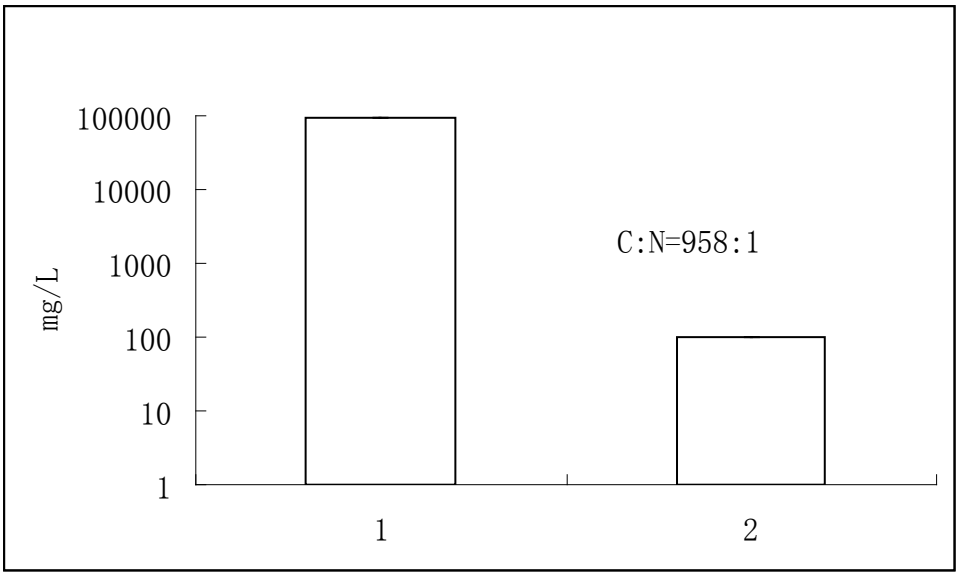
The C: N ratio of 20 or higher was one of the factors for recombinant strain *E. coli/pKSCAB* according to the criteria of PHAs synthesis. The other important factor was 300-350 mg/L nitrogen concentration in the culture medium for the essential nutrient of *E .coli* growth.



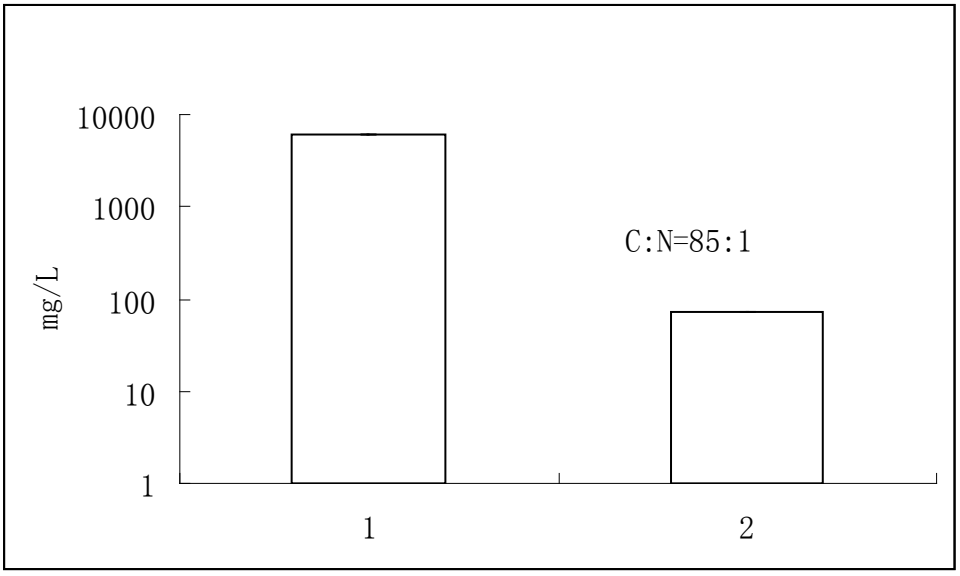
(a) The C: N ratio of tea waste (no heated)



(b) The C: N ratio of tea waste (heated)



(c) The C: N ratio of contaminated candies



(d) The C: N ratio of sugar cane waste

Fig. 6. 1 The C: N ratio of food waste medium

1. TOC 2. TKN

The TOC of the tea waste (no heated and heated), contaminated candies and sugar cane waste were 52, 810 mg/L, 35, 197 mg/L, 92, 960 mg/L and 6, 170 mg/L, respectively. The TKN of the tea waste (no heated and heated), contaminated candies, sugar cane waste and contaminated baked bread were 3, 431 mg/L, 1, 525 mg/L, 97 mg/L, 73 mg/L and 481 mg/L, respectively.

From Fig. 6.1, the highest organic carbon content was determined in contaminated candies which reached nearly 100 g/L of organic carbon. For Tea waste, these two wastes were the same waste source which was handled with two different pre-treatment. Tea waste (heated) was treated with heating process in alkaline digestion. However, tea waste (no heated) was kept at room temperature. The TOC and TKN of tea waste (no heated) were obviously by half lower than tea waste (heated). This phenomenon showed heating process could not raise the organic carbon content but reduce the nitrogen content in the medium. This was a delighted contribution of heating process as C: N ratio could be raised by reducing nitrogen content to satisfy the criteria of PHAs synthesis.

The nitrogen concentration of contaminated candies and sugar cane waste were 70 and 96.6 mg/L, respectively. They could provide sufficient basic nitrogen content during the fermentation stage. This was a favorable condition for the PHAs accumulations of recombinant strain *E. coli/pKSCAB*. Because the PHAs

accumulation was under the condition of limiting the nutrient, in this case, nitrogen was the limiting nutrient.

Contaminated baked bread was an oily hydrolaste. Therefore, TOC could not be obtained by the TOC Analyzer. Therefore, C: N ratio of contaminated baked bread waste was an unknown. Owing to the nature of it, the hydrolyzed starch and oil were expected to provide a relatively high amount of carbon concentration because starch was the major ingredient. Contaminated baked bread waste was subjected to carry out experiments to determine the optimal dilution of substrate for further PHAs fermentation by different dilutions. The ability of strain growth in contaminated baked bread waste could then be done by trial in 1:0, 1:1, 1:4 and 1:7 dilutions to obtain the yield point.

The culture condition of tea waste (heated) should be the most favorable for recombinant strain *E. coli/pKSCAB* to grow and synthesize PHAs since the nitrogen concentration met the minimum requirement and 23:1 of C: N ratio was the nearest fraction to the optimum behavior 20:1 of PHAs synthesis for recombinant *E. coli/pKSCAB*. Tea waste (heated) was expected to give a better harvest amount of cells and PHAs proportion rather than other proposed waste substrates.

6.3 PHAs Production by Food Wastes

6.3.1 The growth ability of *E. coli/pKSCAB* in the different waste medium

In this experiment, all culture media were provided with sufficient nutrients for *E. coli/pKSCAB* growth. The decided dilution of waste concentration was oriented by simulating organic carbon in 10 g/L glucose. The tea waste (no heated) was diluted 40 times. The tea waste (heated) was diluted 25 times. The contaminated baked bread was diluted 7 times. The cell dry weight (CDW) reflects the availability of *E.coli/pKSCAB* growth in different culture medium (Fig. 6.2).

The aim of this experiment was to examine the recombinant strain *E. coli/pKSCAB* growth ability. Nitrogen concentration is excessively supplied in each culture medium. Even contaminated candies waste had a very low nitrogen concentration.

Table 6. 1 Background of the waste medium

	Dilution (waste: water)	TOC in medium (g/L)	C:N ratio
Tea waste (no heated)	1:40	1.3	15:1
Tea waste (heated)	1:25	1.4	23:1
Contaminated candies	1:0	0.93	958:1
Sugar cane waste	1:0	0.88	85:1
Contaminated baked bread	1:7	-	

After fermentation, the mixture was centrifuged and the deposit was to carry out freeze dry process, the harvest CDW was obtained. The data was analyzed by one-way ANOVA and two-sample t-test. The highest was in contaminated candies waste and sugar cane waste reaching 3.27 g/L and 3.19 g/L. The following were in tea waste (no heated), contaminated baked bread waste and tea waste (heated). As contaminated candies waste contained appreciable short chain carbon source which is sucrose ($C_{12}H_{22}O_{11}$) mainly. However, tea waste contained most likely the longer chain of carbon source which is polyphenols/tannic acid ($C_{76}H_{52}O_{46}$). Also, the degree of purity of contaminated

candies waste was much higher than the others. The culture conditions of it facilitated the growth of the recombinant strain *E. coli/pKSCAB* during fermentation.

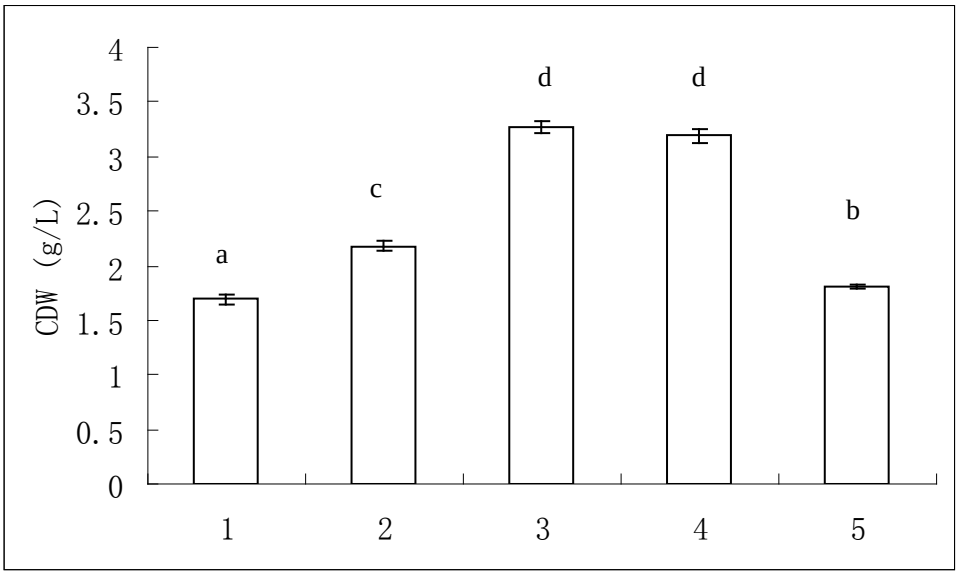


Fig. 6. 2 CDW of *E. coli/pKSCAB* in the different waste medium

1. Tea waste (no heated) 2. Tea waste (heated) 3. Contaminated candies waste 4. Sugar cane waste 5. Contaminated baked bread waste

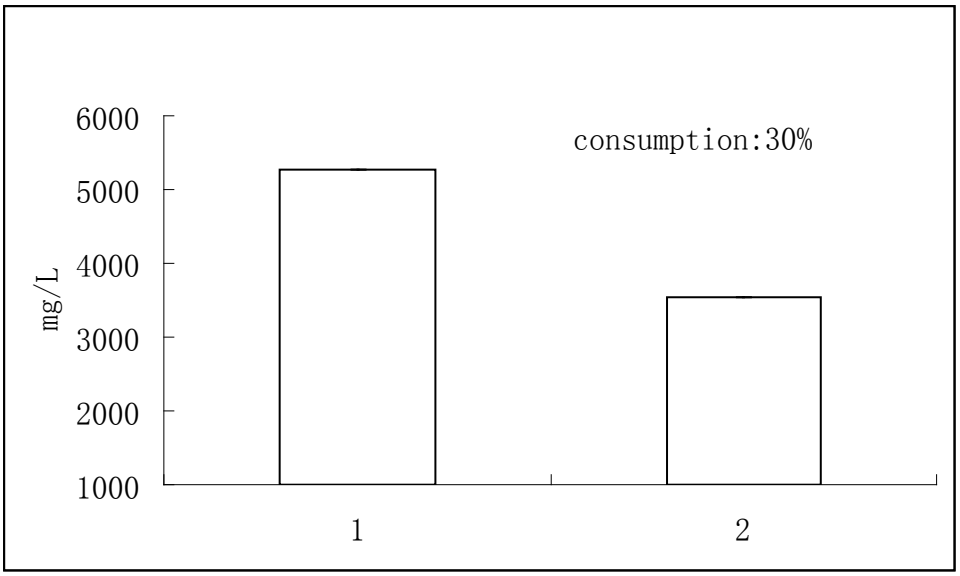
The data was analyzed by one-way ANOVA and two-sample t-test, the different small letters stand for statistical significance at the 0.05 level. Waste 3 and 4 were similar. But both were higher than the others.

