MICROBIAL PRODUCTION OF POLYHYDROXYALKANOATES

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Abstract

Recent interest in biodegradable plastics has prompted growing concern over the recalcitrance of pollutants created by many of the current producers of synthetic polymers. Many countries are introducing more and more stringent environmental regulations on plastic usage. From 1986-1998, about 15% of total domestic, commercial and industrial waste in Hong Kong was petroleum-derived plastics.

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates that are biodegradable, biocompatible, microbial thermoplastics and are regarded as potentially useful in replacing conventional petroleum-derived thermoplastics.

Two PHA producing strains were isolated from activated sludge. Both strains produced PHB most effectively when hydrolyzed malt waste was used as a medium. The PHB can reach up to 37% of cell dry weight. Only homopolymer PHB has accumulated in the cells as other carbon substrates were used and the isolated bacteria were characterized by DNA sequence alignment and PHB inclusion body-associated proteins. The two most abundant proteins have a molecular mass of approximately 20

kDa and 41 kDa, respectively, and are found in all three strains. The biopolymers accumulated were analyzed by GC, ^{1}H NMR, DSC and FT-IR.

Bacillus strains are widely used as industrial production organisms. *Bacillus subtilis* is a Gram-positive non-PHA producer that is nevertheless easy to grow at very high cell density when utilizing inexpensive carbon and nitrogen sources. It is the best-studied member of the genus Bacillus and well characterized in both genetics and physiology. It is generally recognized as nonpathogenic and does not produce endotoxins.

Bacillus megaterium, the first PHA-producing strain to be discovered, was identified in the 1920s. In our laboratory, the *B. megaterium* PHA biosynthetic genes were isolated and cloned into a ϕ-105 prophage-based *B. subtilis* expression system that was shown to be good for protein overproduction. We demonstrated for the first time that the PHA biosynthetic genes from *B. megaterium* were functionally expressed in this *B. subtilis* system. Because the expression in the recombinant *B. subtilis* without heat shock was controlled by the native promoters of the *pha* genes PQRBC, the PHA yield was not very high and the experiments also suggested that the *pha* PQ genes were essential for the PHA production.

Heat shock process in *B. subtilis* 1A304 (ϕ105 MU331) could trigger its strong phage promoter. However, the recombinant *B. subtilis* showed a PHA accumulation after fermentation with the yield lower than the same strain without heat shock. This finding suggests that the heat shock process might impose adverse effects on the expression of the *pha* genes. The use of recombinant *B. subtilis* together with

inexpensive carbon sources, such as soy and malt wastes, as culture media in our laboratory would make PHA production economical and allow easier commercialization of PHA.

The *pha* genes were inserted into the vector pYCL18 and finally transformed into *B.* subtilis 168. The *pha* genes were not integrated into the chromosomal DNA and resulted in a multi-copy of the genes and a higher PHA yield which was expected since the copy number of the *pha* genes is directly proportional to the yield. The expression showed that there was no PHA in the recombinant strains. The *Pseudomonas pseudoalcaligenes* HBQ06 PHA synthase 1 *(pha*C1*)* gene was cloned into the vector pKS- and pUC19 with *pha* AB genes of *Ralstonia. eutrophus* and expressed in *E. coli* LS1298. The target of the subcloning was to produce short chain length and medium chain length PHA copolymers. However, there was also no PHA accumulation. There might be some reasons for the absence of PHA accumulation in both cases, such as plasmid stability and mutation on PCR.

The sub-cloning and expression of the *phaCAB* Gene in *E. coli* XL1-Blue and HMS174 were successful. The expression showed that the majority of biopolymer accumulated in cells was PHB and its yield was much higher in *E. coli* HMS174/PHA than that in *E. coli* XL1-Blue/PHA. In an experiment using fed-batch fermentation in a computer-controlled 3L fermenter, the cell dry weight could reach 10.27 g/L at 59 h and the P(HB-HV) content, as analyzed by GC, could reach 45% (g/g) of the cell dry weight.

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Abbreviation

A_{600}	Absorbance at wavelenth 600 nm
A. eutrophus	**Alcaligenes eutrophus**
B. megaterium	**Bacillus megaterium**
B. subtilis	**Bacillus subtilis**
BHY	Brain heart infusion with yeast extract
bp	Base pair
cat	Chloramphenicol acetyltransferase
Cm	Chloramphenicol
ddH_2O	Distilled deionized water
DNA	Deoxyribonucleic acid
dNTP	Deoxy (G, A, T, C) triphosphates
DSC	Differential scanning calorimetry
EDTA	Ethylene diaminetetraacetic acid
E. coli	*Escherichia coli*
EtBr	Ethidium bromide
Er	Erythromycin
FTIR	Fourier transform infrared
GC	Gas chromatography
h	Hour
HHx	3-hydroxyhexanoate
IPTG	isopropylthio-β-D-galactoside
kb	Kilo-base pairs
kDa	Kilo-daltons
LB	Luria-Bertani medium

Lac Z	β-galactosidase gene
M	Molar
min	minute(s)
M.W.	Molecular weight
NaOAc	Sodium acetate
NMR	Nuclear magnetic resonance
P. oleovorans	*Pseudomonas oleovorans*
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoates
pha	Genes encoding polyhydroxyalkanoates
PHB	Poly(3-hydroxybutyrate)
PHV	Poly(3-hydroxyvalerate)
R. eutrophus	*Ralstonia eutrophus*
RNA	Ribonucleic acid
RNase A	Ribonuclease A
Rpm	Revolutions per minutes
sec	Second(s)
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris-HCl	Tris [hydroxymethyl] aminomethane, adjusted pH with HCl
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis

Chapter 1: Literature Review

1.1) Biodegradable Plastics

Plastics have become an integral part of our life, and the generation of the plastic wastes has been increased dramatically. In 1986 – 1998, about 15% of the total domestic, commercial and industrial waste in Hong Kong was plastic [1]. Biodegradable plastics and polymers are certain to increase in importance because of the severe environmental contamination and waste disposal problems resulting from the chemically synthesized plastics.

The most immediate advantage of making biodegradable plastics is to tackle the problems of litter and marine pollution resulting from plastic disposal, which are difficult to solve in any other way. Moreover, many plastic products, such as packaging film, are not suitable for recovery and recycling, and these could well provide applications for degradable plastics.

1.2) Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates (PHA) are polyesters of hydroxyalkanoates synthesized by numerous bacteria as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cells, [2] usually when an

essential nutrient such as nitrogen or phosphorus is limited in the presence of excess carbon source.[3, 4]

PHA have been drawing much attention because it is a biodegradable, biocompatible, microbial thermoplastic, which is regarded as a potentially useful polyester replacing petroleum-derived thermoplastics.[5]

The first PHA discovered by Lemoigne in 1926 [6] was polyhydroxybutyrate (PHB), which is the most abundant form of PHA in nature. PHB is a highly crystalline thermoplastic sharing many properties with polypropylene. PHB or PHB-like materials are found to be stored in wide ranges of organisms, such as Gram positive and Gram negative bacteria and cyanobacteria. The repeating unit of the homopolymer PHB is 3-hydroxybutyrate. PHA represent a range of polymers and the general structural formula is shown in Fig. 1 [7]; all members of this family contain a backbone consisting of three carbon units connected by ester linkages.

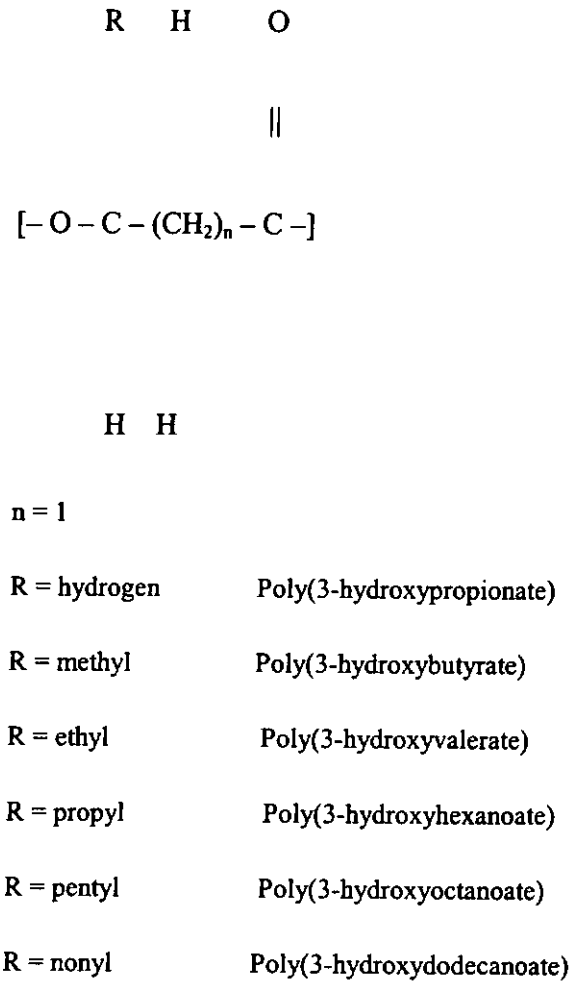


Fig. 1: General Structure of Polyhydroxyalkanoates

PHA can be divided into two groups depending on the number of carbon atoms in the monomer units: short-chain-length (scl) PHA, which consist of 3-5 carbon atoms, and medium-chain-length (mcl) PHA, which consist of 6-14 carbon atoms[2].

1.3) PHA Biocycle

One of the commercially attractive features of PHA is their degradation in the natural environment, effected principally by the enzymic activities of microorganisms[6]. Biodegradation is initiated by the action of microorganisms growing on the surface of the polymer. Several microorganisms, including species of *Aspergillus, Streptomyces, Actinomyces,* and *Pseudomonas,* are known to degrade PHA and certain bacteria, such as *Alcaligenes faecalis*, are capable of growth with PHB as sole carbon source. These microorganisms secrete extracellular enzymes, such as depolymerases and esterases, which solubilize the polymer in the immediate vicinity of the cell. The soluble degradation products are then absorbed through the cell wall and metabolized. The end products of the PHA degradation in aerobic conditions are carbon dioxide and water, but methane, carbon dioxide and water are produced under anaerobic degradation conditions[8]. Thus PHA are environmentally friendly plastics. Their biocycle is shown in Fig. 2.

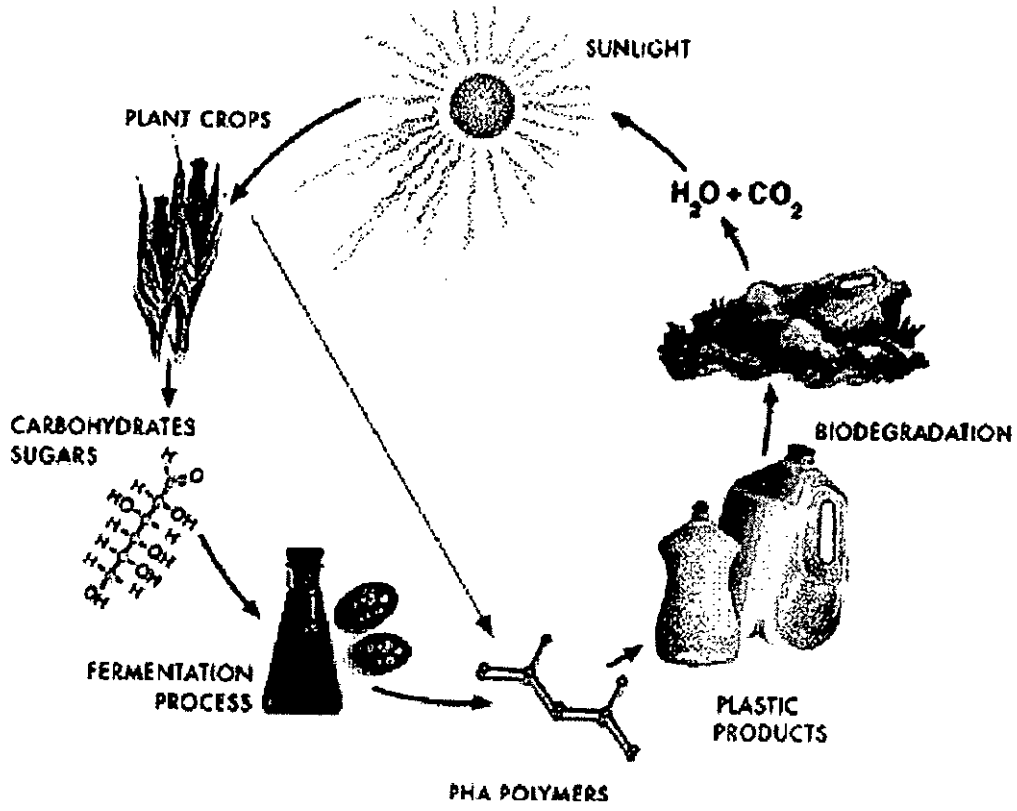


Fig. 2: The biocycle of polyhydroxyalkanoates

The figure shows how plants utilize sunlight to convert CO_2 and H_2O into carbohydrates that can be used in fermentation to make bioplastics and the degradation of these bioplastics back into CO_2 and H_2O.

It shows that photosynthesis in plant crops uses sunlight to convert carbon dioxide and water into carbohydrates that can be acted on as the raw materials for producing PHA by fermentation. A specific microorganism that occurs widely in nature is utilized in a novel transgenic fermentation process to produce PHA polymers, subsequent processing of polymers and conversion into plastic articles. PHA polymers are finally consumed and degraded in an active microbial environment as

they are dicomposed to give the end product of CO_2 and water in aerobic conditions and complete the PHA biocycle.

1.4) Economical Production of PHA

1.4.1) Use of Low Cost Substrates

The price of PHB-co-PHV exceeds 10 US dollars per kilogram, which is much higher than the cost of conventional oil-derived plastic [9]. Substrate cost is one of the major factors in the economical production of PHA. 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), [10] and thus an expensive carbon source is not practical in large-scale industrial production. There are generally two approaches that can be taken in the development of bacterial strains in order to produce PHA from an inexpensive carbon substrate:

1) Substrate utilization genes can be introduced into PHA producers.
2) PHA biosynthesis genes can be introduced into the non-PHA producers, which can utilize cheap substrates[4].

The utilization of waste materials as the substrates is a good alternative to reduce the cost of the production. Several studies had investigated the use of low cost

substrate for PHA production, such as xylose[10], which is a significant component of hemicellulose of hardwoods and crop residues, molasses[11] (a by-product in sugar production), malt waste and soya waste[12].

1.4.2) Production of PHB by Recombinant Bacteria

PHA yield is another major factor of the economical production of PHA. Bacterial species *Escherichia coli* and *Bacillus subtilis* have proven to be powerful tools in the microbial synthesis of bio-products 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 PHA synthetic capacity and to produce novel PHA.[4]

1.4.2.1) Use of recombinant *E. coli*

There are several advantages to employing recombinant *E. coli* for the production of PHA, such as short generation time, the best-understood genetics and biochemistry, and broad substrate utilization ability.

PHA-biosynthesis genes of *Alcaligenes eutrophus* have been sequenced and characterized in detail, and they were found to form an operon[13] which could be expressed from its own promoter in *E. coli*. The use of recombinant *E. coli* harboring

the PHA biosynthesis genes is attractive for the production of PHA since PHB accumulation in the intracellular can be as high as 95% (polymer weight to cell dry weight)[14].

Although acetate can be utilized by wild-type *E. coli* strains, the saturated short-chain fatty acids (SCFAs), such as butyrate, propionate and valerate, cannot directly serve as substrates for the ato enzymes. For these short-chain fatty acids to be metabolized, the ato enzymes are required as well as the fatty acid degradative enzymes encoded by fad genes. Since the expression of the ato and fad structural genes is not induced by saturated SCFAs, two mutations causing constitutive expression of the ato and fad structural genes are necessary for the metabolism of saturated SCFAs [15].

E. coli is supposedly lacking an efficient system for the conversion of propionate to propionyl-CoA, and this 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 atoC and fadR genes, respectively. The mechanism is complex and involves at least two pathways: (I) α-oxidation to pyruvate and (II) metabolism via the hydroxyglutarate pathway [15].

1.4.2.2) Use of recombinant *B. subtilis*

Bacillus subtilis bacteriophage ϕ105, isolated about 40 years ago, is a temperate phage of *B. subtilis* with a unique chromosomal attachment site [16]. The 39.2 kb phage DNA has single-strand cohensive ends that enable circulation to occur *in vitro* and presumably *in vivo*. The structural and functional similarity of phage ϕ105 to coliphage λ made it an obvious candidate for development as a gene cloning and expression vector for *B. subtilis* [17]. The bacteriophage ϕ105 was incorporated into *B. subtilis* chromosomal DNA of which the bacteriophage is only in the mode of lysogenic state. The ϕ105 MU331 prophage is an efficient expression vector based on *B. subtilis* phage ϕ105 [18] and has been proven for high-level protein overproduction in *B. subtilis*. The high level of protein synthesis obtained has been attributed to the presence of a powerful inducible phage promoter upstream from the cloning site in the phage. The promoter is controlled by a temperature sensitive phage repressor and offers temperature inducibility [19]. In this project, the feasibility of PHA production by the use of this system has been tested.