6.3.2 The change of TOC after fermentation

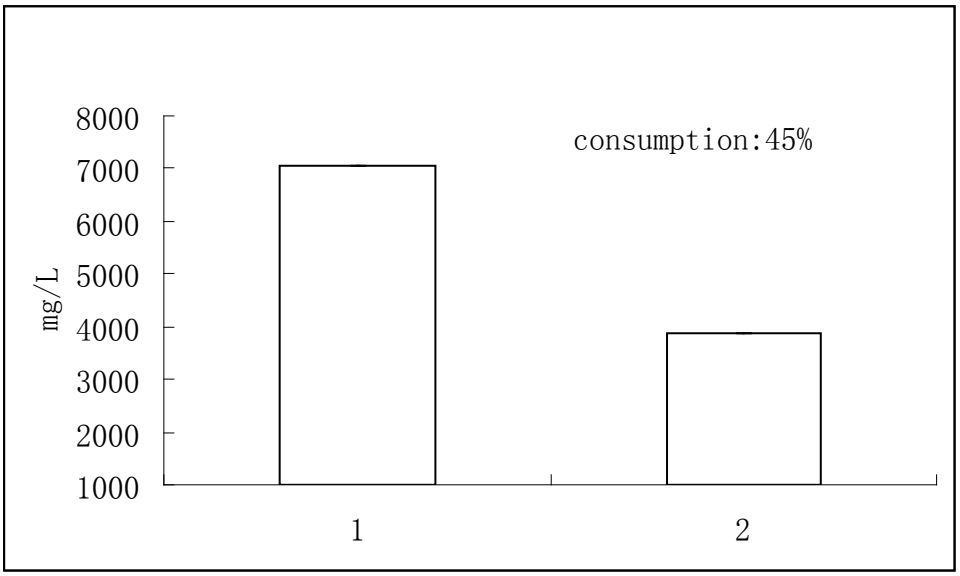
In this experiment, 300-350 mg/L basic nitrogen concentration was oriented according different dilution for each waste substrate. The tea waste (no heated and heated) media were diluted 9 times and 4 times, respectively. After 48 hours fermentation, the mixture was centrifuged and the supernatant was subject to TOC (Fig. 6.3).

The TOC consumption was one of the important parameters to show the ability of the recombinant strain *E. coli/pKSCAB* growth. The tea waste (heated) consumed the most proportion in 45 % to its TOC quantity. And the following were tea waste (no heated), sugar cane waste and contaminated candies which were 30%, 11.3 % and 1.2 %, respectively to their TOC quantity.

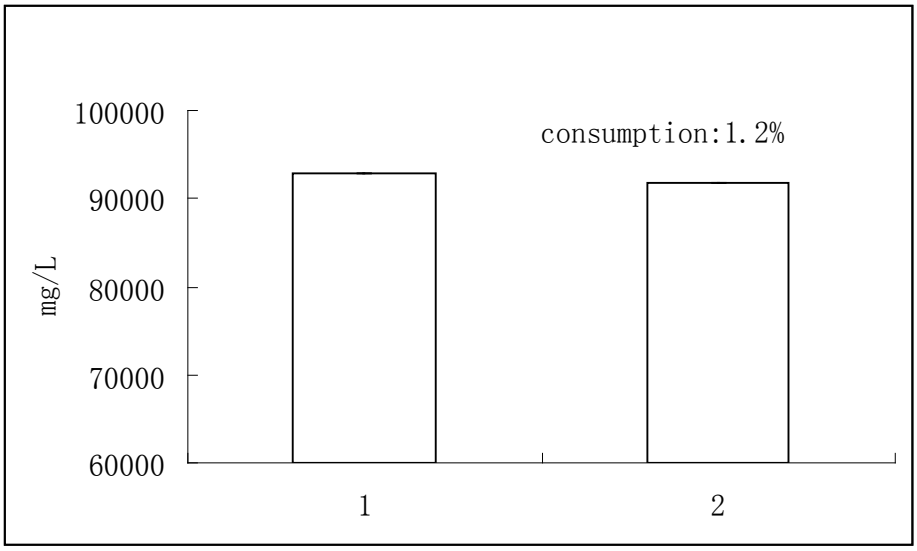
The TOC consumption of PHAs synthesis in contaminated candies was facilitated by the optimal C: N ratio during fermentation. It provided a favorable condition for the recombinant strain *E. coli/pKSCAB* to carry out PHAs synthesis under metabolic stress.



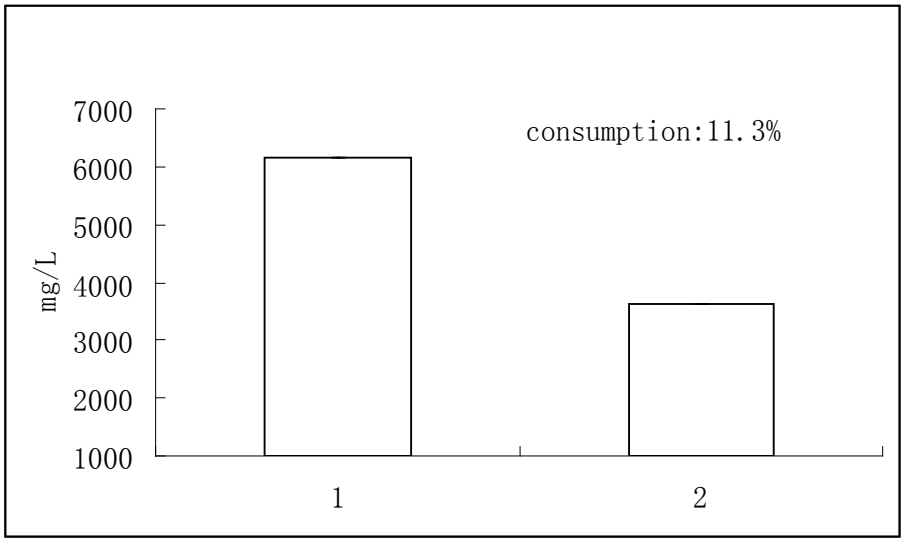
(a) Consumption of tea waste (no heated)



(b) Consumption of tea waste (heated)



(c) Consumption of contaminated candies



(d) Consumption of sugar cane waste

Fig. 6. 3 Consumption of TOC in the different waste medium

1. The TOC before fermentation 2. The TOC after fermentation

The data was analyzed by two-sample t-test. As P-Value < 0.01, there is a significant difference.

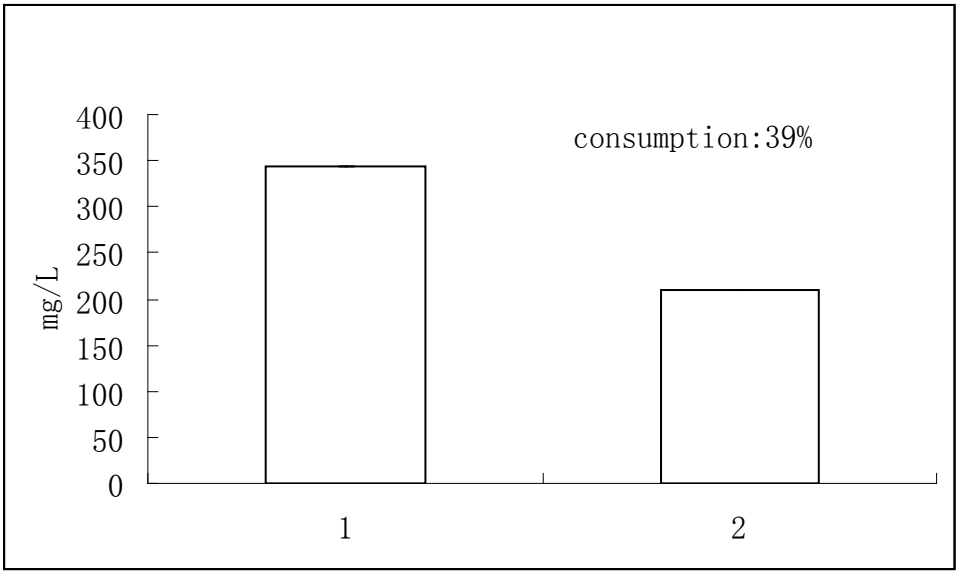
For the extremely low TOC consumption in contaminated candies, the nitrogen concentration was limited. When the nitrogen was consumed completely, there were still so many carbon sources in the medium. So the change of the TOC was not obvious. For the case of sugar cane waste, the concentration of carbon was also much more than that of the nitrogen.

6.3.3 The change of TKN after fermentation

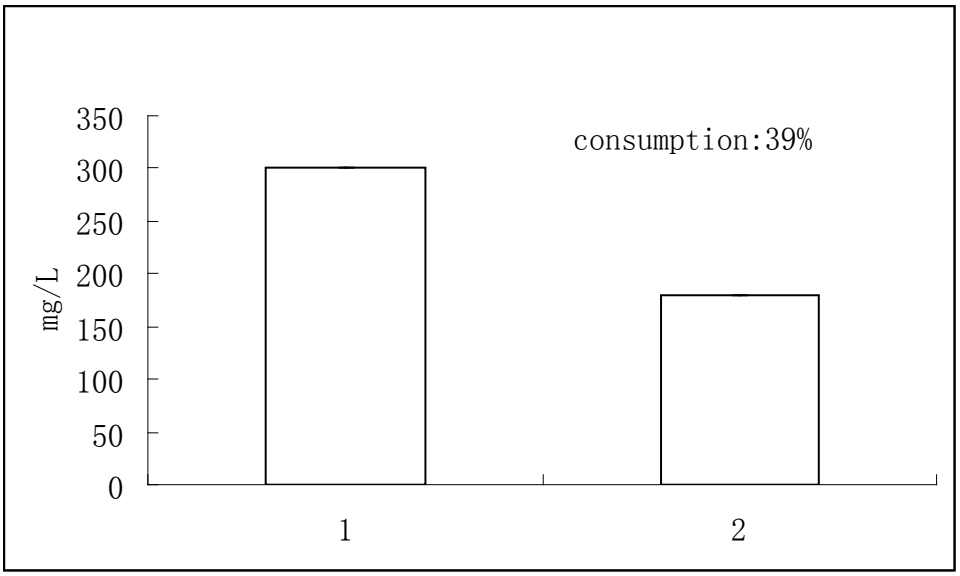
According to the basic nutrient of nitrogen, the tea waste (no heated) was diluted 9 times by ddH_2O for the fermentation and the tea waste (heated) was diluted 4 times by ddH_2O for the fermentation. The contaminated candies, the sugar cane waste and the contaminated baked bread did not need to dilute. Fermentation was operated for 48 hours in this experiment. After the fermentation, the mixture was centrifuged and the supernatant was subject to TKN measure.

From Fig. 6.4, all waste supernatants were in depletion after culture fermentation. The nitrogen consumption was at a very high proportion in contaminated candies and sugar cane waste about 71 % and 74 %, respectively. These two wastes were of very low nitrogen concentration which could sufficiently feed the recombinant

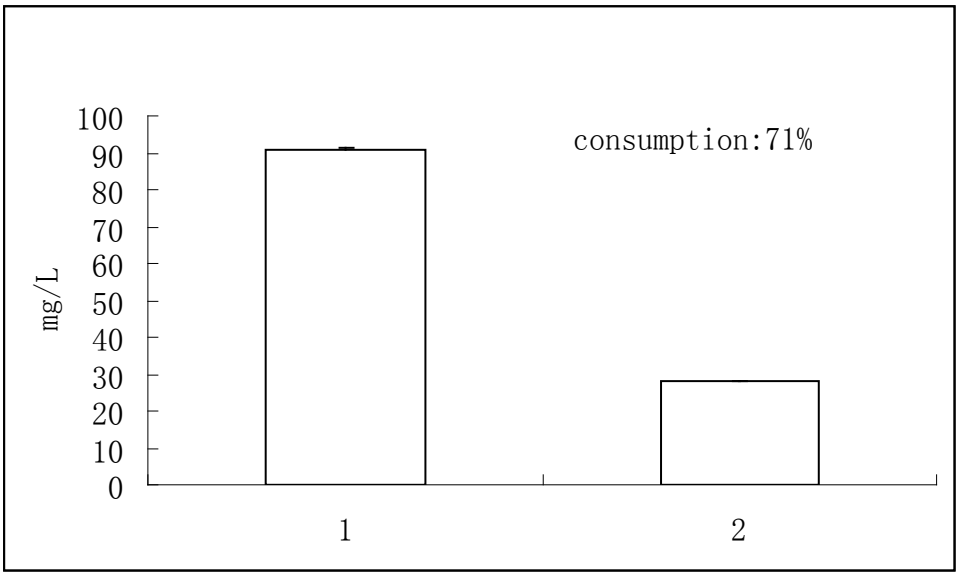
strains for growth at the beginning of the fermentation, so the nitrogen demand from strain was relatively higher than the other wastes. The tea waste (no heated and heated) and the contaminated baked bread showed a similar nitrogen consumption percentage around 39 % and 40 %, respectively. The growth of the strain was decided by the nutrient in the medium. In these waste medium, nitrogen was limited, so the concentration of the nitrogen decided the growth of the strains. The more nitrogen consumption the more strain cells we got. The results of nitrogen consumption in this section were according with the results of the growth ability of the recombinant strains in the different waste medium.



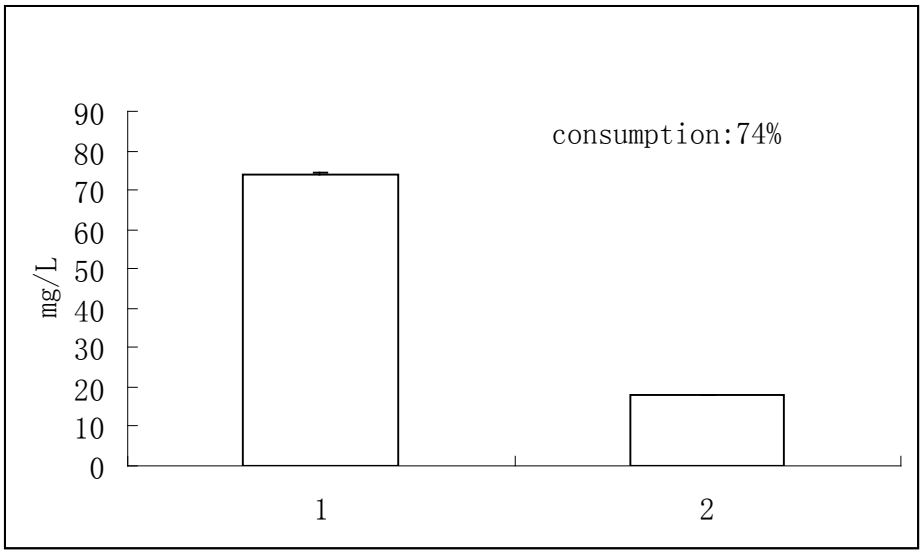
(a) Consumption of tea waste (no heated)



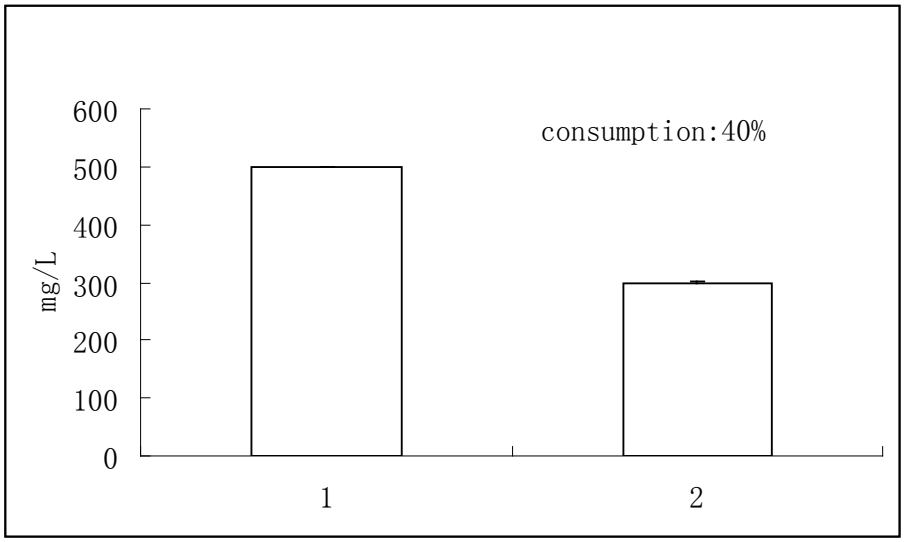
(b) Consumption of tea waste (heated)



(c) Consumption of contaminated candies



(d) Consumption of sugar cane waste



(e) Consumption of Contaminated baked bread

Fig. 6. 4 Consumption of TKN in the different waste medium

1. The TKN before fermentation 2. The TKN after fermentation

The data was analyzed by two-sample t-test. As P-Value < 0.01, there is a significant difference.

6.3.4 The proportion of HB and HV in the different waste medium

In this experiment, the harvest cell was dried in very low temperature. After weighting the mass of the dried cell, the dried cell was prepared for esterification. Accumulated polymeric materials extracted from the microorganisms were analyzed by gas chromatographic methods to contain mainly monomers of hydroxybutyric acid (HB) and hydroxyvaleric acid (HV). Table 6.2 showed the composition of polymers obtained in different waste culture medium. The polymer produced in tea waste (no heated) contained 79.2 mol % of HB monomer. For tea waste (heated), the polymeric materials contained 75.3 mol % of HB. 97.5 mol % of HB was determined in polymer which produced in the contaminated candies. There were 94.1 mol % and 92.9 mol % of HB in the polymer which produced in the sugar cane waste and contaminated baked bread, respectively.

It is well known that the melting point, other physical properties and biodegradability of P (HB-co-HV) are dependent on the mole fraction of monomer unit, HB and HV units in P (HB-co-HV). The polymer produced by tea

waste was more plastic than the other polymer produced by the other waste medium.

Table 6. 2 Composition of PHAs accumulated in the different waste medium

	C:N ratio	HB (mol %)	HV (mol %)
Tea waste (no heated)	15:1	79.2±5.3	20.8±1.6
Tea waste (heated)	23:1	75.3±5.8	24.7±1.8
Contaminated candies	958:1	97.5±7.5	2.5±0.1
Sugar cane waste	85:1	94.1±7.3	5.9±0.4
Contaminated baked bread	--	92.9±8.2	7.1±0.5

Values are means ± S.D. (n=4)

6.3.5 The effect on CDW and PHAs synthesis by citric acid

In this experiment, citric acid (1g/L) was added to observe the variation of CDW and PHAs productivity.

There was no obvious variation of CDW by citric acid, because the wastes can provide sufficient nutrient for the strain growth. Most of the wastes showed a higher PHAs accumulation. For tea waste (no heated), PHAs to CDW fraction

increase about 25 % to 10 %. For tea waste (heated), the fraction of PHAs was promoted to 13 % which was raised about 8.3 %. The PHAs fraction was increased up to 16.7 % to 21 % from the original in contaminated candies waste. For sugar cane waste, there was an obvious change in PHAs fraction which was 13.3 %. For the contaminated baked bread waste, there were 11 % PHAs fraction increased.

Table 6. 3 The effect on CDW and PHAs synthesis by citric acid

	CDW (g/L)		PHAs (%)	
	Absent of Citric acid	Citric acid	Absent of Citric acid	Citric acid
Tea waste (no heated)	1.69±0.08 a	1.70±0.07 a	8±0.7 a	10±0.8 b
Tea waste (heated)	2.18±0.09 a	2.21±0.1 a	12±0.7 a	13±1 b
Contaminated candies	3.27±0.13 a	3.53±0.15 a	18±1 a	21±0.9 b
Sugar cane waste	3.19±0.15 a	3.28±0.16 a	15±0.5 a	17±0.3 b
Contaminated baked bread	1.81±0.08 a	1.92±0.09 a	10±0.7 a	11±1 b

Values are means ± S.D. (n=4). The different small letters stand for statistical significance at the 0.05 level with two-sample t-test.

From the results in this experiment, evidences expressed that citric acid was a useful nutrient for the PHAs accumulation. It was important as an intermediate in the tricarboxylic cycle and therefore occured in the metabolism of microorganism. It directly influences the PHAs production to CDW. On the other hand, citric acid could provide more carbon source, therefore the strains could synthesize more pre-body of the PHAs, as a result, and we obtained more PHAs.

6.3.6 The effect on CDW and PHAs synthesis by yeast extract

In this experiment, 2 g/L of yeast extract was added in every waste medium. The cell growth and polymer accumulation were the focused areas for discovering the differentiation in importance of yeast extract (YE) on the activity of microbial cell and PHAs synthetic ability in different wastes.

CDW was increased for contaminated candies and sugar cane waste which was added 2 g/L yeast extract (YE). The percentage of CDW of the two waste medium were increased 10.7 % and 4.7 %, respectively. And the PHAs fraction to CDW had much increase 25 %, 16.7 %, 27.8 % and 20 %, respectively.

Table 6. 4 The effect on CDW and PHAs synthesis by yeast extract

	CDW (g/L)		PHAs (%)	
	Absent of YE	YE	Absent of YE	YE
Tea waste (no heated)	1.69±0.08 a	1.74±0.04 a	8±0.7 a	10±0.7 b
Tea waste (heated)	2.18±0.09 a	2.28±0.06 a	12±0.7 a	14±0.25 b
Contaminated candies	3.27±0.13 a	3.62±0.11 b	18±1 a	23±1.4 b
Sugar cane waste	3.19±0.15 a	3.34±0.13 b	15±0.5 a	18±0.5 b
Contaminated baked bread	1.81±0.08 a	1.97±0.2 a	10±0.7 a	11±1 a

Values are means ± S.D. (n=4). The different small letters stand for statistical significance at the 0.05 level with two-sample t-test.

Yeast extract fermentations comprise the largest application of microbial technology. Therefore, adding yeast extract to in this experiment aimed at studying how yeast extract affect the efficiency of fermentation. Yeast extract provide enzyme to digest the relative large bonding organic substrates like protein and starch so higher CDW in contaminated candies and sugar cane waste

were expected. Yeast extract enhanced much the fermentation process. The microbial activity was facilitated by enzyme which yeast extract provided. The higher the mass harvest, the higher PHAs produced.

6.4 Discussion

The feasibility of using the proposed wastes for synthesizing PHAs depended on some economical areas such as the carbon sources, technical pre-treatment methods, cultivation condition and PHAs synthesizability.

From all the experiments in this research, tea waste, sugar cane waste and contaminated baked bread were the potential wastes for preparing culture medium for microorganism growth and PHAs synthesis. In addition, they were almost free of charge. In tea waste, the heating process was good for shorten the chain of carbon source to feed the recombinant strain *E. coli/pKSCAB*. This was drawn from the comparison of the TOC consumption in tea waste no heated and heated after fermentation.

For considering the nutrient supplements, citric acid did not affect much on the growth ability, but it could increase the ability of synthesizing of PHAs. Besides, yeast extract was a catalytic agent that could facilitate the fermentation process.

It could offer more strain cells, as a result, more PHAs could be produced.

According to biosynthesis of PHAs, tea waste, sugar cane waste and contaminated baked bread had relatively high development potential on this technology. Also, the C: N ratio of these waste medium provided an optimal C: N ratio and nitrogen concentration met the basic criteria.

CHAPTER 7

PHAs FOR MEDICAL AND PHARMACEUTICAL APPLICATIONS

7.1 Introduction

Polyhydroxyalkanoates (PHAs) was a class of biodegradable polymer synthesized by microorganisms with wide range of commercial and industrial usages. One of the very intense studies of PHAs was its potential in protein drug delivery and tissue engineering. It was found that PHAs nanoparticles had a high loading capacity, high loading efficiency, low toxicity and release in a stable manner which had very high potential in protein drug delivery.

Tissue engineering required cellular components, extracellular matrices and scaffolds, and growth and differentiation factors. Scaffold must act as a substrate for cellular attachment, proliferation and differentiation. For a successful application of such materials, suitable mechanical characteristics and degradation behavior were also essential.

The matrices used as scaffolds should satisfy certain requirements. They should be designed to allow diffusion of nutrients to the transplanted cells and guide cell organization, attachment, and migration. Pore size and porosity were important issues. The matrices had to allow optimum cell, tissue and new blood vessel invasion. The mechanical properties of the material should match that of the tissue and be maintained during healing. Therefore, mechanical properties of scaffolds during degradation must be characterized. There was increasing evidence that changed in scaffold surface chemistry and topography altered cellular activity.

In this study, the preparation, degradation behavior, surface morphologies and surface modification of porous PHAs foams were described to prove that PHAs produced by bacteria could be used to produce nanoparticle and load with pyrene. It was also indicated that PHAs could be the tissue engineering scaffold.

7.2 PHAs for Drug Delivery System

7.2.1 Extraction of PHAs for preparation of nanoparticles

Production of PHAs for preparation of bacterial PHAs nanoparticles was chosen to use *E. coli* in production media since it had high total PHAs (13 %), HV(5 %) and considerable PHB (8 %) accumulation. 7.73 g dry weight of cells was produced by 1L media and 1.53 g PHAs was extracted.

7.2.2 Measurement of sizes

Sample PHAs was dissolved in chloroform in a reflux system. Poloxamer 188 which dissolved in ddH_2O was added to the sample. Oleic acid followed by SPAN 80 was then added. The mixture was subjected to homogenization. Chloroform is evaporated by rotatory evaporator. The mixture was stirred overnight by magnetic stir bar.