1.5) Strategies for cloning of PHA biosynthetic genes

Eight different strategies (Table 1) [20] were applied to identify PHA synthase genes and other genes involved in PHA biosynthesis. The successful application

depends on several prerequisities and constellations.

The enzymatic approach (A) simply screened clones for the expression of active enzymes involved in PHA biosynthesis. A homologous gene probe obtained after transposon mutagenesis was also used to identify the respective intact gene of the same genome (B). Heterologous gene probes, mostly prepared from the well characterized *R. eutropha* PHA synthase gene, were used to identify corresponding genes in genomic libraries prepared from other bacteria (C). Similarly, short oligonucleotides designed according to short highly conserved stretches of PHA synthases were also successfully employed (D). In one case the PHA synthase protein was purified, and oligonucleotides were designed from the N-terminal amino acids sequence to identify the corresponding gene in a genomic library (E). The most successful and widely applied strategy was to screen genomic libraries for phenotypic complementation of a PHA-negative mutant or for conferring the ability to synthesize and accumulate PHA to a PHA-negative wild type (F). Recently, another interesting strategy was employed to clone heterologous phaC genes in a pha C-negative mutant of *Rhodobacter capsulatus* utilizing the detoxification of the medium from fatty acids due to their incorporation into PHA.

Table 1: Strategies for cloning of PHA synthase genes [20]

Strategy	Principle and method applied
A	Enzymatic analysis
B	Homologous gene probes (hybridization) obtained by transposon mutagenesis
C	Heterologous gene probes (hybridization) obtained from well-characterized genes
D	Consense oligonucleotides (hybridization or PCR)
E	Oligonucleotides designed according to N-terminal amino acid sequence of enzyme
F	Opaque colonies in PHA-negative host after heterologous expression
G	Growth after detoxification of media due to removal of fatty acids
H	Analysis of genome sequence and application of PCR

1.6) Poly(3-hydroxyalkanoates) Metabolism

According to the literature, there are mainly four different pathways for the synthesis of PHA [2,6].

1) *A. eutrophus* pathway: two acetyl-CoA moieties condensed to acetoacetyl-CoA by β-ketothiolase. NADPH-dependent reductase reduces acetoacetyl-CoA D(-)-3-hydroxybutyryl-CoA, which is then linked to growing chain of P(3HB) by the PHA synthase.

2) *Rhodospirillum rubrum* pathway: it is similar to the *A. eutrophus* pathway. The difference is in second step where acetoacetyl-CoA is reduced by an NADH-dependent reductase to L(+)-hydroxybutyryl-CoA,which is then converted

to D(-)-hydroxybutyryl-CoA by two enoyl-CoA hydratases.

3) *P. oleovorans* pathway: most pseudomonads belonging to rRNA homology group I synthesize mcl-PHA from various mcl-alkanes, alkanols, or alkanoates. It was suggested that the 3-hydroxyacyl-CoA intermediates of β-oxidation pathway are channeled to PHA synthesis.

4) *P. aeruginosa* pathway: most pseudomonads belonging to rRNA homology group I, except *P. oleovorans*, also synthesize mcl-PHA from acetyl-CoA via the fatty-acid biosynthetic pathway.

Although *Bacillus megaterium* was the first discovered PHA-producing strain, there have been few studies on its molecular basis and its metabolism.

1.6.1) *Alcaligenes eutrophus H16*

The PHB biosynthetic genes of *Alcaligenes eutrophus* H16 consists of the three genes, *pha*C, *pha*A and *pha*B, which encode PHA synthase, -ketothiolase and NADPH-dependent acetoacetyl-CoA reductase, respectively. The promoter is similar to the *E. coli* promoter recognized by ^{70}. [21,22] Synthesis is started from acetyl-CoA and is accomplished by three enzymatic reactions[13], as illustrated in the following diagram (Fig. 3).

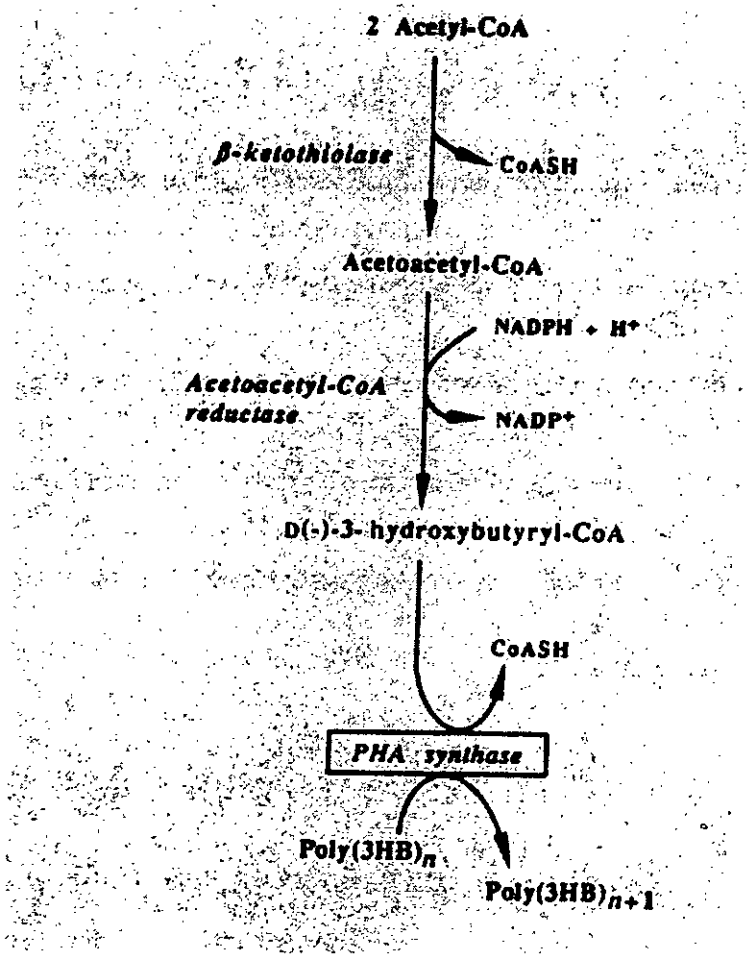


Fig. 3: PHA-biosynthetic pathway in *Alcaligenes eutrophus* H16

i) Condensation of two acetyl-CoA units to acetoacetyl-CoA.

ii) Chiral reduction of acetoacetyl-CoA to D-(-)-3- hydroxybutyl-CoA.

iii) Polymerization of D-(-)-3- hydroxybutyrate units.

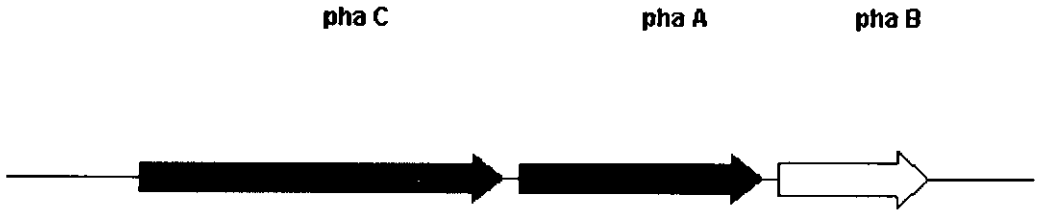
1.6.2) *Bacillus megaterium*

In *A. eutrophus*, three *pha* genes are encoded on the *pha* CAB operon, which

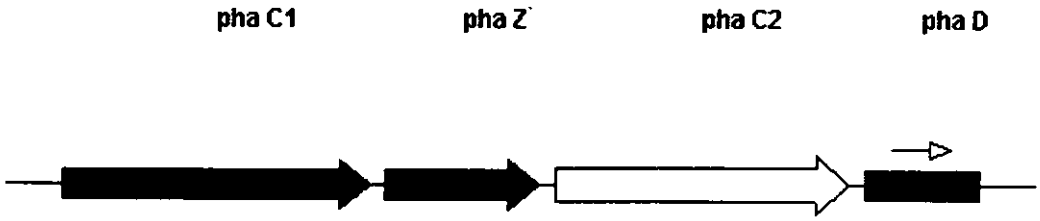
is constitutively expressed, but PHA is not constitutively synthesized. PHA inclusion bodies in *B. megaterium* are 0.2 to 0.5 μm in diameter and were shown to contain 97.7% PHA, 1.87% protein, and 0.46% lipid, with protein and lipid forming an outer layer [23]. *B. megaterium* seems to carry a 4104 bp cluster of 5 *pha* genes, *pha* P, -Q, -R, -B, and –C. The *pha* P and -Q genes were transcribed in one orientation, each from a separate promoter, while immediately upstream, *pha* R, -B, and -C were divergently transcribed as a tricistronic operon. The metabolism of PHA accumulation was still not very clear [24].

The type and arrangement of pha genes are different in different bacteria. The following diagram (Fig. 4) shows some arrangement of *pha* genes in *R. eutrophus* [21], *P. oleovorans* [25] and *B. megaterium* [24].

Ralstonia eutrophus



Pseudomonas oleovorans



Bacillus megaterium

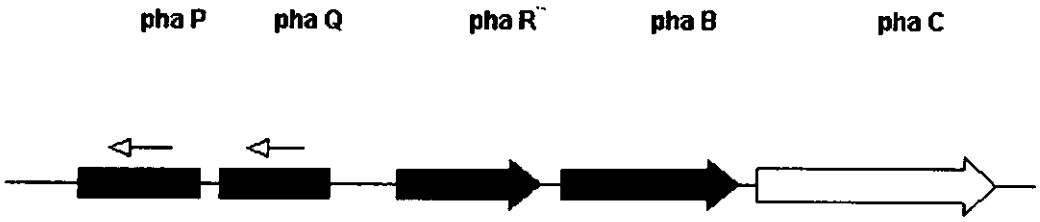


Fig. 4: The PHA gene arrangement in different bacteria

R. eutropus pha gene is composed of 3 genes, *pha*C, *pha*A and *pha*B , which are all in the same direction. *Pseudomonas pha* gene is mainly composed of 4 genes: they are *pha*C1, *pha*Z, *pha*C2 and *pha*D, and are also all in the same direction. *Bacillus megaterium pha* gene is composed of 5 genes: the *pha*P, *pha*Q, *pha*R, *pha*B and *pha*C genes. The gene direction of *pha*P and *pha*Q is opposite to the other three genes.

1.7) Efficient Expression of PHA in Recombinant Bacteria

1.7.1) Genetically Stable Plasmid

Recombinant DNA techniques are well-developed for *E. coli*, allowing easy genetic modification and alternate expression strategies. However, constitutive *phb* expression places a tremendous metabolic burden on the cells, retards the cell growth, and often results in rapid selection of mutations that lead to loss of the PHB-producing phenotype[21]. This genetic instability can be controlled by adding antibiotics during the culture. However, the use of antibiotics is rarely possible in large scale industrial production because of the high cost[26]. Thus the prophage-based expression system in PHB production has been applied in this project.

1.7.1.1) Prophage Expression System Based on *B. subtilis*

Prophage-based expression system for high levels of protein synthesis obtained has been attributed to the presence of powerful inducible phage promoter upstream from the cloning site in the phage. Phage ϕ105 was commonly used as a cloning vector in *B. subtilis* and the *B. subtilis* chromosomal DNA in the prophage was used as a homologous region for recombination to *B. subtilis* chromosome. But the recombination efficiency is low since there are only a few transformants that are

successfully inserted with prophage and ϕ105 lysogen is less competent than non-lysogens. A series of improved phage vectors have been constructed, based on *B. subtilis* bacteriophage ϕ105. The ϕ105 MU331 prophage is an efficient expression vector based on *B. subtilis* phage ϕ105. It has several advantages over plasmid systems. It is stable in the absence of selective pressure because the prophage is covalently inserted, in a single copy, into the host chromosome. The lysogenic state also involves strong repression of phage transcription, minimizing expression of potentially toxic and destabilizing genes during the growth phase. On prophage induction, a strong phage promoter is activated and expression is further enhanced by phage DNA replication giving a rapid increase in copy number [27].

1.7.2) High Plasmid Copy Number

It was suggested that high copy number is required for the accumulation of PHB to a high concentration in recombinant *E. coli* because relatively large amounts of enzymes that seem to be required to direct the carbon flow from acetyl-CoA to PHB synthesis among the several competing metabolic pathways [26].

1.7.3) Heterologous Promoter

Apart from the high gene dosage, high level PHB production can be obtained

through the use of heterologous promoters to increase the number of transcripts from the operon, or a combination. The expression of the PHB operon is generally accomplished by placing the foreign gene into a multicopy plasmid vector under the transcriptional control of either a constitutive or regulatable strong promoter (e.g. lac promoter) [28].

1.7.3.1) lac Promoter Expression System

Transcription from the lac promoter is regulated by the lac repressor, the product of lacI gene. In the absence of an inducer (e.g. IPTG), the lac repressor inhibits transcription from the lac promoter by binding to the operator region of lac operon. When the repressor is bound to the operator, its presence prevents the proper binding of RNA polymerase to the promoter region so that the transcription of the lac genes does not occur [29].

IPTG is commonly used for inducing expression from the lac promoter and it offers a number of distinct advantages. It is a metabolic-free, or gratuitous inducer, because the cell does not metabolize it. This ensures that the level of induction remains constant following the addition of IPTG to the growth medium[22]. But its high cost prevents it from being used practically on an industrial scale.

1.8) Common Analytical Method in PHA Analysis

1.8.1) Quantification of PHA by Gas Chromatography (GC)

The first method in PHB quantification described was a gravimetric method based on PHB extraction [32]. However, the drawback of using this method is that a large amount of polymer is needed to allow an accurate determination. The method described by Braunegg *et. al.*[33] remains the most common one mentioned in the literature. Since this method is based on direct acidic methanolysis on the cells followed by GC analysis of PHA produced, it has the advantages of being quick and reliable.

1.8.2) Fourier Transform Infrared (FTIR) Spectroscopy

In recent years the development of FTIR-spectroscopy and its auxiliary optics has undergone enormous progress. Photometric and wavelength accuracy, spectral quality and reproducibility of modern instruments open up a wide area of new applications. According to quantum mechanics, a molecule can take up an amount of energy to reach the first vibrationally excited state. A molecule that is irradiated with a continuous spectrum of infrared energy may absorb light quanta, which has this energy. The spectrum of the remaining radiation shows an absorption band. Vibrations that modulate the molecular dipole moment are visible in the infrared, and different

forms of vibrations are discriminated, arising from different binding forces and binding angles of atoms in a molecule. Thus, complex molecules display numerous options for internal vibrations[30]. The FTIR spectra of PHA exhibit a strong characteristic band at 1726 – 1740 cm^{-1} [31].

1.8.3) Nuclear Magnetic Resonance (NMR) for PHA Composition Analysis

The composition of the hydroxyalkanoate units in the biopolymer synthesized from microorganisms can be determined by analyzing the nuclear magnetic resonance (NMR) spectra. The advantage of NMR analysis is that the hydrolysis step of the polymer can be avoided and it requires only 2-5 mg of the polymer sample. It is useful in the copolymer mole fractions determination [e.g. hydroxybutyrate (HB) and hydroxyvalerate (HV)], which can calculate from a ratio of the peak areas that result from the 3HV methyl proton resonance and 3HB methyl proton resonance in ^{1}H-NMR. Carbon-13 NMR spectroscopy is also useful for the determination of copolymer compositions [34].

1.9) Properties of Biopolymers

Since these bacterial polyesters are biodegradable thermoplastics, their mechanical properties and physical properties have received much attention as new

environmentally compatible materials. PHB is a relatively stiff and brittle material because of its high crystallinity. However, the properties of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(HB-HV)] and poly (3-hydroxybutyrate-co-3-hydroxy-hexanoate [P(HB-HHx)], including melting point and mechanical strength, vary widely and depend on the molar percentage of 3-hydroxyvalerate (HV) or 3-hydroxyhexanoate (HHx) in the copolymer. The following table (Table 2) shows the thermal and mechanical properties of different polymer samples [35, 36].

Table 2: Thermal and Mechanical Properties of Different Polymer Samples [35,36]

Sample	Tm,°C	Tg,°C	Tensile strength, MPa	Elongation to break, %
P(3HB)	177	4	43	5
P(HB-HV) 10% HV	150	/	25	20
P(HB-HV) 20% HV	135	/	20	100
PHB-HHx(10%)	151	-1	21	400
PHB-HHx(17%)	120	-2	20	850
PHB-HHx(25%)	52	-4	/	/
Polypropylene	176	-10	38	400

In Table 2, the tensile strength of the polymer decreased from 43 to 20 MPa as the 3HHx or 3HV fraction was increased from 0 to 17 mol % and 20 mol %, respectively. In contrast, the elongation to break increased from 5% to 850% and

100% respectively. This result indicates that the copolymer becomes soft and flexible with an increase in the 3HHx and HV fraction.