Pyrene was chosen to imitate protein drug due to its hydrophobicity and fluorescence nature. Nanoparticles were prepared in dispersion of pyrene in ddH_2O. Unloaded pyrene was removed by dialysis in ddH_2O.

Size of nanoparticle and that loaded with pyrene were subjected to zeta sizer to measure the diameter of particles.

The size of the PHAs particles was about 25 nm, and the size of PHAs with pyrene particles was about 31 nm. Both were in nano (10^{-9}) scale, Size of nanoparticles loaded with pyrene was similar to that of no pyrene. The increase in size after loading with pyrene proved that pyrene was successfully encapsulated by PHAs.

The results suggested that the PHAs nanoparticles were able to encapsulate hydrophobic particles, which meant that they had high potential in encapsulating protein drugs. Besides, the PHAs nanoparticles would have quick distribution to target sites and quick entrance into cells due to their small size. Although smaller particle size decreased loading capacity, on the other hand, rate of drug release increased. It was a very encouraging property in potential drug carrier.

7.2.3 Drug loading capacity (DLC) and drug loading efficiency (DLE)

Pyrene was chosen to simulate protein drug due to its hydrophobicity. Its fluorescence nature was also convenient for measurement. The principle of determining DLC and DLE was that, after centrifugation of nanoparticles loaded with pyrene at 14, 000 rpm, pyrene is released from the core shell. As the previous results proved that pyrene was successfully loaded, DLC and DLE were then investigated in order to further characterize the potential as a drug vector. By measuring the fluorescence signal of the supernatant, amount of pyrene loaded and thus, DLC and DLE could be calculated.

DLC = Concentration of loaded pyrene in nanoparticles/ Concentration of nanoparticles

DLE = Concentration of loaded pyrene in nanoparticles/ Concentration of pyrene added X 100%

Concentration of nanoparticles used = 2mg/ml

Concentration of pyrene added = 0.129mg/L

After pre-scan of pyrene standard dissolved in ddH_2O, it was found that the excitation wavelength of pyrene was 332.4 nm. So, intensity at 332.4 of supernatant after centrifugation is measured by luminescence spectrometer. The intensity was then compared with the standard curve of pyrene intensity at 334.2 nm.

Table 7. 1 DLC and DLE of PHAs

Samples	Intensity at 334.2 nm	Weight of pyrene loaded(ug/ml)	Loading capacity (mg/g)	Loading efficiency (%)
PHAs	13.66	125.15±8.33	6.25±0.5	96.90±0.87

Values are means ± S.D. (n=4)

The results showed that the PHAs nanoparticles had a high loading efficiency and good loading capacity. Loading capacity reached over 90 %.

The high DLC suggested that PHAs was a good drug vector since minimum amount of nanoparticles were required to carry required amount of drug into body. It lowered the risk of incompatibility and triggering of immune response

towards the nanoparticles, thus improved safety. The high DLE meant that lower concentration of drugs was required during encapsulation which reduced the cost of preparation.

7.2.4 Drug in vitro release

Rate of release was also an important issue in drug delivery system since rate of release affected their effectiveness. Having a controlled release system was one of the ultimate aims in drug delivering studies.

Extracellular PHB depolymerase was produced and isolated from media of *Pseudomonas lemoigne* for the study of in vitro release of PHAs nanoparticles. The enzyme was able to degrade PHB and thus destroy the core structure. By finding the decrease in fluorescent intensity, rate of pyrene release could be determined. It was found that excitation wavelength of pyrene inside PHAs core shell is 336.4 nm after pre-scan of pyrene loaded PHAs nanoparticles.

Release of pyrene using different volume of enzyme with fixed concentration of nanoparticles and different concentration of nanoparticle with fixed volume of enzyme were both investigated.

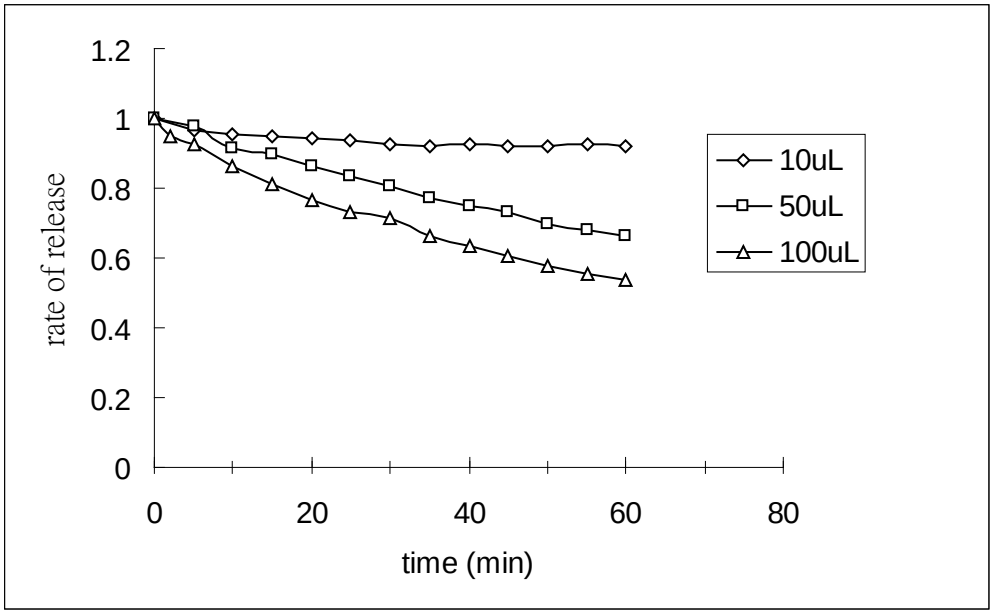


Fig. 7. 1 The effect of different enzyme amount on biodegradation at a constant concentration of nanoparticle dispersion (1 mg/ml)

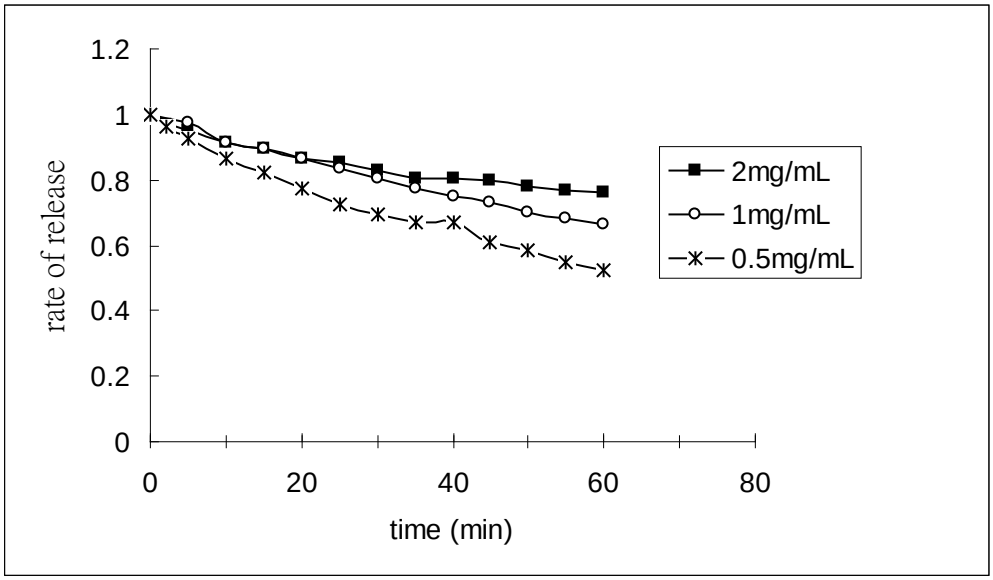


Fig. 7. 2 The effect of a fixed amount of enzyme (50 uL) on biodegradation at different concentration of nanoparticle dispersion

All samples showed decrease in intensity. The pyrene in all samplers decreased faster in the first 10 minutes and decrease in a stable way afterwards. The pyrene decreased in a faster rate when concentration of enzyme increased. It indicated that rate of degradation increased with increased amount of enzyme. The pyrene also decreased faster when concentration of nanoparticle increased which showed that rate of degradation increased with increased amount of nanoparticle.

The pyrene did not reach zero showed that not all pyrene was released in one hour. It was an advantage that the degradation rate was slow. As drug release can be completed before nanoparticles being degrade, the drug release was entirely controlled by enzyme concentration.

The results above showed the possibility in controlled release of PHAs nanoparticles by enzymes. However, since extracellular PHB depolymerase did not present in human body, the release characteristics of PHAs as vector for protein delivery need to be further investigated using other enzymes presented in human body, such as lipase.

7.2.5 Biocompatibility test

Since nanoparticles were delivered into body, biocompatibility and toxicity were important issue for the determination of potential of being drug vector to ensure safety for the patients.

Human breast adenocarcinoma cell line (MCF7) was used to study the toxicity of nanoparticles to cells. Different amount of nanoparticles were added into culture medium and viability of cell after 24 hours was determined by MTS assay. Percentage cell viability is calculated as followed:

$$viability\ \% = \frac{cell\ numbers\ of\ \ control - cell\ numbers\ of\ \ sample}{cell\ numbers\ of\ \ control}\ X\ 100\ \%$$

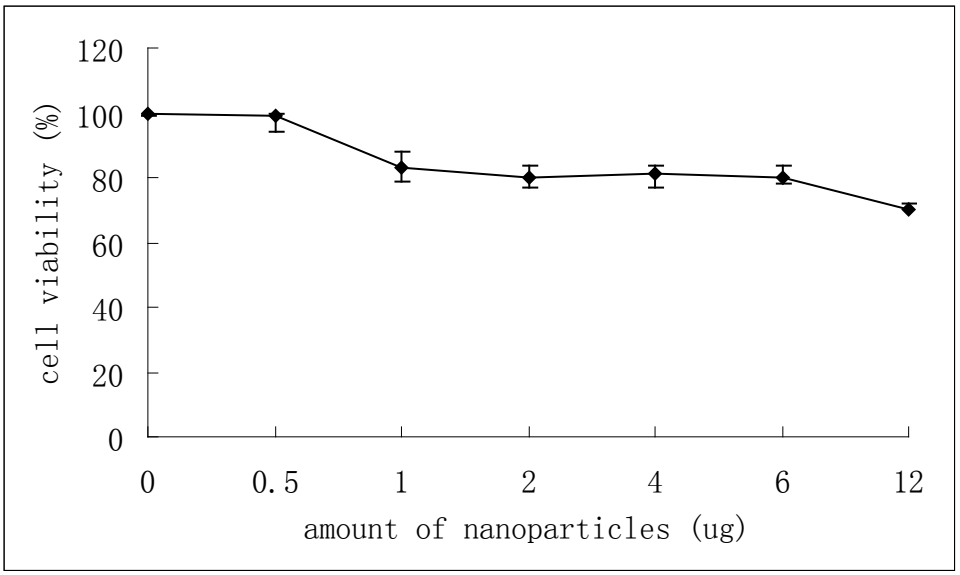


Fig. 7. 3 The effect of different amount of nanoparticles on cell viability (%) of MCF7 after 24 hours

Cell viability was over 80 % except sample with 12 μg nanoparticles. Percentage viability increased as amount of nanoparticle decreases. Percentage viability reached 90 % when amount of nanoparticles were lower than 1μg.

The results showed that cell viability was generally high meaning that nanoparticles had low toxicity. It was because PHB was lipophilic, the PHAs particles might correlate to lipoprotein in cells and in blood and did not cause any effect inside contributing to the biocompatibility of PHAs nanoparticles in cells and blood.

7.3 PHAs for Tissue Engineering

7.3.1 Preparation of the heart valve

PHAs and 89-150 μm sodium chloride particles were dissolved in chloroform. Then the liquid was lain out on the model of the heart valve. The PHAs film and the heart valve model were immersed into excess distilled water for 48 hours to remove the salt and got off the model. The pore size was between 89 and 150 μm, the pore ratio was more than 90 %. The thickness of these PHAs heart valve (Fig. 7.5) was between 1.2 and 1.5 mm.

7.3.2 Cell growth on the PHAs heart valve

The cell was inoculated on the PHAs heart valve. After 5 days, we could observe that the cells began to adhibit on the heart valve. After 20 days, the cell was grown on the 90 % of the heart valve. From the SEM picture, it was known that the cell was about to 20 μm.

When the cells on the PHAs heart valves were stable, they were subject to identify the toughness in the artificial conditions. In all valves, synchronous opening and closing of the three leaflets was observed in the bioreactor, under both low-pressure (35 mm Hg) and high-pressure (150 mm Hg) conditions. The cell appearance showed the most advanced tissue formation after 14 days, without apparent differences at 21 and 28 days. All the leaflets were intact, mobile, and pliable, and all the heart valve constructs were competent during valve closure.

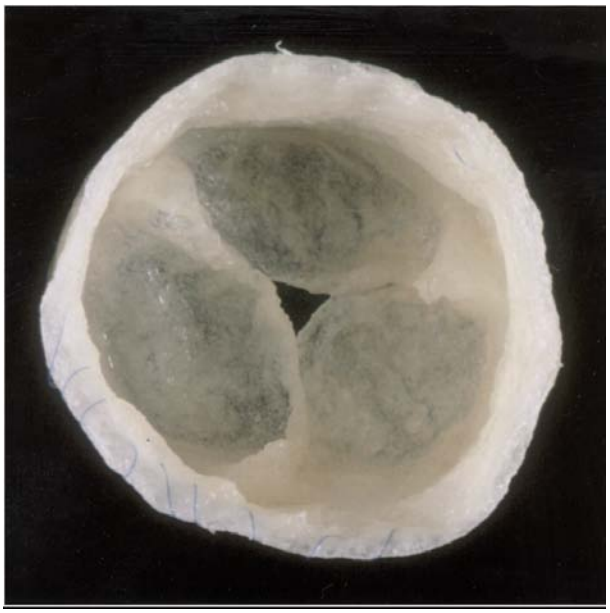
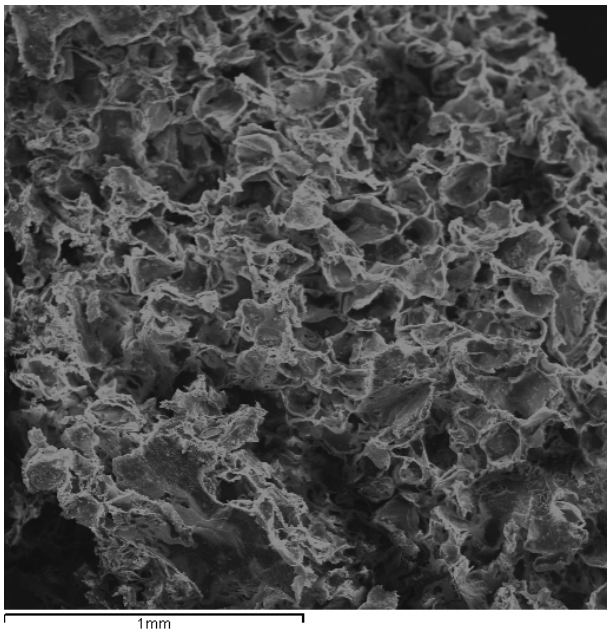
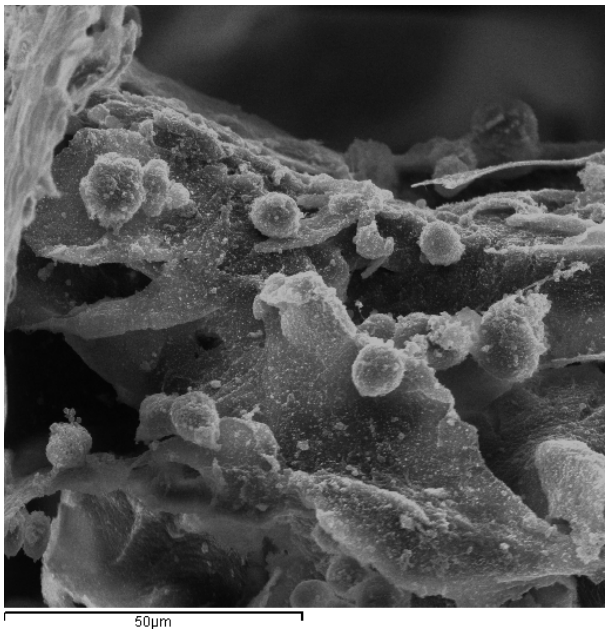


Fig. 7. 4 PHAs heart valve



(a) PHAs heart valve without cells



(b) PHAs heart valve with cells

Fig. 7. 5 SEM resultsof the cells on the PHAs heart valve

7.3.3 Tissue microstructure

Histology of the PHAs leaflets revealed cellular tissue organized in a layered fashion with a dense outer layer and lesser cellularity in the deeper portions after 14 days in the pulse duplicator. Formation of extracellular matrix was demonstrated as collagen. Smooth muscle cells were detectable throughout the tissue. Tissue was maximally organized after 14 days with no further increase after longer culture duration in the pulse duplicator.

7.4 Discussion

The measurement of size proved nanoparticles could be successfully prepared by PHAs. The nanoparticles could also encapsulate pyrene, a hydrophobic chemical, successfully. It suggested that there was high possibility for hydrophobic drugs like protein to be encapsulated in the PHAs nanoparticles, which suggested that other hydrophobic drugs could also be loaded into the shell.

The high drug loading capacity and drug loading efficiency further proved the potential of PHAs as drug carrier. Degradation by enzymes was in a stable manner which suggested the possibility in controlled release. The low toxicity proved that the PHAs nanoparticles were biocompatible and safe for drug delivery. Combining all these properties, PHAs nanoparticles had very good characteristics and high potential to be drug delivering vector.

Heart valve disease was a significant cause of morbidity and mortality world-wide. Valve replacement surgery was efficacious, and the state-of-the-art valves used clinically include mechanical valves and biological valves. The

major drawback of mechanical valves related to the fact that these foreign materials, associated with the risk of infections and complications. Biological valves did not require medication. The majority of biological valves necessitated re-replacement.

The cells were adhered, spread and grown on the PHAs heart valve, which were stable under the high and low pressure condition. This was indicated that PHAs had a wonderful potential in tissue engineering.

CHAPTER 8

CONCLUSIONS

8.1 PHAs Production by the Recombinant Strains

The work was the first report to demonstrate the expression of the mcl-PHAs synthase gene from *Ps. aeruginosa* into *B. subtilis*. And the recombinant strains successfully produce mcl-PHAs. *Ps. aeruginosa* can synthesize mcl-PHAs by ß-fatty acid oxidation which use fatty acid as substrate and de novo fatty acid synthesis pathway which use simple molecule as substrate such as glucose, but production of the latter was lower. In this work, there was no mcl fatty acid but glucose as the substrate in the medium. So, the production of the PHAs was low. We can add some mcl fatty acids to improve the production of the PHAs in the

future work.

As the results showed, the recombinant *B. subtilis*/pBE2C1 and *B. subtilis*/pBE2C1AB can synthesize monomer mcl-PHAs, which accord with the character of PHAs synthase gene *phaC1*. Because the Tm and crystal speed of mcl-PHAs was lower, the recombinant produces P (HB-HD-HDD). Also controlling their percentage was our new research interest. So we subcloned ß-ketothiolase gene (*phbA*) and acetoacetyl-CoA reductase (*phbB)* from *R. eutropha* into plasmid pBE2C1 and obtained plasmid pBE2C1AB. We detected the production of PHB in the recombinant *B. subtilis*/pBE2C1.

Recently, there had been growing interest in P (HB-co-mcl HA) because it had much excellent physical- co-chemical character. Our research results suggested that the recombinant strain *B. subtilis*/pBE2C1AB could produce P (HB-co-mcl HA) polymer.

In the research, we used *B. subtilis* as host because *B. subtilis* had attractive properties such as short generation time, absence of endotoxin, and secretion of both amylases and proteinases that could well utilize food wasters for nutrients, which can further reduce the cost of production of polyhydroxyalkanoates(PHAs).

A PCR cloning protocol was developed to clone the PHAs synthase genes of PHAs loci. This rapid and easy cloning method could be applied to most strains. PCR was the novel technique in the genetic engineering. After sequencing of the cloned fragment, the PCR cloning strategy may be used in the rapid and specific identification of the existence of PHAs synthase genes. A subsequent PCR cloning method using the long and specific primers directly selected from the cloned fragment can lead to the cloning of the complete gene locus. Using the cloning strategy the coding regions of PHAs synthase genes was successfully cloned and functionally expressed in *E. coli* under promoter control.

From these experiments, it was concluded that an genetic engineered strain (recombinant *E. coli*), a high carbon-nitrogen ratio (50:1) by increasing the substrate content (sodium acetate), a medium citric acid amount (0.5 g/L), a higher yeast extract concentration (2 g/L) and a lower tryptone content (0.5 g/L) contributed to the high productivity of PHAs.

8.2 Food Wastes Used as Carbon Source for Cost Reduction

When considering the proposed wastes, C: N ratio could be predicted before laboratory testing. As TOC and TKN were tedious laboratory works. The genius method was to compare the similarity of texture to the wastes proposed in the past research. This could reduce time consuming for understanding the background of wastes. The accurate prediction for feasibility of carbon source could be achieved. It is concluded that contaminated candies can produce certain amount of PHAs.

PHAs can be produced by bacteria and its components can be controlled by variation of contents in the production medium. It was also found that recombinant *E. coli* had better production in total PHAs contents than wild-type strain. The crystallinity of PHB makes it brittle and limits its application; P (HB-co-HV) polymer would have increase flexibility and wider range of application. It was found that *E. coli/pKSCAB* had the highest percentage of total PHAs content in the wastes which suggested that it would be possible to apply this in large scale production.

8.3 PHAs for Drug Delivery System and Tissue Engineering Applications

The measurement of size proved nanoparticles can be successfully prepared by PHAs produced by bacteria. The nanoparticles could also encapsulate pyrene, a hydrophobic chemical, successfully. It suggested that there was high possibility for hydrophobic drugs like protein to be encapsulated in the bacterial PHAs nanoparticles.

The high drug loading capacity and drug loading efficiency further proved the potential of PHAs as drug carrier. Degradation by enzymes was in a stable manner which suggested the possibility in controlled release. The low toxicity proved that the PHAs nanoparticles were biocompatible and safe for drug delivery. Combining all these properties, PHAs nanoparticles had very good characteristics and high potential to be drug delivering vector. Further work including loading with enzyme, enzyme activity assay after release and in vivo test can be done to further prove its ability in drug delivery system.

Mechanical and biological heart valve represent the state-of-the-art replacements in modern cardiac surgery and had tremendously improved the survival and life-quality of millions of patients. However, mechanical prostheses were associated with the adverse side effects of a lifelong anticoagulation medication and biological valves suffer from progressive dysfunctional degeneration. All contemporary valves basically represent non-living replacements and lack the potential of regeneration and growth. In recent years, there was a trend from mechanical valve to biological valves.

The use of minimally processed biological materials as scaffolds for tissue engineering enables the repopulation with cells. The use of biocompatible, biodegradable synthetic material had been well established in various surgical applications. These materials were easy to handle, safe and neither immunogenic reactions nor undesired side effects due to degradation products had been reported so far.

APPENDICES

The gene and amino acid sequences of *Bacillus thuringiensis* HD-81were as follows,

```
1 CCTGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTTGGGGATCCTTAAT

1  P  V  A  C  S  R  P  P  W  R  P  R  E  F  D  L  G  I  L  N

61 TAGAACGCTCTTCAAGCCAATTTCCAATCGTCGGATATGTTTGTTTTACAGCTGTTCCAC

21  *  N  A  L  Q  A  N  F  Q  S  S  D  M  F  V  L  Q  L  F  H

121 CGTAAACGATAGACATATGCCCTGTTGGTAAACATACATATTGTTTATCTGTGCTAGAAA

41  R  K  R  *  T  Y  A  L  L  V  N  I  H  I  V  Y  L  C  *  K

181 TATGGTCTAGTAATGCTTCTACTTGACATGGCAAAGCGATGTGATCACGTTTCCCGGAAA

61  Y  G  L  V  M  L  L  L  D  M  A  K  R  C  D  H  V  S  R  K

241 TATTTAAGACATTCGCCTTAATATTTGCAAGGTCTACCTTTTGTCCGCGAATAACGAGTT

81  Y  L  R  H  S  P  *  Y  L  Q  G  L  P  F  V  R  E  *  R  V

301 CACCCTTAACCGACTTATTATTTTGATAAAAATCACGAATCCACTGTCTGTATGATTCAC

101  H  P  *  P  T  Y  Y  F  D  K  N  H  E  S  T  V  C  M  I  H
```