1.10) Applications of PHA

Biodegradable plastics and polymers are becoming more significant as environmental contamination and waste disposal problems associated with plastics become more severe. The three areas in which the exploitation of degradable plastics receives the most attention are medical, agriculture and packaging.

PHB and poly(3HB-co-3HV) are nontoxic and biodegradable, giving rise to several applications of the polymers for controlled drug release, such as the release of buserelin. Other possible medical applications are as surgical sutures that do not require surgical removal, surgical swabs, wound dressings or lubricants for surgeons' gloves[37]. In addition, the range of biomaterials available for use in tissue engineering as temporary three-dimensional scaffolds, which can support cell growth and then degrade away leaving viable tissue, has been limited. The PHA polymers promise to extend significantly the range of biomaterials suitable for tissue engineering [38].

In agriculture, degradable plastics have been used in agricultural mulch films. Because of the short life property of packaging film, the use of the biodegradable plastic is more environmentally friendly.

Industrial application of PHB has been hampered owing to its low thermal stability and excessive brittleness upon storage. The copolymer PHB-co-PHV is more flexible and tougher, and can be used to make various products, including film, coated paper and boards, compost bags, disposal food service-ware, and molded products such as bottles[4, 7]. As PHA are optically active, the monomers derived from the polyesters have also been considered as a source for the synthesis of enantiomeric pure chemicals[37].

Aims of Project

1. To screen and identify the isolated novel *Bacillus* species from activated sludge

2. To construct recombinant *Bacillus subtilus* the using *pha* genes isolated from *Bacillus megaterium* ATCC 11561

3. To construct recombinant *Escherichia coli* for rapid and high-yield production of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate)

4. To optimize and characterize PHA production from recombinant bacteria in shake flask

Chapter 2: Materials and Methods

2.1) Materials

2.1.1) Bacterial Strains

a) *E. coli* XL-1 Blue obtained from Dr. Thomas Leung.

b) *E. coli* HMS174 (recA1 hsdR; Rifr) obtained from the *E. coli* Genetic Stock Center at Yale University

c) *E. coli* XL-1 Blue harboring the plasmid pKS- (2.96 kb) and the entire PHA operon (5.2 kb) from *Ralstonia eutrophus* H16 was obtained from Dr. Thomas Leung (Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University, China). It is ampicillin resistant (80 μg/ml) and possesses a strong lac promoter.

d) Recombinant *E. coli* HMS174 harboring the plasmid pJM9131 was given by Dr. Doug Dennis (Department of Biology, James Madison University, USA). The multicopy plasmid is kanamycin resistant (50 μg/ml) and it contains the entire *pha* operon (5.2kb) from *Ralstonia eutrophus* H16. It possesses the promoter similar to the *E. coli* promoter recognized by ^{70}. Restriction enzymes *Eco*RI and *Hin*dIII can be used to cut the *pha* operon out.

e) *E. coli* LS1298 (C600 fadB::Kan) was given by Professor Alexander Steinbuchel

(Institut für Mikrobiologie, University of Münster, Germany). It is kanamycin resistant (50 μg/ml).

f) *E. coli* HB101 harboring the plasmid pGEM-HBQ06 (5.94 kb) that including *Pseudomonas pseudoalcaligenes* HBQ06 PHA synthase 1 (*pha*C1) gene isolated from soil, was obtained from Professor GQ Chen (Department of Biology, Tsinghua University, China). It is ampicillin resistant (80 μg/ml).

g) *Bacillus megaterium* ATCC 11561 was given by Dr. Maura C. Cannon (Department of Biochemistry and Molecular Biology, University of Massachusetts, USA).

h) *E. coli* XL1-Blue harboring the plasmid pGEM10 (10.86 kb) that includes the entire PHA operon from Bacillus megaterium ATCC 11561, was given by Dr. Maura C. Cannon (Department of Biochemistry and Molecular Biology, University of Massachusetts, USA).

i) ϕ-105 prophage-based *B. subtilis* was obtained from Dr. Thomas Leung. It is erythromycin resistant (5 mg/ml).

j) *E. coli* RR1 harboring the plasmid pYCL18 (8.2 kb) was given by Dr. Thomas Leung. It is ampicillin resistant (80 μg/ml), chloramphenicol resistant (15 μg/ml) and also erythromycin resistant (100 μg/ml). This plasmid is a shuttle plasmid and can replicate in both *E. coli* and *B. subtilis*.

k) *Bacillus subtilus* 168 was kindly provided by Dr. Thomas Leung.

2.1.2) Culture media

a) Food Waste Medium

Malt waste, mostly semisolids of spent barley and millet refuse, was obtained from Carlsberg Company and San Miguel Ltd., both local beer breweries. Soy waste, chiefly semisolid cellular residues of soy beans, was collected from Vitasoy International Holdings Ltd, a local soy milk company. The ratio of the C and N contents of the malt and soy wastes were 7:1 and 8:1, respectively, as determined by total organic carbon (TOC) and total Kjeldahl nitrogen (TKN) methods, which were carried out according to the standard methods. [39]

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 14333 × 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.

b) Subculture Media

All media were autoclaved at 121 °C for 20 min.

i. 2XYT Medium

16 g/L tryptone, 10 g/L yeast extract and 5 g of NaCl, pH 7.0

ii. Luria-Bertani (LB) Medium

10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0

iii) BHY Medium

37 g/L brain heart infusion, 5 g/L yeast extract, pH 7.0

iv) Nutrient-rich Growth Medium

Medium A: 20 g/L LB broth and 10 g/L glucose

Medium B: 7 g/L of NH_4Cl, 1.5 g/L of KH_2PO_4, 1.5 g/L of Na_2HPO_4, 0.35 g/L of K_2SO_4, 0.17 g/L of $MgSO_4 \cdot 7H_2O$, 10 g/L of selected carbon source, pH7.0

c) Nitrogen-limited PHA Production Medium (Medium C)

It contains 3.57 g/L Na_2HPO_4, 0.25 g/L $(NH_4)_2SO_2$, 1.50 g/L KH_2PO_4, 0.20g/L $MgSO_4 \cdot 7H_2O$ and 20g/L glucose) with 0.1 ml of trace elements solution (100 ml water consisting of 0.60 g $FeCl_3 \cdot H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.03 g H_3BO_3, 0.002 g $CoCl_2 \cdot 6H_2O$, 0.010 g $ZnSO_4 \cdot 7H_2O$, 0.003 g $MnCl_2 \cdot 4H_2O$, 0.003 g $Na_2MoO_4 \cdot 2H_2O$, 0.0024 g $NiCl_2 \cdot 6H_2O$, 0.001 g $CuSO_4 \cdot 5H_2O$), pH 7.0

d) Fermenter Fermentation Media

i) Start Medium

It contains 15 g/L of glucose, 7 g/L of KH_2PO_4, 1 g/L of $MgSO_4$, 1.5 g/L citrate, 6 g/L tryptone, 2 g/L yeast extract and 1.5 g/L propionate; pH 7.0

ii) Feeding Medium

It contains contained 750 g/L glucose and 15 g/L $MgSO_4 \cdot 7H_2O$, and 75 g/L propionate.

e) *B. subtilis* Transformation Media

i) Pre-transformation Medium (PTM)

SMM	10 ml
40% w/v glucose	0.25 ml
Solution P	0.1 ml
Casamino acid (2 g/ 10ml)	0.2 ml
Tryptophen (10 mg/ 5ml)	0.1 ml
Isoleucine (100 mg/ 5 ml)	0.1 ml
Valine (100 mg/ 5 ml)	0.1 ml
Leucine (100 mg/ 5 ml)	0.1 ml
Methionine (25 mg/ 5 ml)	0.1 ml

ii) Transformation Medium (TM)

SMM	10 ml
40% w/v glucose	0.15 ml
2.465 g $MgSO_4$ / 10 ml	0.05 ml
Casamino acid (2 g/ 10ml)	5 ul
Tryptophen (10 mg/ 5ml)	0.1 ml
Isoleucine (100 mg/ 5 ml)	0.1 ml
Valine (100 mg/ 5 ml)	0.1 ml
Leucine (100 mg/ 5 ml)	0.1 ml
Methionine (25 mg/ 5 ml)	0.1 ml

iii) Spizizen Minimal Medium (SMM)

0.2% w/v $(NH_4)_2SO_4$, 1.4% w/v K_2PO_4, 0.6% w/v KH_2PO_4, 0.1% w/v sodium citrate dihydrate and 0.02% w/v magnesium sulphate.

iv) Solution P

2.465 g $MgSO_4$ / 10 ml	2.5 ml
0.147 g $CaCl_2$ / 10 ml	0.5 ml
0.11 g $MnSO_4$ / 10 ml	0.1 ml
ddH2O	7 ml

2.1.3) Buffer

a) TBE Buffer

108 g/L Tris base, 55 g/L boric acid and 20 ml 1 M EDTA, pH8.0.

b) 6x Agarose Gel Loading Buffer

40% (w/v) sucrose, 0.25% (w/v) bromophenol blue.

c) TE Buffer

10 mM Tris-base, 0.1 mM EDTA, pH was adjusted by HCl to pH8.0

2.1.4) Regents Used in Midipreps

a) Buffer P1

50 mM Tris-Cl, pH8.0; 10 mM EDTA; 100 μg/ml RNase A

b) Buffer P2

200 mM NaOH, 1% SDS

c) Buffer P3

3 M potassium acetate, pH 5.5

d) Buffer QBT

750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Triton X-100

e) Buffer QC

1.0 M NaCl; 50 mM Tris-Cl, pH 8.5; 15% isopropanol

f) Buffer QF

1.25 M NaCl; 50 mM Tris-Cl, pH 8.5; 15% isopropanol

2.2) General Methodology

2.2.1) Extraction of Biopolymers

An appropriate amount of freeze-dried cell powder was treated with a dispersion of chloroform and 10% sodium hypochlorite solution (1:1 in volume). After the cell powder was incubated at 37 with 250 rpm agitation for 1 hr, the mixture was centrifuged at 2610 × g for 15 min., which resulted in three separate layers. The upper phase was a hypochlorite solution, the middle phase contained the non-PHB cell material and undisrupted cells, and the bottom phase was chloroform containing PHB [40].

The upper phase was first removed with a pipette, and the bottom phase chloroform layer was filtered by glass fiber filter paper GC50 (Advantec). The chloroform is allowed to air evaporate in order to reduce the volume. Finally, pure

PHB was obtained by nonsolvent precipitation (chloroform: methanol in ratio 1: 9).

2.2.2) Analytical Methods

i) Gas Chromatography (GC) Analysis of the PHB

1 ml esterification solution (3 ml 95-98% H_2SO_4, 0.29 g benzoate and 97ml methanol), freeze-dried cells or extract polymer and 1 ml chloroform were heated at 100 for 4 h, distilled deionized water (dH_2O) was added and the solution was vortexed to enhance phase separation. 1ml of ddH_2O was added to the cooled mixture, which was vortexed for phase separation. 1 µl 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-foot-long Supelco (10% Carbowax 20 M with 80/100 in mesh size Chromosorb WAW) packed column. Nitrogen was chosen as the carrier gas at a flow rate of 20 ml/min. A flame ionization detector was used. The sample injection volume was 1 µl.

The oven temperature for GC was initially set at 135 and the temperature was kept stable for 15 min to determine both the content and composition of the polymer by comparing it to the purchased PHB and PHV standards. The fractionated products were detected by flame ionization. The temperature of the injection port was 260 , and the detector temperature was 300 . The composition of the PHA samples was

determined from the peak areas and retention times.

ii) Analysis by Differential Scanning Calorimetry (DSC)

A Mettler DSC 30 Thermal Analysis System (Mettler) was used. 3 to 4 mg of extracted biopolymers were encapsulated in aluminum pans for the measurements. The sample was first annealed at 200°C for 3 min, quenched to 50°C and then scanned at a rate of 10°C/min from 50°C to 200°C. Melting point was determined. Dry nitrogen was used as flow gas at a flow rate of 30 ml/min.

iii) ^{1}H Nuclear Magnetic Resonance (^{1}H NMR)

The ^{1}H NMR analysis was carried out on a Bruker DPX-400 Spectrometer. ^{1}H NMR spectra of a deuteriated chloroform ($CDCl_3$) solution of the extracted biopolymers were recorded at room temperature. 10 mg to 20 mg samples were put into the NMR tube and 1 ml of $CDCl_3$ was added to dissolve the sample for measurement. Tetramethylsilane was used as an internal reference [44].

iv) Fourier Transform Infrared (FTIR) Spectroscopy

2-5 ml of the cell culture was centrifuged at 2610 × 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 autogain, spectra were recorded at wavenumbers (cm^{-1}) from 400 to 4000 using a Mangna-IR spectrometer 750 (Nicolet) [41]. The height ratio (Fig. 5) of the PHA peak with wavenumbers about 1726 cm^{-1} [34,45,46] and the protein peak next to it with wavenumbers about 1650 cm^{-1} (a/b) could be used for the semi-quantitative analysis of the PHA content in cells [41]. This measurement is not as accurate as GC analysis, but the measurement is simple and fast.

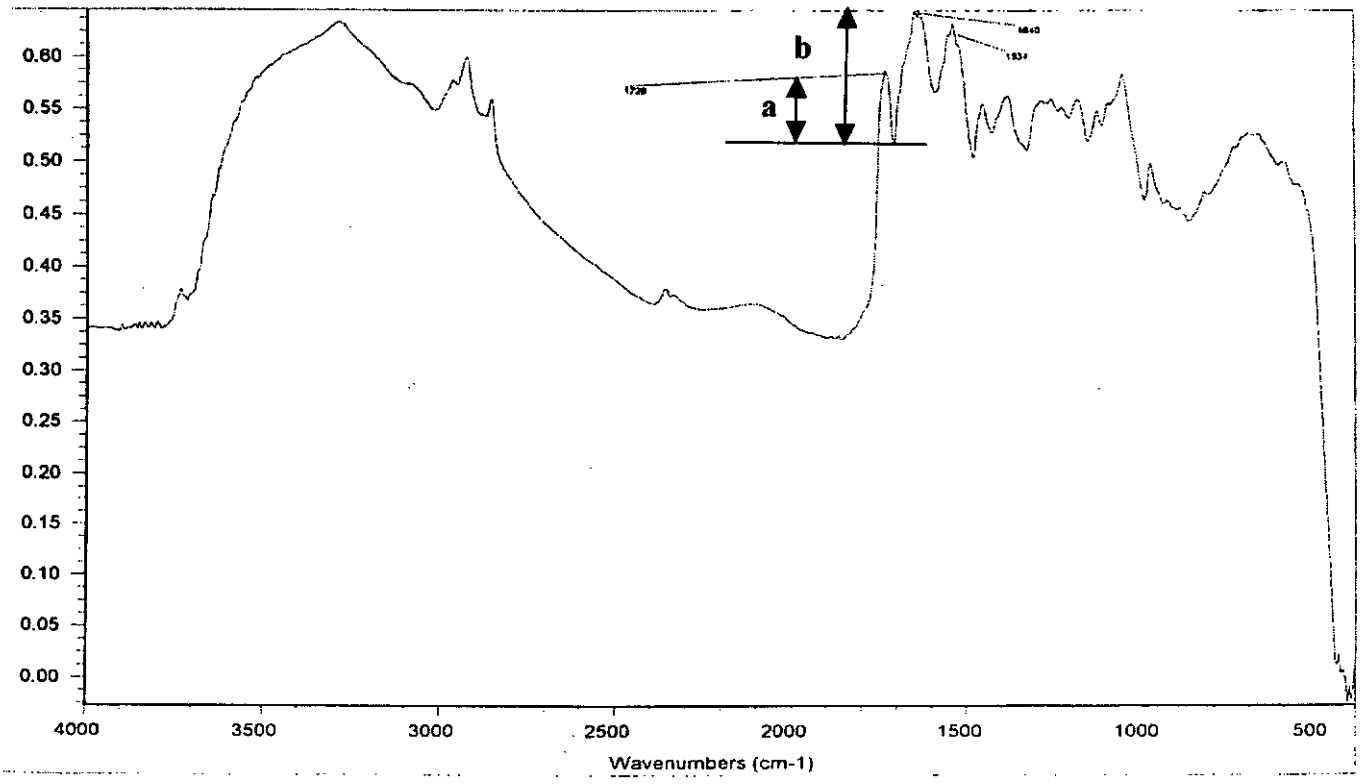


Fig. 5: The Measurement of Peak Height Ratio on FTIR Spectrum

2.2.3) SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The proteins in inclusion bodies were examined by SDS-PAGE, according to the method of Laemmli [42]. It was performed by using 7 cm x 8 cm x 0.75 mm (length x width x thickness) slab gel Mini-Protein II SDS-PAGE apparatus (Bio-Rad). After polymerization, the gels were subjected to electrophoresis in 1x running buffer at 200 volts for 1 hour. The gels were stained with Coomassie Brilliant Blue (250 ml ddH_2O, 250 ml methanol, 100 ml acetic acid and 0.6 g Comassie Blue R-250) and destained with destaining solution (10% w/v acetic acid, 10% w/v methanol).