361 CTGGGAACGGAATACCATCACCAACCCACTTTTGAACCAATCTCCAGCTTTCGACGAAGC

121 L G T E Y H H Q P T F E P I S S F R R S

421 GCTCATTCTCTGAACGATCTACTAAAGCAACATATGGACCAACAAAGTTCGTAATTGGCT

141 A H S L N D L L K Q H M D Q Q S S * L A

481 TTAACATTTTGTTTCCGAAATCAATCATTTCTGGCGGAATATTTCCAAATGTATCAACCG

161 L T F C F R N Q S F L A E Y F Q M Y Q P

541 CTTTATCTAAATTGAAGTATTTCTCATCTAATAAAGGGCCATACAATCCTGTTTCAGAGA

181 L Y L N * S I S H L I K G H T I L F Q R

601 AATCAAAAGGACTTGTCATGAAAATTAAATTACGAATTGGCATGTGCGGATGAAGTGCTG

201 N Q K D L S * K L N Y E L A C A D E V L

661 CATAAATAGAAGTTAGCGTTCCACCCATGCAATAACCAAGTAAAGAAATCTCGTCCGATT

221 H K * K L A F H P C N N Q V K K S R P I

721 TTGCAGTTCGCATTACTTTTTTTACTGCTTTTGCAATATAATCAAACACGAAATCATCAA

241 L Q F A L L F L L L L Q Y N Q T R N H Q

781 ATTTCAAATGACTATCTTCTAAACCAAATGTGCCCCAATCAAGCATATACACATCAAAAC

261 I S N D Y L L N Q M C P N Q A Y T H Q N

841 CACGATCCACTAGATATTCCACTAAACTATTTCCAGGAGTTAAATCCATAATATATGGTT

281 H D P L D I P L N Y F Q E L N P * Y M V

901 TATTAATAAGAGCATATATTAATAGAATTGGAACTCTTTGTGTTTTTTCTTGTTTTGGAA

301 Y * * E H I L I E L E L F V F F L V L E

961 TGTAGCGATAAAGCTTCGTCTTATTCTTCGTCCAAATAACCTCTTTCGGCGTTAATCCGA

```
321  C  S  D  K  A  S  S  Y  S  S  S  K  *  P  L  S  A  L  I  R

1021 CTTGGGGCTCTGGTTCACGTAATAAAATTTCACTCGCCCTTTTCACTCGGCGGTATGCTT

341  L  G  A  L  V  H  V  I  K  F  H  S  P  F  S  L  G  G  M  L

1081 TTCGGTACTCTTCTGGGTATAGCTCTAATTGCTTTTCCCATTCTGTTGCGAATGTAGTCA

361  F  G  T  L  L  G  I  A  L  I  A  F  P  I  L  L  R  M  *  S

1141 TTTTTCTATCTCCTTTTTGGTCGATTTCCTTCTAAAAAAAGAAGTGCATTCCTGCTATGG

381  F  F  Y  L  L  F  G  R  F  P  S  K  K  R  S  A  F  L  L  W

1201 ATATGCACTTTTTTCTTACTTCATTACATATATAATCCGCCGTTAATGTTTAATTGTTGA

401  I  C  T  F  F  L  L  H  Y  I  Y  N  P  P  L  M  F  N  C  *

1261 CCTGTGATATACGCACCGTCACGGCATAGGTATACTACACCTTTTGCAATTTCATCAGCT

421  P  V  I  Y  A  P  S  R  H  R  Y  T  T  P  F  A  I  S  S  A

1321 TGACCAAAACGTTTTTTCGGGATTTTTGCAACGATTTTTTGACGTACTTCTTCTGGTACT

441  *  P  K  R  F  F  G  I  F  A  T  I  F  *  R  T  S  S  G  T

1381 TCTGCTACCATTTCAGTATCAACAAATCCTGGGCAAATAGCGTTTACAGTGACATTTGTT

461  S  A  T  I  S  V  S  T  N  P  G  Q  I  A  F  T  V  T  F  V

1441 TTTGCAAGTTCTAACGCTAATGATTTTGTAAATCCTAACATACCCGCTTTTGCTGCTGAG

481  F  A  S  S  N  A  N  D  F  V  N  P  N  I  P  A  F  A  A  E

1501 TAGTTTGTTTGGCCAAATCCACCTGCTTGACCAATAATAGAAGAAATGCTAATAATTCTT

501  *  F  V  W  P  N  P  P  A  *  P  I  I  E  E  M  L  I  I  L

1561 CCTTCTTCTGCTTCCGTTATGTATGGAAGAACCGCGCTTGTCGTATTAAATACGCTACTT

521  P  S  S  A  S  V  M  Y  G  R  T  A  L  V  V  L  N  T  L  L
```

1621 AAATTCACGTCAATTACGCGCTCCAATCTTCACGATTTAACTTTTTGGATGTACGATCTC

5 4 1 K F T S I T R S N L H D L T F W M Y D L

1681 TTGTAATACCAGCATTATTAACAAGAATATCAACTTTACCAAAATGATTCACAGCTTCTT

5 6 1 L * Y Q H Y * Q E Y Q L Y Q N D S Q L L

1741 CTACAAGTCGGTTTGCATCTTCTACTTTAGAAACATCCGCTTGCACTGCATAAACGTCAT

5 8 1 L Q V G L H L L L * K H P L A L H K R H

1801 GTCCGTCTTTTCCTAATTCATTTACTAAATTTTCAGCTGCTTCTTTACTGCTATTATAGT

6 0 1 V R L F L I H L L N F Q L L L Y C Y Y S

1861 TAATAACTACTTTCGCTCCCTCTTGTGCTAATGCTACTGTAATTGCTTTTCCAATTCCTT

6 2 1 * * L L S L P L V L M L L * L L F Q F L

1921 TTGCTCCACCTGTTACGATTGCTACTTTTCCATTTAATTGAACCATTTCTTTTCCCCCTT

6 4 1 L L H L L R L L L F H L I E P F L F P L

1981 AAATGAATTTGTGAACGATTGTTATGAATCATTTTGGTACTCATACTATASAGTCCATAT

6 6 1 K * I C E R L L * I I L V L I L X S P Y

2041 ATAATTATGTTTCGCTGTTTATACACGAAAAGAGATTCTCCCTCTCTCTTTTACATGTTA

6 8 1 I I M F R C L Y T K R D S P S L F Y M L

2101 TAAACAAGCGCTTTTATCATTTTTTATTTTCTGGTTATTCGCAGGCTTTACCTCTTCTTT

7 0 1 * T S A F I I F Y F L V I R R L Y L F F

2161 TACAGGTACTTGTAGTTTAGCTAGTTCTGCATTTTGCTTTTCTAGCAATTCTAAAATTTG

7 2 1 Y R Y L * F S * F C I L L F * Q F * N L

2221 ATCTACTTTTGTTTCAAGCGTACGAATATCTTGTTTTACTTTCGTCACATCACGCTTTAA

```
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761  C  R  S  L  S  *  R  I  Y  F  L  L  *  K  L  L  N  V  I  Y

2341 TTTATTTTCTAAGTTAATAACAAGCGTAGCCACTCTTGCGATATCCTCTTTCGTCGGCAC

781  F  I  F  *  V  N  N  K  R  S  H  S  C  D  I  L  F  R  R  H

2401 ATTTACTTGTTCTAAATAGCTTTTTGTCGTATCATTTAATGCTTTTTGATAAAACAAATT

801  I  Y  L  F  *  I  A  F  C  R  I  I  *  C  F  L  I  K  Q  I

2461 CAAATCTAGAACGCTGCCCATCCAAGCAGAATATTCTTCTGTTTTAATTGTTTCATTGAG

821  Q  I  *  N  A  A  H  P  S  R  I  F  F  C  F  N  C  F  I  E

2521 CGCTTTTCCCCAAAATGTTTCGGTTTGTTCATAAGCATTTTTCCATGCTTGTAGTGGATC

841  R  F  S  P  K  C  F  G  L  F  I  S  I  F  P  C  L  *  W  I

2581 GAATTTTTGATCAATCACTTTACCTACACTCCTTTTTTCGTTTTTGAAACAATTCAACCT

861  E  F  L  I  N  H  F  T  Y  T  P  F  F  V  F  E  T  I  Q  P

2641 ATTCACTATTTCCTAATATTCTGAATAAAACTTCAAGTCTATATTTTATTTTTTTCTATT

881  I  H  Y  F  L  I  F  *  I  K  L  Q  V  Y  I  L  F  F  S  I

2701 TTCTAACAAATGTGTTGACTTTAAAAACAGATAGGTGTAAATTGCACTTATAAGCAAAAA

901  F  *  Q  M  C  *  L  *  K  Q  I  G  V  N  C  T  Y  K  Q  K

2761 ATTCCATCTAACTGATTCATGAGGTGATGCACAATCATGCTACAAAATGAACCAGAACAA

921  I  P  S  N  *  F  M  R  *  C  T  I  M  L  Q  N  E  P  E  Q

2821 CGCGAAAGTTTGGAGAGCAACCAAGCGAAACAACCAAACTCCATGCCAAAAAACTTCTTA

941  R  E  S  L  E  S  N  Q  A  K  Q  P  N  S  M  P  K  N  F  L
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2881 GTTCCCTTCTTACTTCTCTGTCTAAAAGACTGGAGTCTTCATGGTTACAAACTTATTCAA

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981 M L M D I G F S S V D Q G N V Y R T L R

3001 AAGTTAGAAAAAGAAAACCTTATTTCTTATACTTGGGATACAAGCGATGGAGGGCCAGCA

1001 K L E K E N L I S Y T W D T S D G G P A

3061 AAAAGAATTTATTCTTTAACTGAATATGGAGAGCAATATTTAACAACATGTGCGACTTCT

1021 K R I Y S L T E Y G E Q Y L T T C A T S

3121 TTTGAACATTATCAAAATATGTTGCGAACGTTTTTCACGTTATACACCAATGCATTCTTT

1041 F E H Y Q N M L R T F F T L Y T N A F F

3181 CCATTTTCTACTTCTCCAGAAAAGGATGAAAAGGATTCTTCATCTTCACCTGGTGGTACA

1061 P F S T S P E K D E K D S S S S P G G T

3241 GCAGAGTAATCTGCGTTCACAATATGCAAAAAAACATTTGGGGGTCAAACAATGGAAACT

1081 A E * S A F T I C K K T F G G Q T M E T

3301 AAACCATACGAATTAGTCGATGCATTTTGGAAAAACTGGTCTCAATCACTTTCCCTTTTC

1101 K P Y E L V D A F W K N W S Q S L S L F

3361 TCTTCAGCTGGGAAACAGTTAGAGCAACTTACTTTAGAAACATTAAAACAACAACAAGAC

1121 S S A G K Q L E Q L T L E T L K Q Q Q D

3421 GCTTTGCATAAGTTAACATCAGGAGTAGATGAACTAGAAAAAGAACTACAACAACTCACT

1141 A L H K L T S G V D E L E K E L Q Q L T

3481 GCTCAGTTCAATAACCAATATACAGATTACGTGAAACAATTAACTGGAAACTCCTTAAAT

1161 A Q F N N Q Y T D Y V K Q L T G N S L N

3541 GATCAAATTAACGAGTGGCAAGACAAATGGAAAGAACTTTCTGCTCATATGCATCAGCTT

1181 D Q I N E W Q D K W K E L S A H M H Q L

3601 ACCGTTTCTCCTACAAAAACTTCTTTGTCAATTCTTACACAAACAAGCGGTCAATTTGAA

1201 T V S P T K T S L S I L T Q T S G Q F E

3661 GAAACAACAAAACAATTTATTGAACAACAACAATTACAACGTGAAGAGGCTCAAAAACAG

1221 E T T K Q F I E Q Q Q L Q R E E A Q K Q

3721 TTAGAGGGTTTTTTGGAAGAGTTCAAGTCAAAACAGTTGGAACTCGCAAAAAAGTTCGAG

1241 L E G F L E E F K S K Q L E L A K K F E

3781 GAAAACTCAAAAAATCTATTTACTTCCATCAAGTAAGAGCTCACCAATCACTAGTGAATT

1261 E N S K N L F T S I K * E L T N H * * I

3841 CGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCAAGC

1281 R G R L Q V D H M G E L P T R W M Q

REFERENCES

Abe, H., Doi, Y., Fukushima, T., Eya, H. (1994) Biosynthesis from gluconate of a random copolyester consisting of 3-hydroxybutyrate and medium-chain-length 3-hydroxyalkanoates by Pseudomonas sp. 61-3. *International journal of Biological Macromolecules*. **16**:115–119.

Anderson, A. J., Dawes, E. A. (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiological Reviews.* **54**: 450-472.

Baptist, J. N (1962) Process for preparing poly-beta-hydroxybutyric acid. United States Patent 3044942.

Beom, S. K. (2000) Production of poly (3-hydroxybutyrate) from inexpensive substrates. *Enzyme and Microbial Technology*. **27**: 774-777.

Berger, E., Ramsay, B.A., Ramsay, J. A., Chavarie C. (1989) PHB recovery by hypochlorite digestion of non-PHB biomass. *Biotechnology Technique*. **3**(4): 227-232.

Brandl, H., Gross, R. A., Lenz, R. W., Fuller, R. C. (1988) Pseudomonas oleovorans as a source of poly(b-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Applied Enviroment Microbiology*. **54**:1977–1982.

Byrom, D (1987) Polymer synthesis by micro-organisms: technology and economics. *Trends Biotechnology*. **5**:246–250.

Byrom, D. (1994). Polyhydroxyalkanoates. In: D. P. Mobley (ed.), Plastic From Microbes: Microbial Synthesis of Polymers and Polymer Precursors (pp. 5-33). Munich: Hanser.

Cammas, S., Bear, M..M., Moine, L., Escalup, R., Ponchel, G, Kataoka, K., Guerin, P. (1999) Polymers of malic acid and 3- alkylmalic acid as synthetic PHAs in the design of biocom- patible hydrolyzable devices, *International journal of Biological Macromolecules*.**25**: 273–282.

Chan, P.L., Yu, V., Wai, L., Yu, H.F. (2006) Production of medium-chain-length polyhydroxyalkanoates by Pseudomonas aeruginosa with fatty acids and alternative carbon sources. *Applied Biochemistry and Biotechnology.* **129-132**: 933-41.

Chen, C., Yu, H., Cheng, Y.C., Yu, P.H., Cheung, M.K (2006) Biodegradable nanoparticles of amphiphilic triblock copolymers based on poly(3-hydroxybutyrate) and poly(ethylene glycol) as drug carriers. *Biomaterials*. **27**(27):4804-14.

Choi, J., Lee, S. Y. (1999). Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Applied Microbiology and Biotechnology*. **51**:13-21.

Curley, J. M., B. Hazer, R. W. Lenz, and R. C. Fuller (1996) Production of poly(3-hydroxyalkanoates) containing aromatic substituents by *Pseudomonas oleovorans*. *Macromolecules*. **29**:1762–1766.

Dawes, E. A., Senior, P. J. (1973) The role and regulation of energy reserve polymers in microorganisms. *Advances in Microbial Physiology*. **10**:135-266.

De, K., Kellerhals, C., Witholt, B. (1997) A process for the recovery of poly (3-hydroxyalkanoates) from pseudomonads. Process development and economic evaluation. *Bioprocess Engineering*. **17**:15–21.

De, S., Eggink, B., Witholt, J. Kingma, H. (1983) Characterization of intracellular inclusions formed by Pseudomonas oleovorans during growth on octane. *Journal of Bacteriology*. **154**(2): 870-878.

Dionisi, D., Majone, M., Papa, V., Beccari, M. (2004) Biodegradable Polymers From Organic Acids by Using Activated Sludge Enriched by Aerobic Periodic Feeding. *Biotechnology and Bioengineering*. **85**(6): 569-579.

Doi, Y., Kawaguchi, N. ,Koyama, S., Nakamura, M., Hiramitsu, Y. (1992). Synthesis and degradation of polyhydroxyalkanoates in Alcaligenes eutrophus. *FEMS Microbiology Reviews*. **103**:103-108.

Fritzsche, K., Lenz, R. W., Fuller, R. C. (1990) An unusual bacterial polyester with a phenyl pendant group. *Makromology Chemistry* **191**:1957–1965.

Fukui, T., Ito, M., Saito, S., Tomita, K. (1987) Purification and characterization of NADP-linked acetoacetyl-CoA reductase from *Zoogloea ramigera* I-16-M. *Biochimica et Biophysica Acta*. **917**:365–371.

Gagnon, K. D., Lenz, R. W., Farris, R. J., Fuller, R. C.(1994) Chemical modification of bacterial elastomers. 1. Peroxide crosslinking. *Polymer* **35**: 4358–4367.

Gaurivaud, P., Laigret, F., Garnier, M., Bove, J. (2000) Fructose utilization and pathogenicity of *Spiroplasma citri*: characterization of the fructose operon. *Gene*. 11: 61-69.

Guo, X. H., Xiong, Z., Zhou, M., Jia, S. F., Xu. Y. (1991) The construction of multifunctional shuttle vectors of *Bacillus subtilis-Escherichia coli*. *Chinese Journal of Biotechnology*.**7**:224-229.

Haywood, G. W., Anderson, A. J., Chu, L. Dawes, E. A. (1988) Characterization of two 3-ketothiolases possessing differing substrate specificities in the polyhydroxyalkanoate synthesizing organism *Alcaligenes eutrophus*. *FEMS Microbiology Letter*. **52**: 91–96.

Haywood, G. W., Anderson, A. J., Chu, L.,. Dawes, E. A (1989) The role of NADH- and NADPH-linked acetoacetyl-CoA reductases in the poly-3-hydroxybutyrate synthesizing organism *Alcaligenes eutrophus*.

FEMS Microbiology Letter. **52**:259–264.

Hazer, B., Lenz, R. W., Fuller, R. C. (1994) Biosynthesis of methylbranched poly (b-hydroxyalkanoate)s by Pseudomonas oleovorans. *Macromolecules*. **27**:45–49.

Hiraishi, A., Khan, S. T. (2003) Application of polyhydroxyalkanoates for denitrification in water and wastewater treatment. *Applied Microbiology and Biotechnology*. **61**:103-109.

Hong, K., Leung, Y.C., Kwok, S.Y., Law, K.H., Lo, W.H., Chua, H., Yu, H.F. (2000) Construction of recombinant Escherichia coli strains for polyhydroxybutyrate production using soy waste as nutrient. *Applied Biochemistry and Biotechnology*. **84–86**: 381–390.

Huijberts, G. N. M., Eggink, G., Waard, P., Huisman, G. W., Witholt, B. (1992) Pseudomonas putida KT2442 cultivated on glucose accumulates poly(3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. *Applied Environmental Microbiology*. **58**:536–544.

Huisman, G. W., Leeuw, O., Eggink, B. (1989) Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent *pseudomonads*. *Applied Environmental Microbiology*. **55**:1949–1954.

Huisman, G. W., Wonink, E., Koning, G., Preusting, H., Witholt, B. (1992) Synthesis of poly(3-hydroxyalkanoates) by mutant and recombinant Pseudomonas strains. *Applied Microbiology and Biotechnology*. **38**:1–5.

Huisman, G. W., Wonink, R., Meima, B., Kazemier, P., Terpstra, B. (1991) Metabolism of poly(3-hydroxyalkanoates) (PHAs) by Pseudomonas oleovorans. Identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA. *Journal of Biology and Chemistry*. **266**:2191–2198.

Jan, S., Roblot, G., Goethals, J., Courtois, B., Courtois, J. E., Nava S, Seguin, J., Barbotin (1995) Study of parameters affecting poly(3-hydroxybutyrate) quantification by gas chromatography. *Analytical Biochemistry*. **225**: 258-263.

Jendrossek, D., Handrick, R. (2002) Microbial degradation of polyhydroxyalkanoates. *Annular Review of Microbiology*. **56**: 403–432.

Jendrossek, D., Schirmer, Schlegel, G. (1996) Biodegradation of polyhydroxyalkanoic acids. *Applied Microbiology and Biotechnology*. **46**: 451-463.

Kaneko, T., Sato, H., Kotani, A., Tanaka, E., Asamizu, Y., Nakamura, N., Miyajima, M., Hirosawa, M., Sugiura, S., Sasamoto, T., Kimura, T., Hosouchi, A., Matsuno, A., Muraki, N., Nakazaki, K., Naruo, S., Okumura, S., Shimpo, C., Takeuchi, T., Wada, A., Watanabe, M., Yamada, M., Yasuda, S., Tabata (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and

assignment of potential protein-coding regions. *DNA Research*. **3**(l): 109–136.

Kassab, A.C., Xu, K., Denkbas, E.B., Dou, Y., Zhao, S., Piskin E (1997) Rifampicin carrying polyhydroxybutyrate microspheres as a potential chemoembolization agent. *Journal of Biomater Science Polymer*. **8**: 947–961.

Kichise, T., Taguchi, S., Doi, Y. (2002) Enhanced accumulation and changed monomer composition in polyhydroxyalkanoate (PHA) copolyester by in vitro evolution of Aeromonas caviae PHA synthase. *Applied and Environmental Microbiology*. **68**: 2411-2419.

Kim, B.S., Chang, H.N. (1995) Control of glucose feeding using exit gas data and its application to the production of PHB from tapioca bydrolysate by Alcaligenes eutrophus. *Biotechnology Techniques*. 311-314.

Kim, O., Gross, R. A., Rutherford, D. R. (1995) Bioengineering of poly (b-hydroxyalkanoates) for advanced material applications: incorporation of cyano and nitrophenoxy side chain substituents. *Canada Journal of Microbiology*. **41**(1):32–43.

Kim., B. S., Lee, S. Y., Chang, H. N. (1992) Production of poly-β-hydroxybutyrate by fed-batch culture of recombinant Escherichia coli. *Biotechnology Letters*. **14**: 811-816.

Kraak, M. N., Kessler, B., Witholt, B. (1997) In vitro activities of granulebound

poly[(R)-3-hydroxyalkanoate) polymerase C1 of Pseudomonas oleovorans. Development of an activity test for medium-chain-length-poly (3-hydroxyalkanoate) polymerases. *Europe Journal of Biochemistry*. **250**:432–439.

Laffferty, R.M., Korsatko, B., Korsatko, W. (1988) Microbial production of poly-3-hydroxybutyric acid. In: H.J. Rehm and G. Reed (Eds), Biotechnology: A Comprehensive Treatise, Special Microbial Processes. *VCH Verlagsgesellschaft Weinhheim*. **6**:135-176.

Lageveen, R. G., Huisman, G. W., Preusting, P., Ketelaar, G., Eggink, B., Witholt (1988) Formation of polyesters by Pseudomonas oleovorans: effect of substrates on formation and composition of poly(R)-3-hydroxyalkanoates and poly(R)-3-hydroxyalkenoates. *Applied Environmental Microbiology*. **54**: 2924–2932.