2.2.4) 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 an eppendorf for 3 minutes at 10000 × g; 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 µg/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 for less than 5 min. 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), then inverted several times and centrifuged at 13200 × g in a microcentrifuge for 10 min.

The cleared lysate was transferred to the spin column with a 2ml collection tube. After centrifugation at 13200 × g for 1 min., the flow-through was discarded. 750µL of the Column Wash Solution (60 mM potassium acetate, 10 mM Tris-HCl, pH7.5 and 60% ethanol) was added and the column was centrifuged at 13200 × g for 1 min. and the flow-through was also discarded. 250 µl Column Wash Solution was added again and the column was centrifuged at 13200 × g for 5 min. The spin column was then transferred to a autoclaved 1.5 ml eppendorf, 100µl nuclease-free water was added into spin column and centrifuged at 13200 × g for 1 min. to elute the DNA.

2.2.5) 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 an appropriate antibiotic and incubated for 8 hours at 37°C with 280 rpm shaking. 1% of the culture was inoculated into 100 ml LB medium and incubated at 37°C with 280

rpm shaking overnight. 100 ml culture was centrifuged at 6000 × g for 15 min at 4°C. The cell pellets were re-suspended in 4 ml Buffer P1, 4 ml of Buffer P2 was then added and the solution was incubated for 5 min at room temperature. 4 ml of chilled Buffer P3 was added and incubated in ice for 15 min. It was centrifuged at 20000 × g for 45 min at 4°C and the supernatant containing the plasmid DNA was transferred to the QIAGEN-tip 500 column that was pre-equilibrated using 4 ml Buffer QBT. 10 ml Buffer QC was then applied twice to the column and the DNA was eluted with 5 ml of Buffer QF. 3.5 ml of isopropanol was added to the eluted plasmid DNA and the solution was centrifuged at 15000 × g for 30 min at 4°C. The supernatant was removed and 1.5 ml 70% ethanol was added and the solution was centrifuged at 15000 × g for 10 min. The ethanol was carefully removed and the pellet was air-dried. Finally, 100 µl deionized distilled water was added to re-dissolve the plasmid DNA.

2.2.6) 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 1x TBE buffer. The DNA sample was mixed with an appropriate amount of 6x agarose gel loading buffer to give 1x final concentration. The samples were loaded onto slots of the gel placed in the electrophoresis tank submersed in 1x TBE buffer. 100 volts

was applied in electrophoresis and the gel was examined under UV after the bromophenol blue with the sample was migrated for an appropriate distance through the gel. The DNA marker used was 1kb DNA Ladder (Promega).

2.2.7) 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 h or longer. After digestion, the reaction mixture was analyzed by agarose gel electrophoresis.

2.2.8) Dephosphorylation of Plasmid Vector

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

2.2.9) Chromosomal DNA Isolation

5 ml culture was centrifuged down and the cell pellet was resuspended by TE buffer, pH 8 and the suspension was frozen using 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 13200 × g for 30 s and the supernatant was collected. In order to obtain the genomic DNA at a higher level of purity, the supernatant was underwent phenol-chloroform extraction and ethanol preoccupation to remove contaminants.

2.2.10) Primer Design

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

2.2.11) Polymerase Chain Reaction (PCR)

The Expand High Fidelity PCR System (Boehringer Mannheim) or Taq DNA

Polymerase (Boehringer Mannheim) was used for the polymerase chain reaction (PCR). The PCR was used to select and amplify the *pha* gene cluster of *B. megaterium* 11561 by using a pair of oligonucleotide primers, each of which was complementary to one end of the target DNA sequence. Two primers were extended toward each other by thermostable DNA polymerase in a reaction cycle of denaturation, primer annealing and polymerization.

Final concentration of 1x 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 Expand High Fidelity Enzyme Mix or 2.5 units of Taq Polymerase were 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 min,

Step 2: 94°C cycle denaturation for 1 min;

Step 3: 54°C primer annealing for 1 min;

Step 4: 68°C extension for the time according the size of the amplification fragment (1 min for each kilobase of DNA)

Step 5: Repeat steps 2 to 4 for 35 times;

Step 6: 72°C final elongation for 7 min;

Step 7: 4°C for infinity.

2.2.12) Phenol / Chloroform Extraction

Equal volume of phenol/chloroform mixture (phenol: chloroform: isoamyl alcohol in ratio of 25: 24: 1) was added to the reaction mixture after CIAP hydrolyzation. The mixture was gently mixed by hand agitation until an emulsion was formed. The mixture was then centrifuged at 16100 × g for 30 s in a microcentrifuge.

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

2.2.13) Ethanol Precipitation

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

The eppendorf was deep-frozen at - 80 for 30 min. in order to precipitate the DNA, and then was subjected to centrifugation at 16100 × g for 10 min. The supernatant was discarded and the pellet was washed in cold 70% ethanol (without disturb the pellet) and re-spun for 2 minutes. The supernatant was discarded and the pellet from the eppendorf was dried by DNA SpeedVac (Savant Model SC100) for 20 min. 20 µl sterile water was added to dissolve the dried pellet and incubated at 37 for 5 min. to enhance the detachment of DNA pellet from the wall of eppendorf.

2.2.14) Purification of DNA Fragments from Agarose Gel

The DNA samples were electrophoresed on a 1x TBE agarose gel. The desired DNA was purified by QIAEX II Gel Extraction Kit (Qiagen). The 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 for 10 min. with vortex mixing 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 16100 × g for 30 s and the resin was pelleted with the discarded supernatant. 500 µl of Buffer QX1 was added to remove any residual agarose contaminants and the pellet was resuspended by vortexing, and the above centrifugation steps were repeated. The above wash steps were also repeated twice 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 resuspended and incubated at 50 for 5 min. The eppendorf was finally centrifuged at 16100 × g for 30 s, and the supernatant containing desired DNA was transferred to a clean autoclaved eppendorf.

2.2.15) Preparation of Competent Cells

Appropriate *E. coli* from glycerol stock was inoculated in 5 ml sterilized 2XYT and incubated at 37 overnight with 250 rpm shaking. 200 µl portion of the culture was added into 20ml sterilized 2XYT and then incubated at 37 until the A_{600} reached the range of 0.3 – 0.4. The cells were collected by centrifugation at 2500 rpm for 15 min. at 4 . The supernatant was discarded and 10 ml cold 100 mM sterilized $CaCl_2$ was added to resuspend the pellet by mild vortexing. The tube was incubated in ice for 25 min. and the cells were pelleted again by 2500 rpm for 15 min. in 4 and resuspended in 2ml cold 100mM sterilized $CaCl_2$ solution. The resulting competent cells were incubated in 4 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 .

2.2.16) Ligation Reaction

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

2.2.17) Transformation of Ligation Reaction Mixes or Plasmid DNA into *E. coli*

Frozen competent cells were incubated on ice until the cell suspension was just thawed. Ligation reaction mixes or plasmid DNA 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 for 2 min and immediately chilled on ice for 2 min. 200 µl 2XYT or LB medium was added and incubated at 37 for 1.5 hr. Portions of 50µL and 200µL transformed cells were spread on nutrient agar plates with an appropriate concentration of antibiotics, and the plates were incubated at 37 overnight.

2.2.18) Transformation of *Bacillus subtilis*

Bacillus subtilis strain was first streaked on a nutrient agar plate with

appropriate antibiotic and incubated overnight at 37°C. Cells from the overnight incubated plate were heavily inoculated (about 0.9 at A_{600}) into a 100 ml Erlenmeyer flask containing 5 ml pre-transformation medium (PTM). The culture was incubated at 37°C with 280 rpm shaking. After the A_{600} reached 3.0 (3.0 to 3.5 is acceptable), the stationary phase of the culture was reached. 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 (5 µg/ml). The plates were incubated at 37°C overnight or 2 days and the colonies on agar plates were counted.

2.2.19) Thermo-induction of derivatives of *B. subtilis* 1A304 (ϕ105 MU331)

The strain *B. subtilis* 1A304 (ϕ105 MU331) with *pha* operon was transferred from glycerol stock to 5 ml of BHY, and 5 µg/ml chloramphenicol was used. The culture was incubated overnight at 37°C with 280 rpm shaking. A volume of 1 ml of overnight incubated culture was inoculated into a 100 ml Erlenmeyer flask containing 15 ml sterilized BHY broth without antibiotics added. The flask then incubated at 37°C shaking at 280 rpm. After the A_{600} reached 3.5, the culture was

incubated at 50°C for 3 min with vigorous shaking. Then the culture was incubated at 37°C with 280 rpm shaking. The cells were collected at different time intervals and subjected to analysis.

2.2.20) Cycle Sequencing

ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was used in cycle sequencing. The reaction contained 500 ng of the template DNA, 4 μl of the Terminator Ready Reaction Mix and 3.2 pmol of the primer, and sterile water was used to fill up the reaction mixture to 10 μl when necessary. The reaction mixture then underwent the cycle sequencing on the PCR machine and the conditions were listed below:

96°C for 10 s

50°C for 50 s

60°C for 4 min

Total cycles: 25 cycles

The 10 μl of the reaction mixture was purified as the protocol of ethanol precipitation mentioned before. But 95% ethanol was used instead of absolute ethanol and 20 minutes was used to precipitate the DNA at room temperature instead of 30 min at -80°C. Absolute ethanol absorbs water from the atmosphere, gradually

decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, and extensive precipitation will increase the precipitation of unincorporated dye terminator. Finally, the pellet was resuspended in 10 μl of Template Suppression Reagent to prevent the formation of bubbles at the tip of the capillary, and the mixture was denatured at 95°C for 3 min and incubated in ice immediately. The reaction mixture was then subjected to sequencing using the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequence assembly was performed by using Advanced BLAST (National Center for Biotechnology Information).

2.3) PHA-producing Strain from Activated Sludge

2.3.1) Screening of PHA Producing Bacteria from Activated Sludge

Activated sludge (50 ml) was transferred into conical flasks. The flasks were placed in a water bath at 87°C for 8 min to kill all non-spore forming cells, leaving heat-resistant survival *Bacillus* spores. The sludge sample was centrifuged at 2610 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in 100 ml of sterile Medium A and incubated for 18 h at 37°C with 250 rpm shaking. The culture from Medium A (25 ml) was centrifuged at 2610 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in 100 ml Medium A. The culture was incubated at 37°C for 16 h with 250 rpm shaking for the PHB

accumulation.

Serial dilutions of 10^{-1} to 10^{-7} of cell culture from media B were prepared (the culture was diluted with 0.9% NaCl solution). The culture from each dilution was streaked on Production Agar (Media C, 1 ml/L trace elements solution and 1.5% agar) plate. The plates were incubated for 16 h at 37°C. The cells from each single colony were subjected to FT-IR analysis, Gram's staining, endospore staining and microscopic morphology examination.

2.3.2) Identification of Selective Strains

The isolated strains were further identified by the Microbial ID, Inc. (125 Sandy Drive, Newark, DE 19713, USA). The identification was based on the fatty acid composition profiles of the bacteria and the Similarity Index was used to express its similarity of to the other known bacteria.

2.3.3) Fermentation of Selective Strains

Single stage fermentation process was employed. A loopful of selective strain was inoculated in 5 ml of LB medium in a universal bottle and incubated at 37 with 250 rpm shaking for 16 h. 1% total culture volume of seed culture cells was then transferred to the food waste media or Medium B, in which glucose, maltose, sucrose

were used as the carbon source. The cultivation was maintained at 37 and agitated at 250 rpm. The samples were collected and periodically analysed.

2.3.4) PHB Inclusion Bodies Isolation

The proteins in inclusion bodies of *B. megaterium* 11561, HF-1 and HF-3 after fermentation were isolated for analysis. The culture was pelleted at 6000 × g in 4 °C for 20 min and resuspended in 5 ml of 10 mM Tris-HCl, pH 8, 1 mM EDTA, 20 mM $MgSO_4$ and 0.25 M sucrose at 4°C. Lysozyme was added to a final concentration of 1.5 mg/ml and incubated at 37°C for 15 min and at room temperature for 10 min. The cells were broken by sonication for 10 min.

Aliquots of 1 ml of lysate were loaded on sucrose step gradients in 5 ml ultracentrifuge tubes and consisted of 0.9 ml of each of the following sucrose concentrations: 2.0 M, 1.66 M, 1.33 M, 1.0 M and 0.4 ml of 0.66 M in TE (10 mM, Tris-HCl, pH 8, 1 mM EDTA). The tubes were centrifuged in 150,000 × g (Hitachi automatic preparative ultracentrifuges CP70G) for 1 h at 10°C. The inclusion bodies, which banded about mid-tube, were collected, washed in 20 volumes TE, and pelleted at 20000 × g. The sucrose gradient steps were repeated for further purification and purified inclusion bodies were stored in TE buffer at 4°C [43].

The pelleted inclusion bodies were resuspended in TE buffer with 2% sodium

dodecyl sulfate (SDS). An equal volume of 2× sample buffer was added prior to boiling for 5 min, samples were centrifuged for 3 min to pellet PHA and the supernatant was loaded on 12% SDS polyacrylamide gel. Coomassie Brilliant Blue R-250 was used to stain the gel after electrophoresis.

2.3.5) Further Identification of the Strains HF-1 & -3

Both strains were suggested to be *B. megaterium* as identified by Microbial ID, Inc. (Newark, DE, USA) It was also suggested that the HF-1 was more likely to be *B. megaterium* than HF-3 (see Results). Therefore, the genomic DNA of HF-1 and HF-3 were isolated and subjected to PCR reaction. The primer sequences used for identification of HF-1 & 3 were listed below, which were designed according to the sequence of *pha* P and Q genes (total about 1.2 kb) of *B. megaterium* ATCC 11561. LKH 1 was the forward primer and LKH 7 was the reverse primer.

The 5 to 6 arbitrary bases (blue colour) were designed before the restriction sequence (red colour bases), BamHI was designed in primer LKH 4 and SacI was designed in LKH 7 to make it easier to recognize the sequence by the restriction enzyme.

Target	Primer Name	Sequence
PQ	LKH 4	ACT CGT GGA TCC TAC TGA ACC GTT GCT GCG TG
	LKH 7	GGC GCC TCG AGT ACA CAT ATG GAA AAC AAA TTC TC

2.3.6) Brief Outline for the Experiment (Fig. 6)

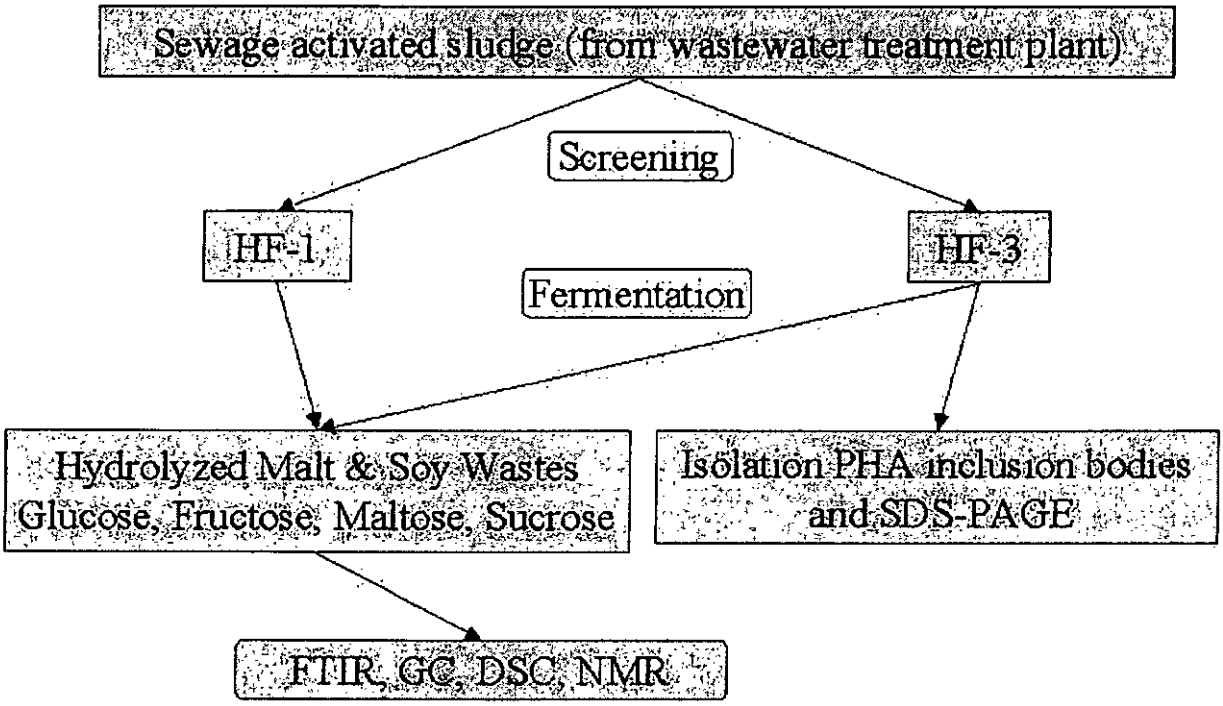


Fig. 6: A brief outline for isolation of the PHA-producing strain from activated sludge

2.4) Sub-cloning and Expression of *B. megaterium* PHA Gene in *B. subtilis*

2.4.1) Sub-cloning of *B. megaterium pha* Genes into *B. subtilus* 1A304 (ϕ105 MU331)

Genomic DNA of *B. megaterium* ATCC 11561 was extracted by the method mentioned above. The PHA gene PQRBC and RBC (both included their native promoter) were isolated by PCR and the primer sequence used were:

Target	Primer Name	Sequence
PQRBC	LKH 1	ACT CGT GAG CTC TAC TGA ACC GTT GCT GCG TG
	LKH 2	CAG CTA TCT AGA CAT ACT CAT TGT TAC ATG AAT AGC
RBC	LKH 3	GGC GCC CCG GGG TAC ATG TAG GTA ATT TTT ATA AC
	LKH 2	CAG CTA TCT AGA CAT ACT CAT TGT TAC ATG AAT AGC

*Sac*I was designed in primer LKH 1, *Xba*I was designed in LKH 2 and *Sma*I was designed in LKH 3.

Two different PCR products were digested by appropriate restriction enzymes and purified and finally inserted into the vector pSG703 (Fig. 7).

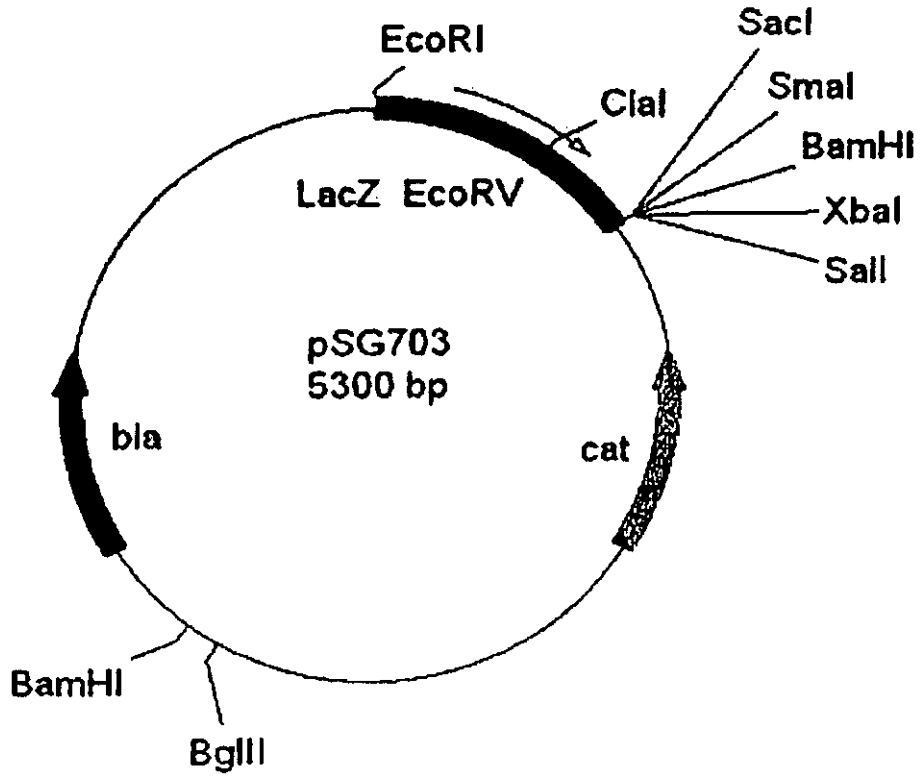


Fig. 7: The Gene Map of pSG703

The final plasmids were amplified in *E. coli* and finally transformed into *B. subtilis* 1A304 (ϕ105 MU331) by homologous recombination and expressed by heat

shock strategy as mentioned in "General Methodology" and expressed by two-stage fermentation.

The fermentation was separated into two stages:

1) The selected strain was first grown in BHY for 16 h with 280 rpm shaking at 37°C (Growth phase).

2) The cells were centrifuged down and washed by sterile ddH_2O and resuspended in Medium C. The culture was then incubated at 37°C with 280 rpm shaking (Production phase).

2.4.2) Outline for the Experiment (Fig. 8)

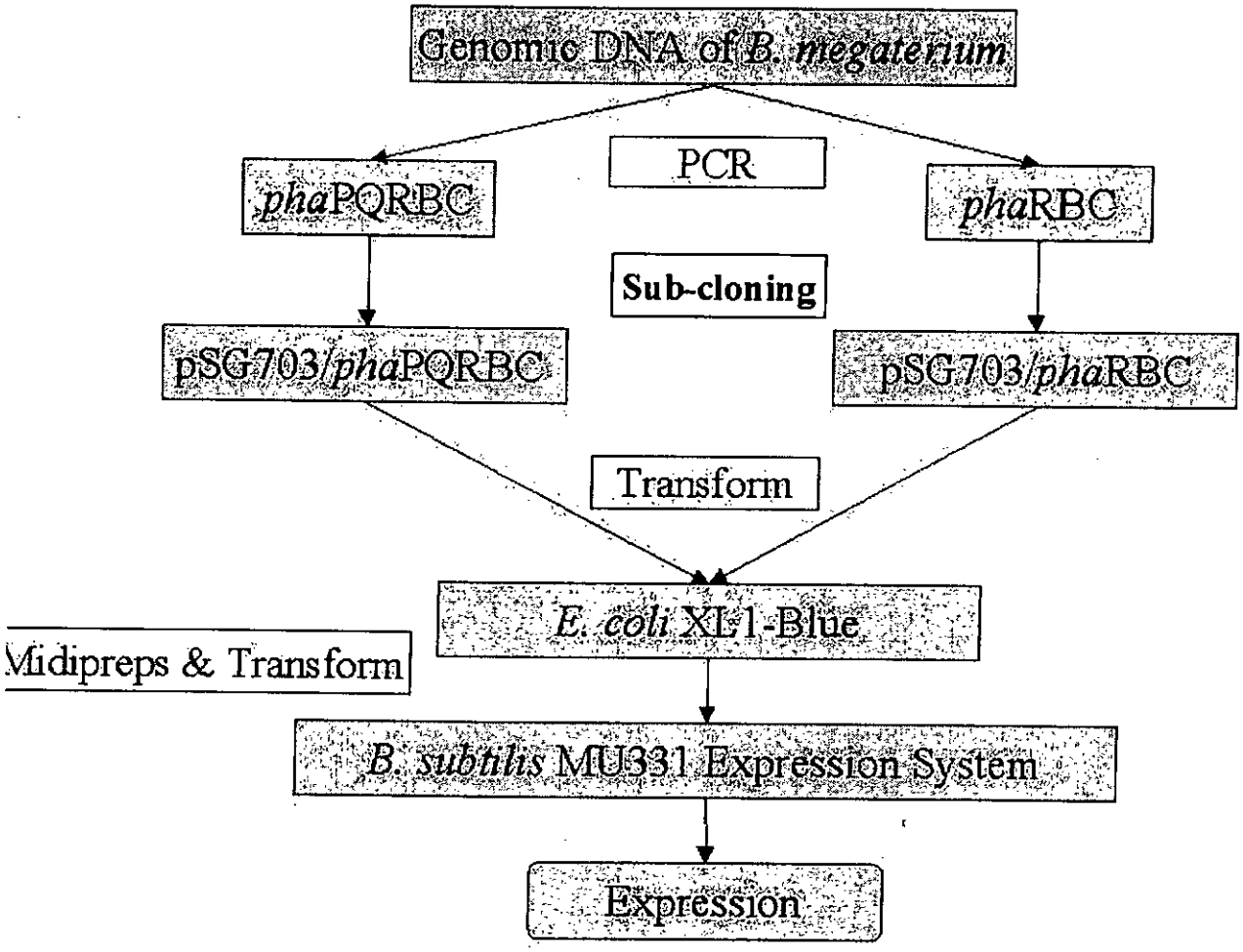


Fig. 8: A brief outline for sub-cloning and expression of *B. megaterium* PHA gene in *Bacillus* System

2.4.3) Sub-cloning of *B. megaterium* PHA Gene in *B. subtilus* 168

Plasmid pGEM10 with whole PHA genes in *B. megaterium* was used in this part of the experiment. The PHA gene PQRBC and QRBC were isolated by PCR and restriction digestion, respectively. The restriction enzymes used for the isolation of

PHA gene QRBC were *Eco*RI and *Sca*I. The isolated gene was then purified and sub-cloned into vector pYCL17. The gene map of plasmid pGEM10 and pYCL17 is shown in Fig. 9.

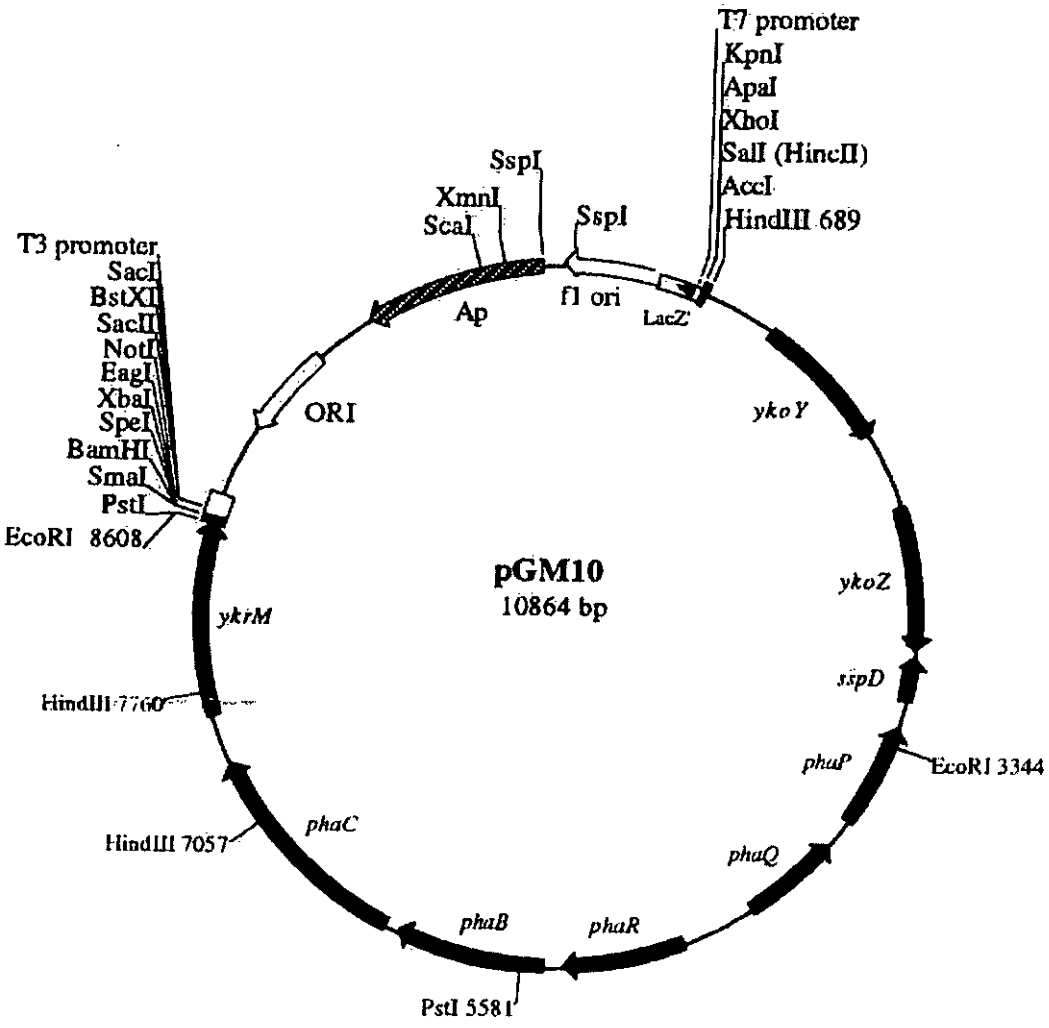


Fig. 9 (a)

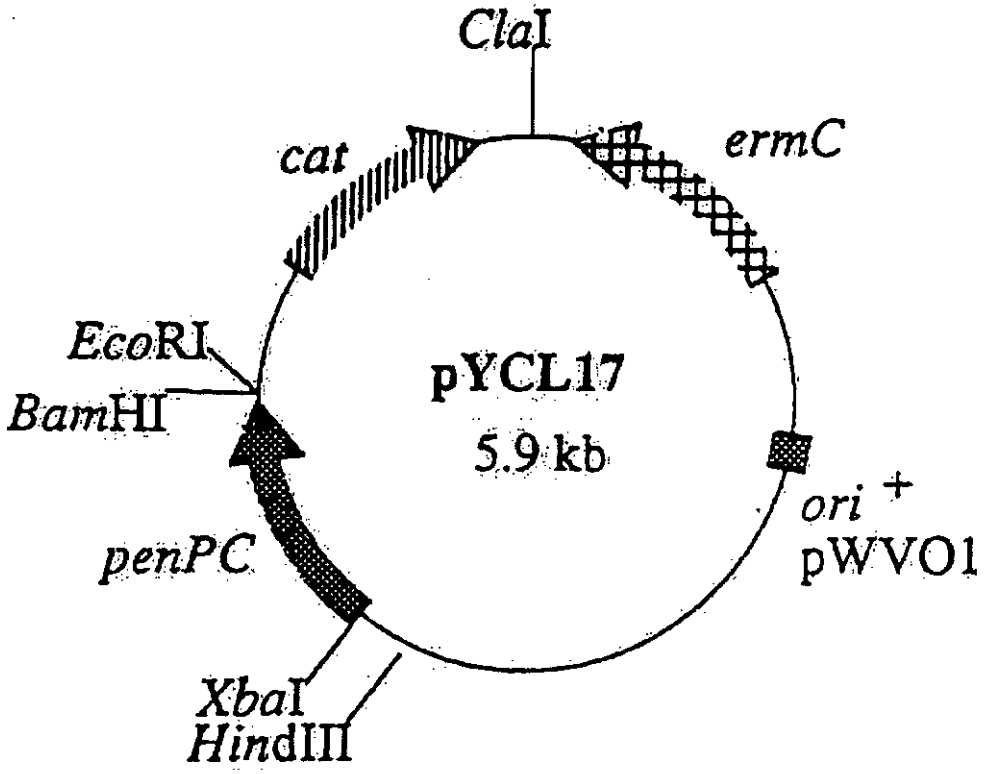


Fig. 9 (b)

Fig. 9: Gene Map of (a) pGEM10 and (b) pYCL17

The primer sequence used for the isolation of genes PQRBC was:

Target	Primer Name	Sequence
PQRBC	LKH 4	ACT CGT GGA TCC TAC TGA ACC GTT GCT GCG TG
	LKH 2	CAG CTA TCT AGA CAT ACT CAT TGT TAC ATG AAT AGC

PCR products were digested by BamHI and XbaI respectively and purified and finally sub-cloned into the vector pGK13 (Fig. 10).