Langenbach, S., Rehm, B.H., Steinbuchel, A. (1997) Functional expression of the PHA synthase gene phaC1 from Pseudomonas aeruginosa in Escherichia coli results in poly (3-hydroxyalkanoate) synthesis. *FEMS Microbiology Letters*. **150** (2): 303-309.

Lee, B., Pometto, A. L., Fratzke, T. B. (1991) Biodegradation of degradable plastic polyethylene by Phanerochaete and Streptomyces species. *Applied and Environmental Microbiology*. **57**(3): 678-685.

Lee, S. Y (1996) Bacterial Polyhydroxyalkanoates. *Biotechnology*

Bioengineering. **49**: 1–14.

Lee, S. Y., Chang, H. N. (1994) Effect of complex nitrogen source on the synthesis and accumulation of poly-3-hydroxybutyric acid by recombinant Escherichia coli in flask and fed-batch cultures. *Journal of Environmental Polymer Degradation*. **2**:169-176.

Lee, S. Y., Choi, J. (1997) Production and degradation of polyhydroxyalkanoates in waste environment. *Waste Management*. **19**:133-139.

Lee, S. Y., Choi, J. (2001) Production of microbial polyester by fermentation of recombinant microorganisms. *Advances in Biochemical Engineering, Biotechnology*. **71**: 183-207.

Lee, S. Y., Choi, J., Wong, H. H. (1999) Recent advances in polyhydroxyalkanoate production by bacterial fermentation: mini-review. *International Journal of Biological Macromolecules*. **25**:31–36.

Li, W., Shimizu, H., Shioya, S., Suga, K. (1990) Optimal cultivation condition for production of biodegradable poly-beta-hydroxybutyric acid by Alcaligenes eutrophus H16. *Annual Reports of International Centre of Cooperative Research in Biotechnology, Osaka University*. **13**:113-120.

Liebergesell, M., Sonomoto, M., Madkour, F., Mayer, A., Steinbuchel (1994) Purification and characterization of the poly(hydroxyalkanoic acid) synthase from *Chromatium vinosum* and localization of the enzyme at

the surface of poly(hydroxyalkanoic acid) granules. *Europe Journal of Biochemistry*. **226**:71–80.

Liebergesell, M., Steinbuchel, A. (1992) Cloning and nucleotide sequences of genes relevant for biosynthesis of poly(3-hydroxybutyric acid) in *Chromatium vinosum* strain D. *Europe Journal of Biochemistry*. **209**:135–150.

Liebergesell, M., Steinbuchel, A. (1993) Cloning and molecular analysis of the poly(3-hydroxybutyric acid) biosynthetic genes of *Thiocystis violacea*. *Applied Microbiology and Biotechnology*. **38**:493–501.

Liu, F., Li, W., Darin, R., Tingyue, G. (1998) Porduction of poly-β-hydroxybutyrate on molasses by recombinant Escherichia coli., *Biotechnology letters*. **20**: 345-348.

Loosdrecht, M.C.M., Pot, M.A., Heijnen, J.J. (1997) Importance of bacterial storage polymers in bioprocesses. *Water Science Technology*. **35**(1): 41-47.

Lusty, C.J., Doudoroff, M. (1966) Poly-fl-hydroxybutyrate depolymerase of Pseudomanas Iemoignei. *Biochemistry*. **56**, 960-965.

Lynen (1953) Functional group of coenzyme A and its metabolic relations, especially in the fatty acid cycle. *Fed Process*. **12**:683–691.

Madison, L. L., Huisman, F. W. (1999) Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. *Microbiology and*

Molecular Biology Reviews. **63**: 21-53.

Marchessault, R. H. (1996) Tender morsels for bacteria: recent developments in microbial polyesters. *Trends Polymer Science*. **4**:163–168.

Mas, A., Jaaba, H., Schue, F., Belu, A.M., Kassis, C.M., Linton, R.W., Desimone, J. M. (1997) Surface modification of poly(hydroxybutyrate-co-9% hydroxyvalerate) by allyl alcohol plasma polymerization. *Europe Polymer Journal*. **33**: 331–337.

Masamune, S., Palmer, R., Gamboni, S., Thompson, J. T., Davis, S. F., Williams, O. P., Peoples, A. J., Sinskey, C. T., Walsh (1989) Bio-Claisen condensation catalyzed by thiolase from *Zoogloea ramigera*. Active site cysteine residues. *Journal of American Chemistry* . **111**:1879–1881.

Masamune, S., Walsh, A. J., Sinskey, O. P., Peoples (1989) Poly-(R)-3-hydroxybutyrate (PHB) biosynthesis: mechanistic studies on the biological Claisen condensation catalyzed by b-ketoacyl thiolase. *Pure Applied Chemistry*. **61**:303–312.

Mergaert, J., Webb, A., Anderson, C., Wouters, A., Swings, J. (1993) Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils. *Applied Environmental Microbial*. **59**:3233-3238.

Merrick, J.M., Doudoroff, M. (1964) Depolymerization of poly-3-hydroxybutyrate by an intracellular enzyme system. *Journal of*

Bacterial. **88**: 60-71.

Muller, W. R., Heinemann, C., Schafer, J., Wurmthaler, T. (1992) Aspects of PHA (poly-ε-hydroxy-butyric-acid) as an H-donor for denitrification in water treatment process. *Water Supply*. **10**:70-90.

Ojumu, T. V., Yu, B., Solomon, O. (2004) Production of polyhydroxyalkanoates, a bacterial biodegradable polymer. *African Journal of Biotechnology*. **3**(1):18-24.

Page, W. J., Manchak, J. (1995) The role of b-oxidation of short-chain alkanoates in polyhydroxyalkanoate copolymer synthesis in Azotobacter vinelandii UWD. *Canadian Journal of Microbiology*. **41** (Supplement 1): 106-114.

Palmer, M. A. J., Differding, R., Gamboni, S. F., Williams, O. P., Peoples, C. T., Walsh, A. J., Sinskey, S. (1991) Biosynthetic thiolase from *Zoogloea ramigera*. Evidence for a mechanism involving Cys378 as the active site base. *Journal of Biological Chemistry*. **266**:8369–8375.

Park, S. J., Ahn, P. R., Green, S. Y. (2001) Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) by metabolically engineered Escherichia coli strains. *Biomacromolecules*. **2**: 248-254.

Peoples, O. P., Sinskey, A. J. (1989a) Poly-b-hydroxybutyrate biosynthesis in *Alcaligenes eutropha* H16. Characterization of the genes encoding b-ketothiolase and acetoacetyl-CoA reductase. *Journal of Biological Chemistry*. **264**:15293–15297.

Peoples, O. P., Sinskey, A. J. (1989b) Poly-b-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (phbC). *Journal of Biological Chemistry*. **264**:15298–15303.

Poirier, Y., Nawrath, C., Somerville, C. (1995) Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. *Biotechnology*. **13**: 142-150.

Pouton, C. W., Akhtar, S. (1996) Biosynthetic polyhydroxyalkanoates and their potential in drug delivery, *Advanced Drug Delivery Review*. **18**: 133–162.

Preusting, H., Kingma, G., Huisman, A., Steinbuchel, B. (1993) Formation of polyester blends by a recombinant strain of Pseudomonas oleovorans: different poly(3-hydroxyalkanoates) are stored in separate granules. *Journal of Environmental Polymer. Degrad*. **1**:11–21.

Preusting, H., Nijenhuis, A., Witholt, B. (1990) Physical characteristics of poly(3-hydroxyalkanoates) and poly(3-hydroxyalkenoates) produced by Pseudomonas oleovorans grown on aliphatic hydrocarbons. *Macromolecules*. **23**:4220–4224.

Punrattanasin, W. (2001) The utilization of activated sludge polyhydroxyalkanoates for the production of biodegradable plastics. PhD Thesis. Virginia Polytechnic Institute and State University.

Ramsay, B. A., Lomaliza, K., Chavarie, C., Dube, B., Bataille, P., Ramsay, J. A. (1990) Production of Poly-(β-Hydroxybutyric-Co-β-Hydroxyvaleric) Acids. *Applied and Environmental Microbiology.* **56**: 2093-2098.

Ramsay, J. A., Berger, R., Voyer, C., Chavarie, B. A. (1994) Extraction of poly-3-hydroxybutyrate using chlorinated solvents. *Biotechnology Techniques*. **8**(8):589-594.

Rehm, B. H. A., Kruger, N., Steinbuchel, A. (1998) A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. *Journal of Biological Chemistry*. **273**: 24044–24051.

Reis, M.A.M., Lemos, P.C., Martins, M.J., Costa, P.C., Gonçalves, L.M.D. and Carrondo, M.J.T. (1991) Influence of sulfates and operational parameters on volatile fatty acids concentration profile in acidogenic phase. *Bioprocess Engineering*. **6**: 145 – 151.

Reusch, R.N (1995) Low molecular weight complexed poly(3-hydroxybutyrate): a dynamic and versatile molecule in vivo. *Canada Journal of Microbiology*. **41**: 50-54.

Schubert, P., Kruger, N., andSteinbuchel, A. (1991) Molecular analysis of the *Alcaligenes eutrophus* poly(3-hydroxybutyrate) biosynthesis operon: identification of the N terminus of poly(3-hydroxybutyrate) synthase and the identification of the promoter. *Journal of Bacteriology*. **173**:168–175.

Schubert, P., Steinbuchel, A., Schlegel, H. G. (1988) Cloning of the *Alcaligenes eutrophus* genes for the synthesis of poly-b-hydroxybutyrate (PHB) and synthesis of PHB in *Escherichia coli*. *Journal of Bacteriology*. **170**: 5837–5847.

Sendil, D., Gursel, I., Wise, D.L., Hasirci, V. (1999) Antibiotic release from biodegradable PHBV microparticles, *Journal of Control Release*. **59**: 207–217.

Slater, S. C., Voige, W. H., Dennis, D. E. (1988) Cloning and expression in Escherichia coli of the Alcaligenes eutrophus H16 poly-b-hydroxybutyrate biosynthetic pathway. *Journal of Bacteriology*. **170**:4431–4436.

Sodian, R., Sperling, J. S., Martin, D. P., Egozy, A., Stock, U., Mayer, J. E., Vacanti, J. P. (2000) Fabrication of a trileaflet heart valve scaffold from a polyhydroxyalkanoate biopolyester for use in tissue engineering, *Tissue Engineering*. **6**: 183–188.

Steinbuchel, A. (1996) PHB and other polyhydroxyalkanoic acids. In: H. J. Rehm and G. Reed (eds.), Biotechnology (pp. 403-464). New York: VCH Publishers.

Steinbuchel, A. (2001) Perspectives for biotechnological production and utilization of biopolymers: metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a successful example.

Macromolecular Bioscience. **1**(1):1-24.

Steinbuchel, A., Schlegel, H. F. (1991) Physiology and molecular genetics of poly(beta-hydroxy-alkanoic acid) synthesis in Alcaligene eutrophus. *Molecular Microbiology*. **5**: 535-542.

Sudesh, K., Abe, H., Doi, Y. (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in Polymer Science*. **25**(10):1503-1555.

Suzuki, T., Yamane, T., Shimizu, S. (1986) Mass production of poly-beta-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. *Applied Microbiology and Biotechnology*. **24**: 370-374.

Thompson, S., Mayer, F., Peoples, O. P., Masamune, S., Sinskey, A. J., Walsh, C. T. (1989) Mechanistic studies on b-ketoacyl thiolase from *Zoogloea ramigera*: identification of the active site nucleophile as Cys89, its mutation to Ser89, and kinetic and thermodynamic characterization of wildtype and mutant enzymes. *Biochemistry*. **28**:5735–5742.

Timm, A., Steinbuchel, A. (1990) Formation of polyesters consisting of medium-chain-length 3-hydroxyalkanoic acids from gluconate by Pseudomonas aeruginosa and other fluorescent pseudomonads. *Applied Environmental Microbiology*. **56**:3360–3367.

Timm, A., Steinbuchel, A. (1992) Cloning and molecular analysis of the poly

(3-hydroxyalkanoic acid) gene locus of Pseudomona aeroginosa PAO1. *Europe Journal of Biochemistry*. **209**:15–30.

Tsuchikura, K. (1994) 'BIOPOL' properties and processing. *Biodegradable Plastics and Polymers*. 362-364.

Valentin, H. E., Schonebaum, A., Steinbuchel, A. (1996) Identification of 5-hydroxyhexanoic acid, 4-hydroxyheptanoic acid and 4-hydroxyoctanoic acid as new constituents in bacterial polyhydroxyalkanoic acids. *Applied Microbiology and Biotechnology*. **46**: 261–267.

Valentin, H. E., Steinbchel, A. (1994) Application of enzymatically synthesized short-chain-length hydroxy fatty acid coenzyme A thioesters for assay of polyhydroxyalkanoic acid synthases. *Applied Microbiology and Biotechnology*. **40**: 699–709.

Wang, J. G., Bakken, L. R. (1998) Screening of soil bacteria for polyb-hydroxybutyric acid production and its role in the survival of starvation. *Microbiological Ecology*. **35**: 94–101.

Ward, P. G., Roo, G., O'Connor, K. E. (2005) Accumulation of polyhydroxyalkanoate from styrene and phenylacetic acid by Pseudomonas putida CA-3. *Applied and Environmental Microbiology*. **71**(4): 2046-2052.

Williams, S. F., Martin, D. P. (2002) Application of PHAs in Medicine and

Pharmacy. *Biopolymers*. **4**: Poly ester III.

Williams, S. F., Martin, D. P., Horowitz, D. M., Peoples, O. P. (1999) PHA applications: addressing the price performance issue I. Tissue engineering. *International Journal of Biological Macromolecules*. **25**:111-121.

Witholt, B., Kessler, B. (1999) Perspectives of medium chain length poly(hydroxyalkanoates), a versatile set of bacterial bioplastics Curr Opin. *Biotechnology*. **10**(3): 279-85.

Wong, A. L., Chua, H, Lo, W. H., Yu, P. H. F. (2000) Synthesis of bioplastics from food industry wastes with activated sludge biomass. *Water Science and Technology*. **41**(12): 55-59.

Yabutani, T., Maehara, A., Ueda, S., Yamane, T. (1995) Analysis of b-ketothiolase and acetoacetyl-CoA reductase genes of a methylotrophic bacterium, *Parracoccus denitrificans*, and their expression in *Escherichia coli*. *FEMS Microbiology Letter*. **133**: 85-90.

Yagmurlu, M.F., Korkusuz, F., Gursel, I., Korsuz, P., Ors, U., Hasirci, V. (1999) Sulbactam-cefoperazone polyhydroxy-butyrate-co-hydroxyvalerate (PHBV) local antibiotic delivery system: in vivo effectiveness and biocompatibility in the treatment of implant-related experimental osteomyelitis. *Journal of Biomedia Material Research*. **46**: 494–503.

Yoshiharu, D., Yasushi, K., Yoshiyuki, N., Masao, K. (1989) Nuclear magnetic

resonance studies of poly (3-hydroxybutyrate) and polyphosphate metabolism in Alcaligenes eutrophus. *Applied and Environmental Microbiology*. **55**: 2932–2938.

Yu, P. H. F., Chua, H., Huang, A. L., Lo, W. H., Ho, K. P. (1999) Transformation of industrial food wasters into poly- hydroxyalkanoates. *Water Science and Technology*. **40**(1): 365-370.

Yu, R. F., Liaw, S. L., Chang, C. N., Lu, H. J., Cheng, W. Y. (1997) Monitoring and control using on-line ORP on the continuous-flow activated sludge batch reactor system. *Water Science and Technology*. **35**(1): 57-66.

Zinn, M., Witholt, B., Egli, T. (2001) Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. *Advanced Drug Delivery Review*. **53**(1): 5-21.