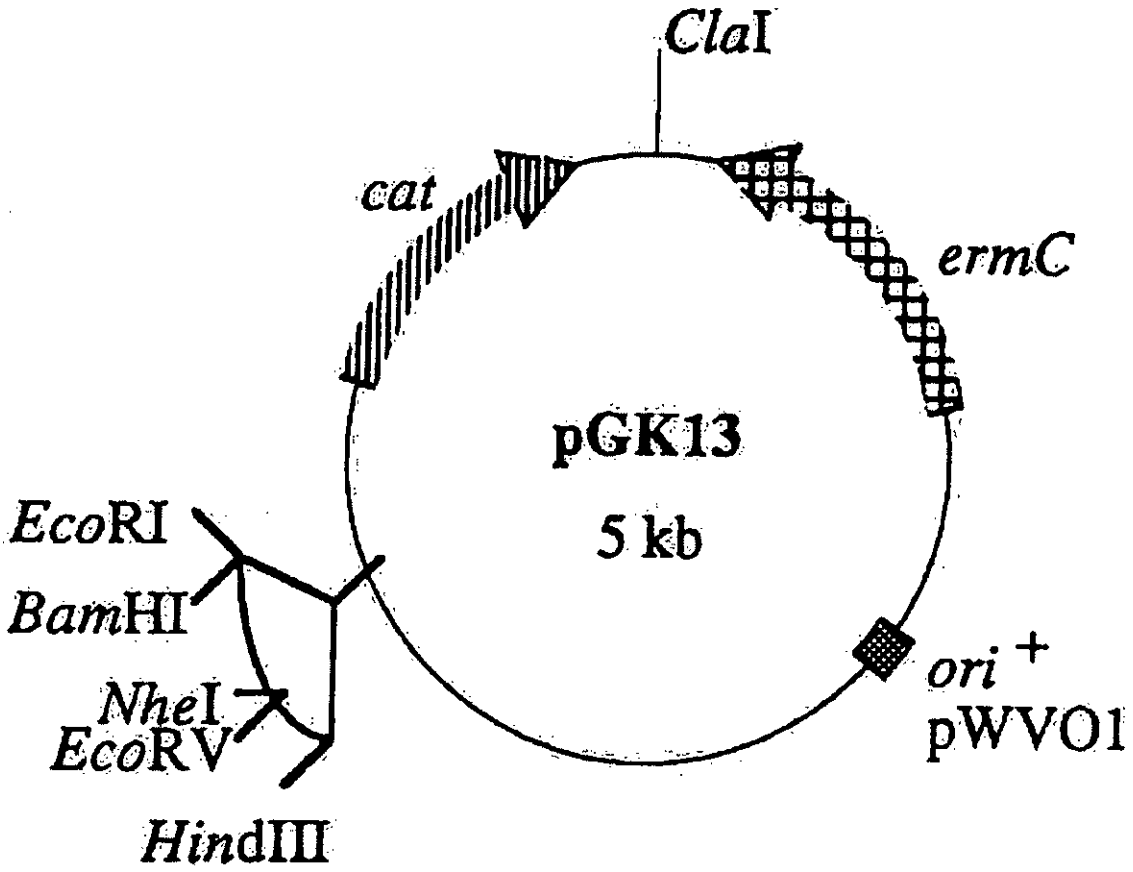


Fig. 10: Gene Map of Vector pGK13

2.4.4) Brief Outline of the Experiment (Fig. 11)

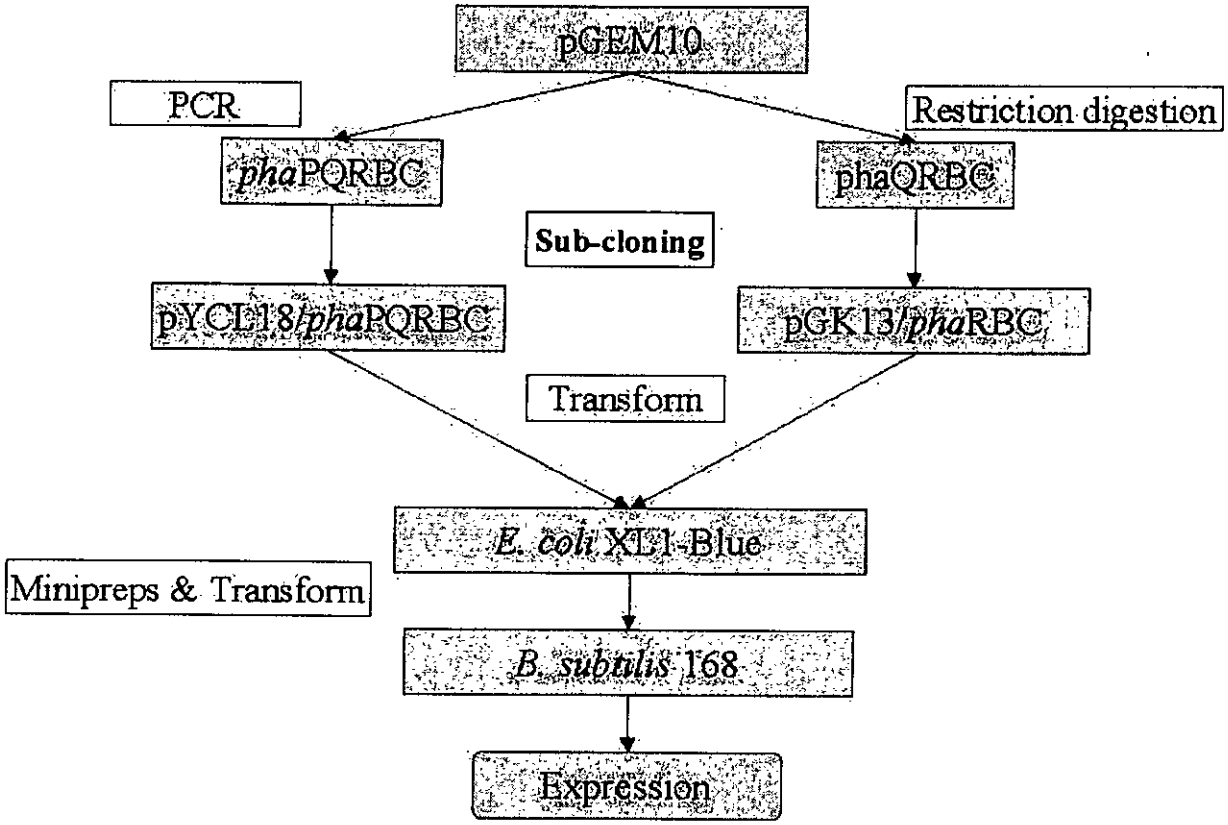


Fig. 11: A brief outline for the sub-cloning the *B. megaterium* PHA gene into *B. subtilus* 168

The final plasmids were amplified in *E. coli* and finally transformed into the *B. subtilis* 168 by the same method as *B. subtilis* 1A304 (ϕ105 MU331) and expressed by the use of a two-stage fermentation.

2.5) Sub-cloning & Expression of the *phaC1AB* Gene in *E. coli*

2.5.1) Joining the *phaC1* Gene from *Pseudomonus* to *phaAB* Gene in *Alcaligenes eutrophus*

Plasmid with *Pseudomonas pseudoalcaligenes* HBQ06 PHA synthase 1 (*pha*C1) gene (Fig. 12) was used. The *phaC1* gene was isolated by PCR, and the primers used are listed below. The restriction sites designed in primer were *Hin*dIII and *Eco*RI.

Target	Primer Name	Sequence
C1	LKH 5	ACT GAG AAG CTT ATT GGG CCC GAC GTC GCA TG
	LKH 6	GTA TCT GAA TTC TAC TCA AGC TAT GCA TCC AAC G

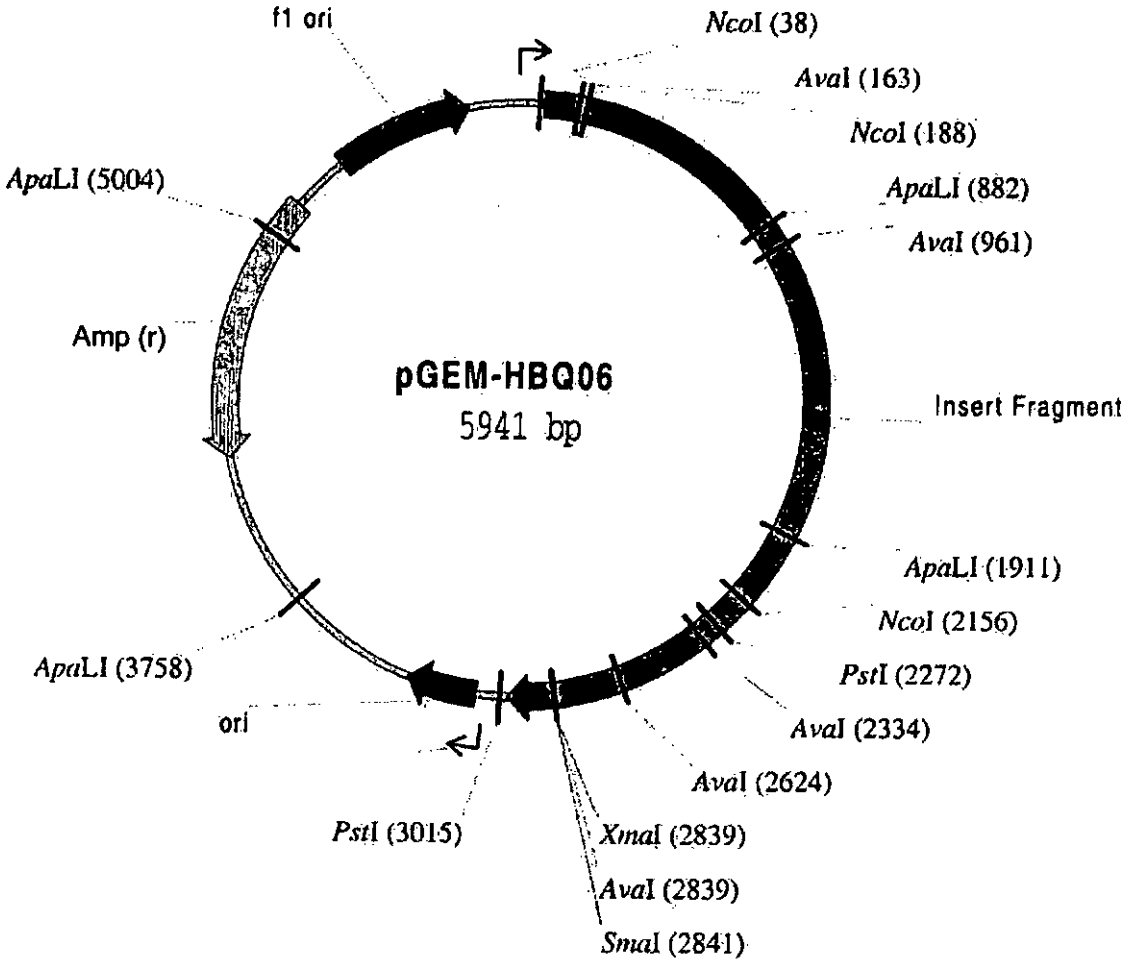
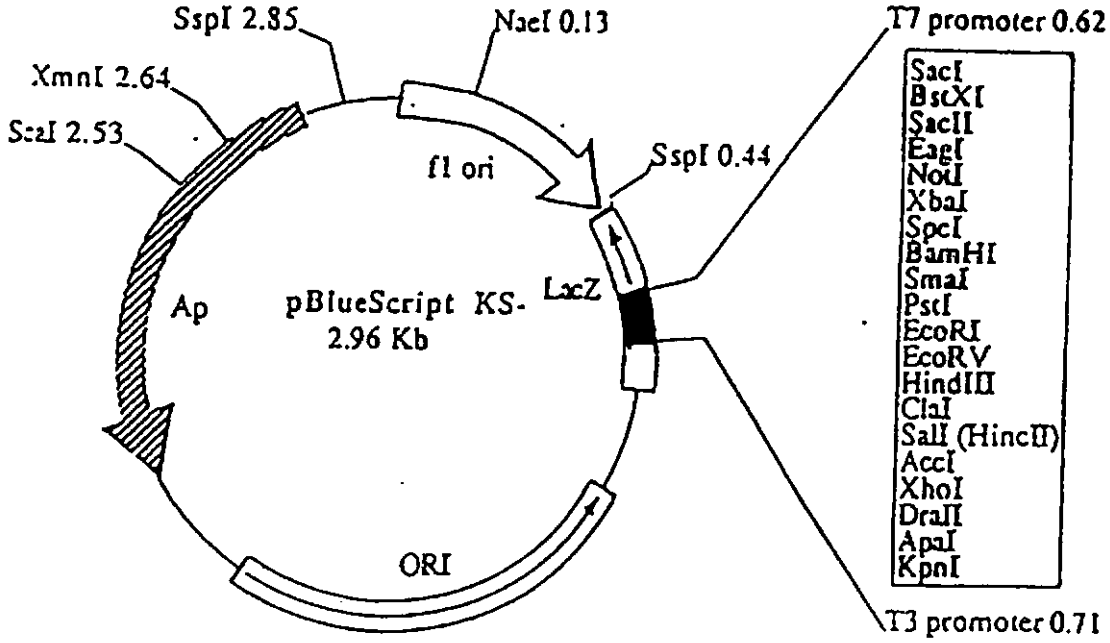
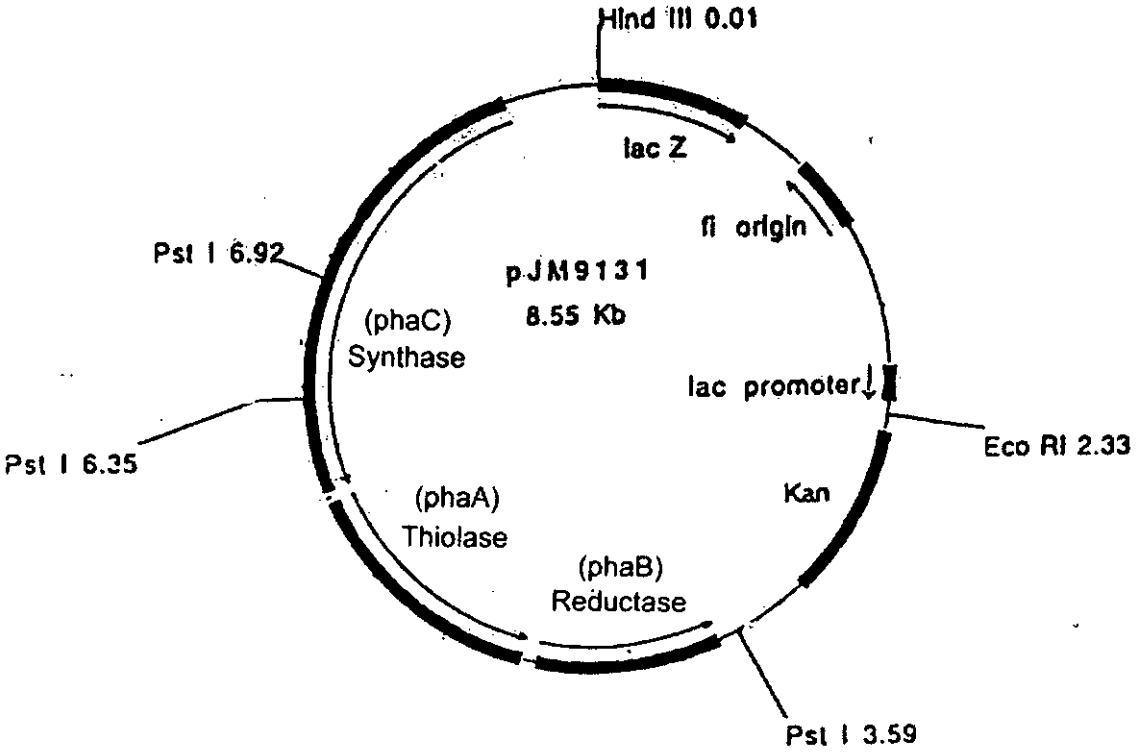


Fig. 12: Gene Map of Plasmid pGEM-HBQ06

The *pha*BA gene of *R. eutrophus* was isolated from the plasmid pJM9131 (Fig. 13b) by restriction digestion (*Pst*I). The *pha*AB gene was purified and cloned into the vector plasmid pBluescript KS- (Fig. 13a) and then transformed into the *E. coli* XL1-Blue and LS1298 for the amplification



(a)



(b)

Fig. 13: Gene Map of Plasmid Vectors (a) pKS- & (b) pJM9131

The *pha*C1 gene was then sub-cloned into the vector plasmid pKS-/*pha*AB (Fig 14) at the restriction sites *Eco*RI and *Hind*III.

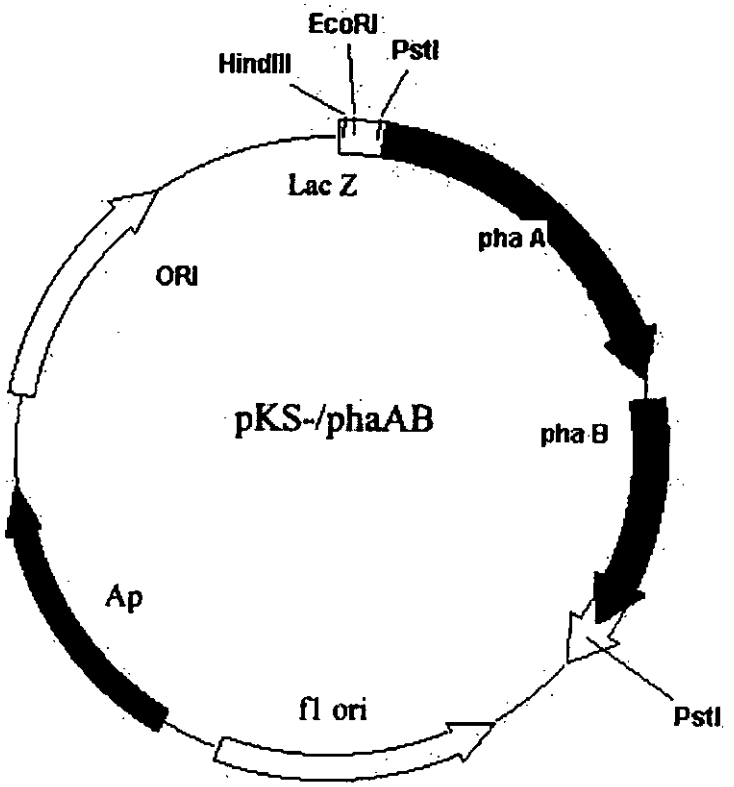


Fig. 14: Gene Map of Plasmid pKS-/phaAB

The cloned plasmid pKS-/*pha*C1AB gene was amplified in *E. coli* XL1-Blue and *pha*C1AB gene was then isolated from the restriction digest. The *pha*C1AB gene was then sub-cloned into plasmid vector pUC19 (Fig. 15).

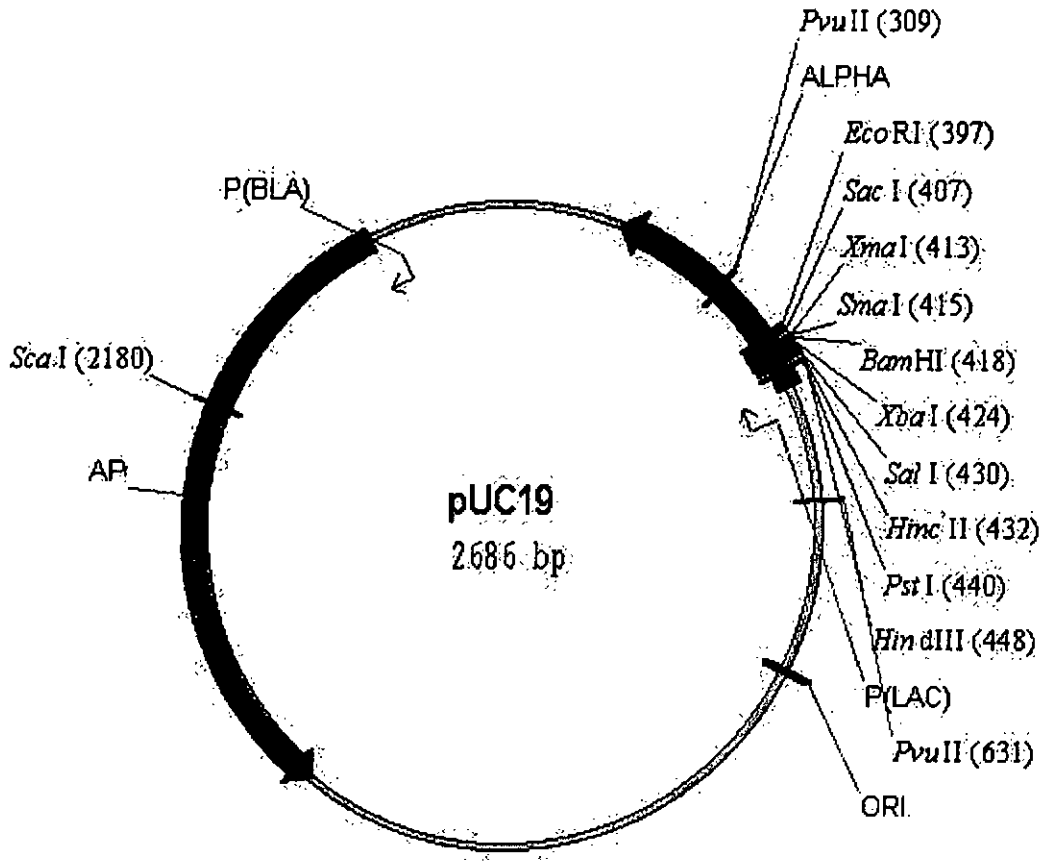


Fig. 15: Gene Map of Vector pUC19

The plasmid pKS-/*pha*C1AB and pUC19/*pha*C1AB in *E. coli* XL1-Blue and LS1298 were finally subjected to expression. They were subjected to 2-stage fermentation as well as cultivation by nutrient-rich medium (Medium B) with 0.5 mM IPTG induction when A_{600} attended unity.

2.5.2) Brief Outline of the Experiment (Fig. 16)

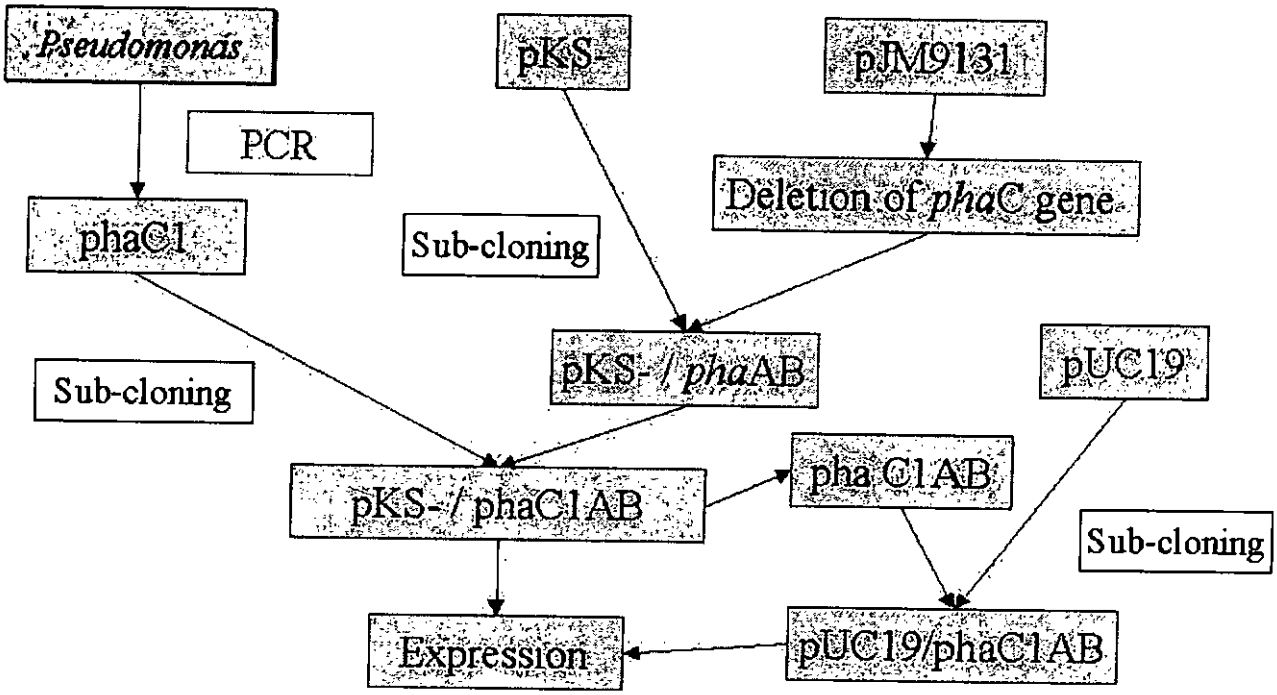


Fig. 16: A brief outline for the Sub-cloning & Expression of *phaC1AB* Gene in *E. coli*

2.6) Sub-cloning & Expression of the *phaCAB* Gene in different *E. coli*

2.6.1) Construction of recombinant E. coli strains

Plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). The DNAs were analyzed by electrophoresis in 0.8% (w/v) agarose horizontal slab gel and 1 kb DNA ladder (Promega, Madson, WI) as used as a marker. DNA fragments were isolated from agarose gel using the Concert Rapid Gel Extraction System (Pharmacia). Restriction enzymes and T4 DNA ligase (Pharmacia) were used according to the instructions provided by the supplier. A 5.2 kb long *phb* operon, were cut out from the pKS-/PHA by using the *HindIII* and *EcoRI*

and these three genes form an operon in the order *phaC*, *phaA* and *phaB* coding for PHA synthase, β-ketothiolase, and reductase, respectively, was used as an insert, and pUC19 carrying the lac promoter was employed as vector. Ligation product was transformed into *E. coli* cells XL1-Blue and HMS174 (recA1 hsdR; Rifr).

2.6.2) Fed-batch Fermentation in a computer-controlled 15 L fermenter

Fed-batch fermentation was performed in a computer-controlled 15 L stainless steel fermenter (B. Braun, Germany). With the conditions of 20% dissolved oxygen (DO), 37°C, pH 7.0 and initial stirrer speed 300 rpm. The media for fed-batch fermentation was reported in the Methodology section.

The start medium in the fermenter was inoculated with 1% (v/v) inoculum, and feeding was started when the glucose in medium was completely consumed with a rise in pH, since the medium pH increases as a result of depletion of glucose and the use of organic acid as the carbon source.

2.6.3) Brief Outline of the Experiment (Fig. 17)

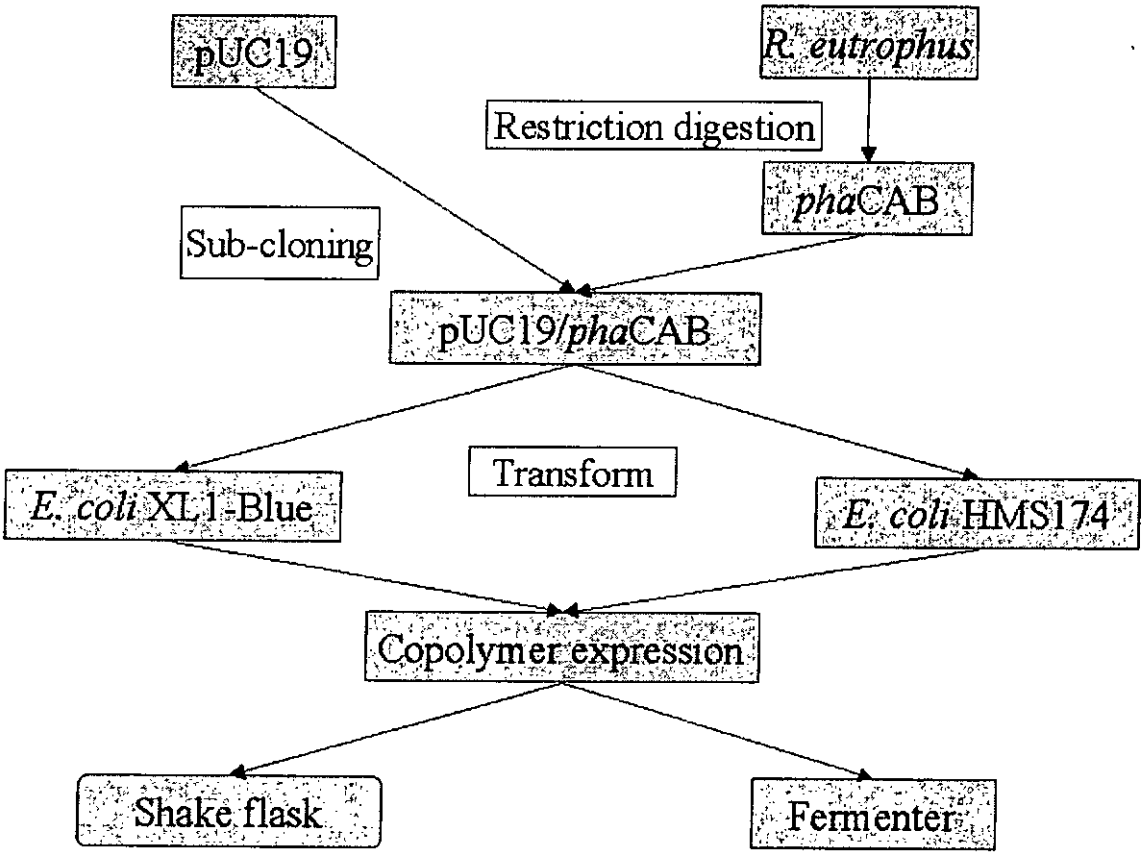


Fig. 17: A brief outline for the Sub-cloning & Expression of *phaCAB* Gene in different *E. coli*

Chapter 3: Results

3.1) Isolation of the PHA-producing Strain from Activated Sludge

3.1.1) Screening of PHA-producing strains from activated sludge

Two PHA-producing microbial strains (HF-1 and HF-3) were successfully isolated from the activated sludge. The two strains isolated were rod-shaped when observed under the microscope, and both of them were Gram positive as shown by Gram's staining.

The isolated strains were further identified by the Microbial ID, Inc. (Newark, DE, USA) as described in the Methodology section. The identification was based on the fatty acid composition profiles of the bacteria and expressed as a similarity index to known bacteria. Both HF-1 and HF-3 were identified as *Brevibacillus laterosporus* (0.745 in HF-1, but 0.552 in HF-3) and *Bacillus megaterium* (0.736 in HF-1, but 0.544 in HF-3).

3.1.2) Fermentation of the Isolated PHA-producing Strains

3.1.2.1) Fermentation in Food Waste Media

The changes in optical density of both isolated strains (A_{600}) [54, 55] during the

fermentation in malt and soy wastes are shown in Fig. 18. The two strains showed similar A_{600} values, but a large variation in their ability to produce PHA when comparing their yield of PHA accumulation.

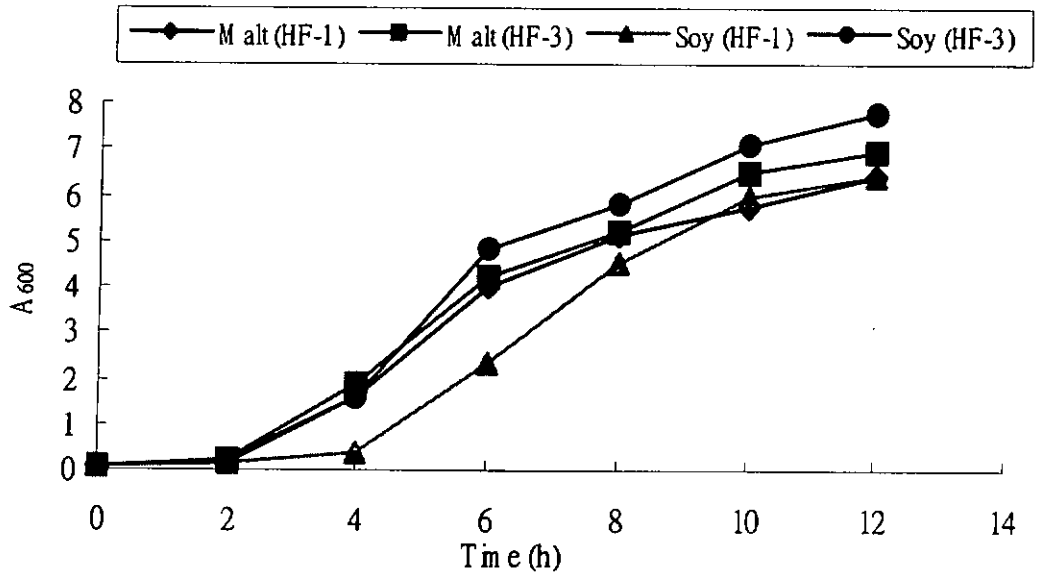


Fig. 18: Time profile of the absorbance of HF-1 and HF-3 during flask culture in malt and soy wastes

During the fermentation, the PHA content of the cells was monitored by FT-IR and the yield of PHA was estimated by the height ratio of PHA peak to protein peak [31,41], as shown in the absorption spectra of FT-IR in Fig 19.

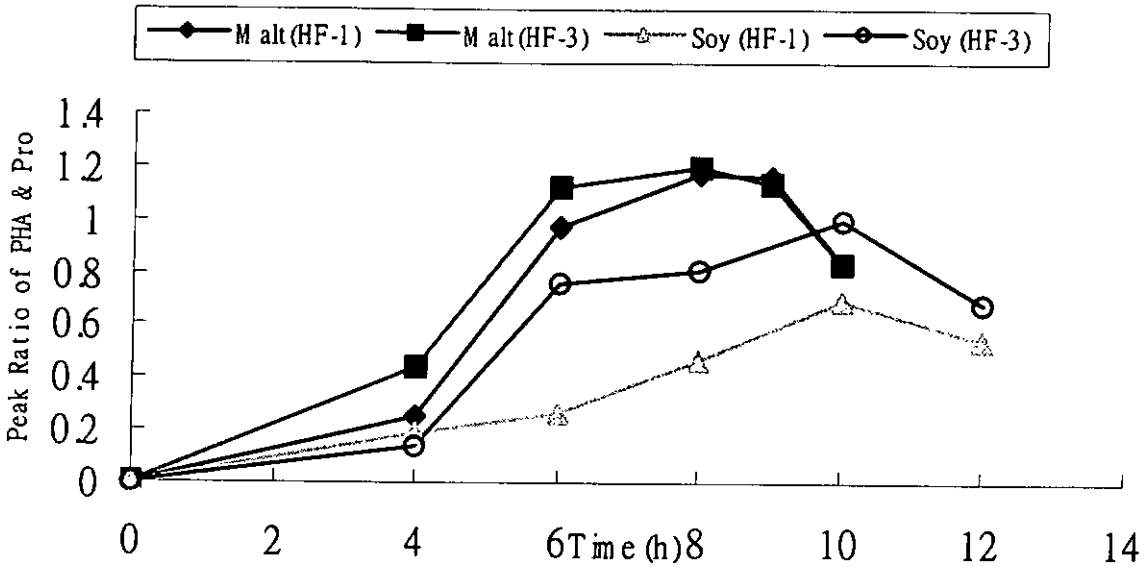


Fig. 19: Time profile of the height ratio of PHA peak to protein peak from FT-IR absorbance spectra during the flask culture of HF-1 and HF-3 in malt and soy wastes

Fig. 19 shows that the highest PHA content in cells occurred at 8 h when hydrolyzed malt waste was used as the medium. The FT-IR spectra show a characteristic PHA absorption peak at a narrow range of wavenumber around 1726 cm^{-1} to 1740 cm^{-1}. This pattern is similar to those of the previous studies [44,45,46].

3.1.2.2) Fermentation in Different Carbon Sources Synthetic Media

Both HF-1 and HF-3 were selected to ferment several types of carbon sources, including sucrose, fructose and maltose. The changes in A_{600} of fermentation media are shown in Fig. 20.

Both HF-1 and HF-3 were poor in utilizing fructose as the carbon source as shown in Fig 18, but were good in utilizing sucrose and maltose with similar A_{600} profiles, so they grew only slightly faster in sucrose than in maltose. The height ratio of PHA peak to protein peak on FT-IR absorption spectra is shown in Fig. 21.

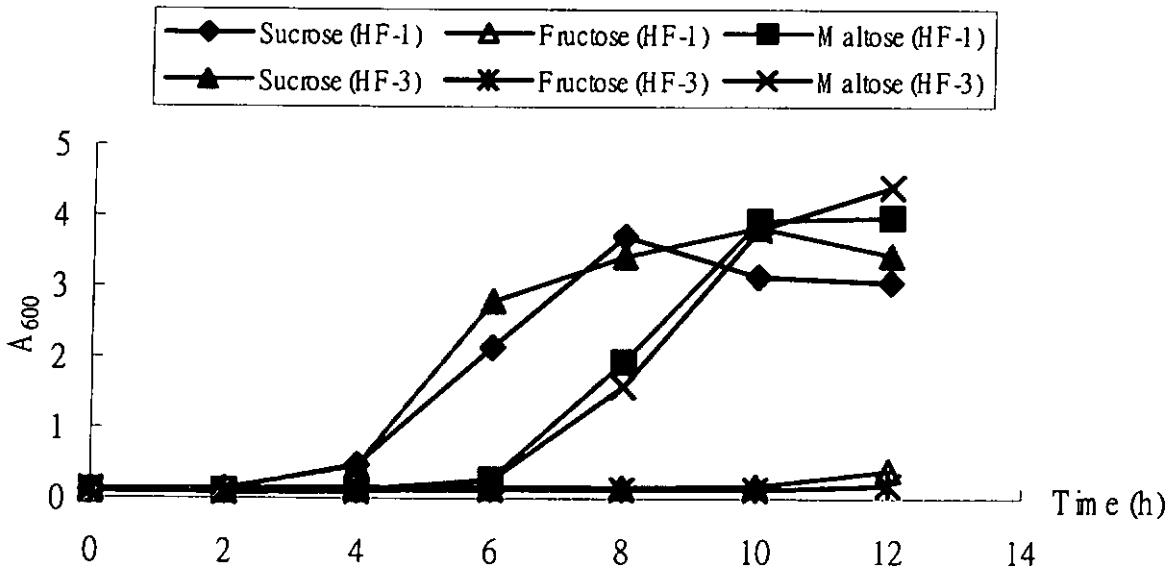


Fig. 20: Time profile of the absorbance of HF-1 and HF-3 during the flask culture in different carbon sources

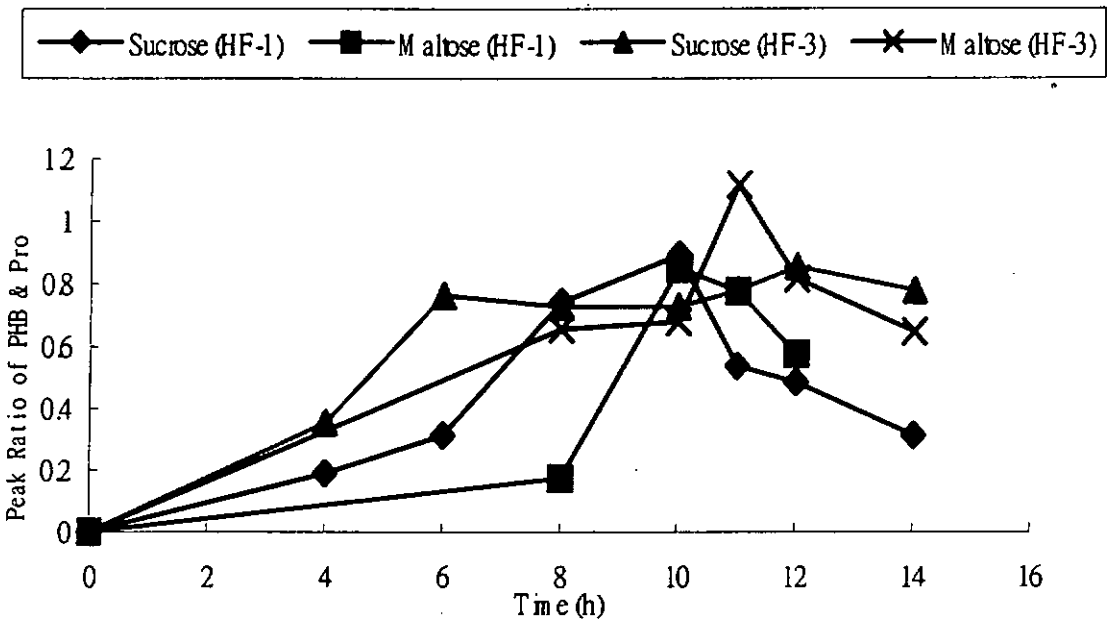


Fig. 21: Time profile of the height ratio of PHA peak to protein peak from FT-IR absorbance spectra during the flask culture of HF-1 and HF-3 in media utilizing sucrose and maltose as carbon sources

Fig. 21 shows that the highest PHA content in HF-3 cells occurred at 10-11 h when maltose was used as the carbon source.

3.1.3) Physicochemical Properties of Extracted Biopolymer

3.1.3.1) ^{1}H-NMR Spectra of Extracted Biopolymer

The composition of the hydroxyalkanoate units in extracted products was determined by analyzing the nuclear magnetic resonance (NMR) spectra. ^{1}H-NMR

spectrum showed the presence of three groups of characteristic signals (1.26 ppm, 2.52 ppm and 5.25 ppm) for the homopolymer PHB. Fig.22a shows the ^{1}H-NMR spectrum of biopolymer extracted from HF-1 whereas Fig. 22b shows the ^{1}H-NMR spectrum of biopolymer extracted from HF-3.

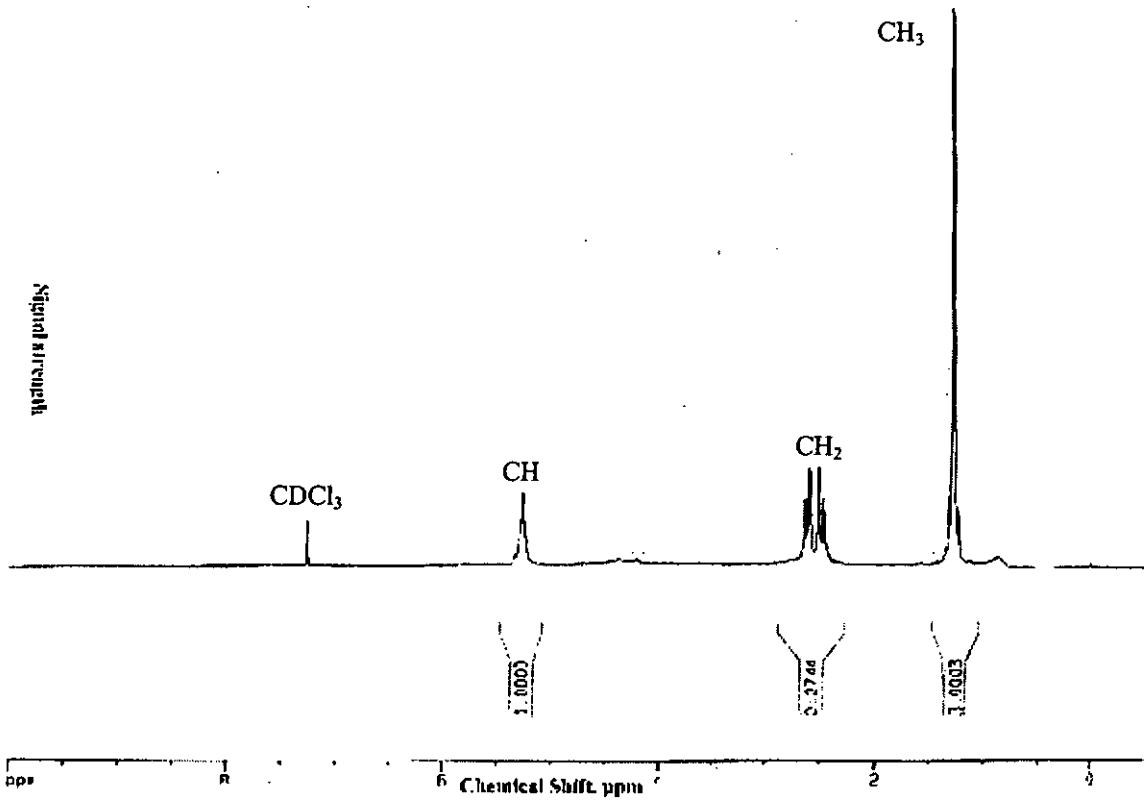


Fig. 22 (a)

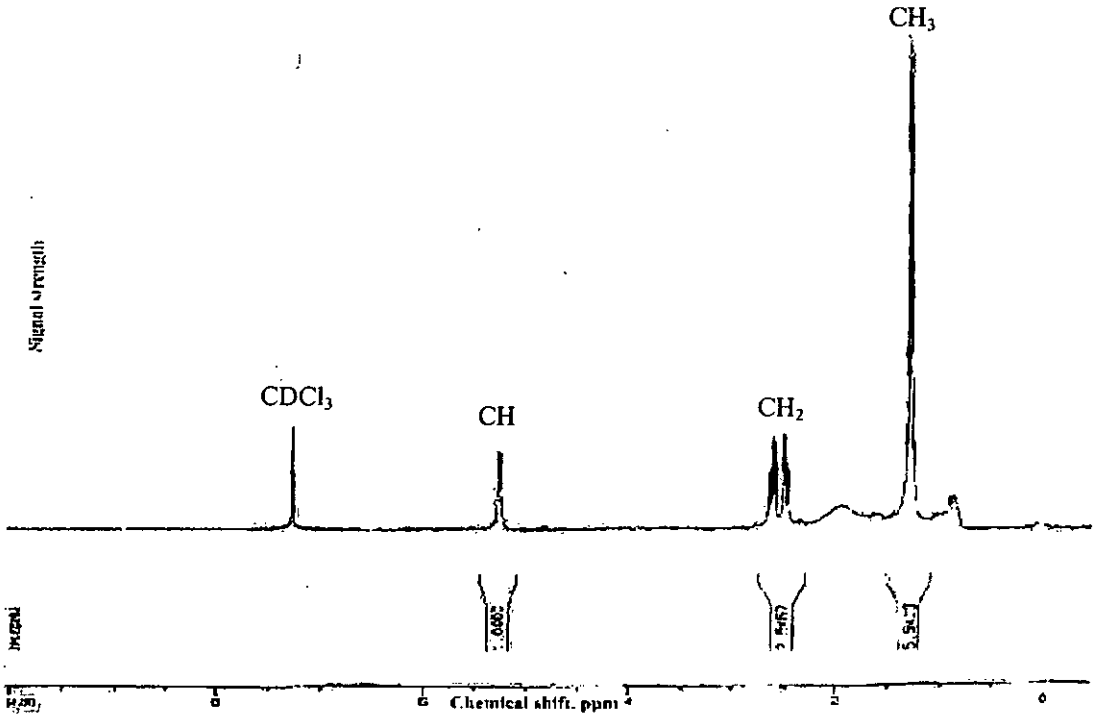


Fig. 22 (b)

Fig. 22: The ^{1}H-NMR spectra of biopolymer produced by HF-1 (a) and HF-3 (b) using malt waste as medium. The represented carbonyl groups are illustrated on the figure.

3.1.3.2) GC Spectra of Biopolymer

The extracted biopolymers were subjected to GC analysis to determine its purity and composition. The recovery yield and purity of PHA are strongly dependent on PHA content and hence the production cost. There was the presence of the PHB peak at retention time around 4.77 min but absence of PHV peak at around 6.56 min when compared to the standards (Fluka).

Data from the GC analysis from freeze-dried cells of HF-1 after fermentation was illustrated in Fig. 23. There was a PHB peak at a retention time of 3.363 min but no PHV peak when compared to the spectra of the standards.

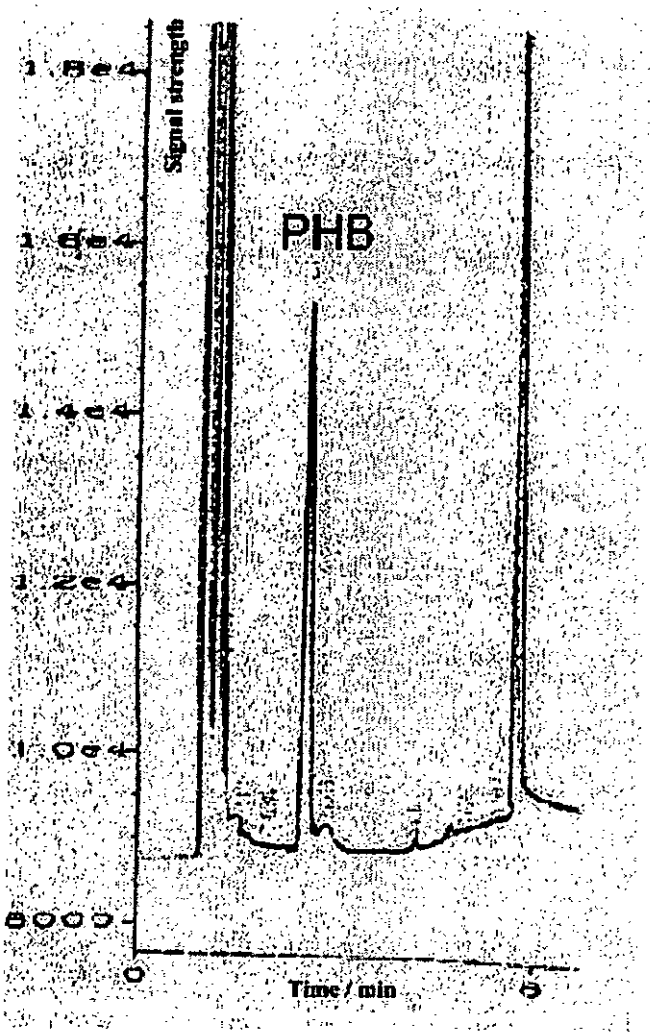


Fig. 23: GC spectrum of the freeze-dried intact HF-1 cells after fermentation.

In order to calculate the PHA purity of the sample (e.g. extracted biopolymer or freeze-dried cells), it was necessary to standardize the results to make them comparable. Although the concentration of internal standard benzoic acid in each sample was the same, the areas of benzoic acid signal peaks might be different that may due to the human error on injection. It can standardize all the areas of benzoic acid signals to the same value by its particular multiplying factor of each sample. Then the area of target signal (e.g. PHB) of each sample is then multiplied by its

multiplying factor. The calculation of percentage yield of biopolymer (e.g. PHB) in freeze-dried cells is calculated by the simple ratio of area between the PHA standard peak and PHA peak of samples as shown in Table 3 and Table 4.

Table 3: The standardization of the PHB signal area by internal standard benzoic acid

Sample	Benzoic acid signal area	Benzoic acid signal area × factor	Area of PHB signal	Area of PHB signal × factor
Std. PHB	1.13162×10^7	$1.13162\times10^7 \times 1.2360 = 1.39863\times10^7$	5761892	7121432
HF-1 freeze-dried cells	1.34821×10^7	$1.34821\times10^7\times1.0374 = 1.39863\times10^7$	1.13936×10^7	1.18197×10^7
HF-3 freeze-dried cells	1.39863×10^7	$1.39863\times10^7\times1 = 1.39863\times10^7$	1.57408×10^7	1.57408×10^7

Amount of PHB in sample = (standardized area of PHB signal / standardized area of purchased PHB signal) × amount of purchased PHB used

% yield of PHB in cells = (amt. of PHB in sample / amt. of freeze-dried cells used in GC) × 100%

Table 4: The percentage yield of the PHB inside the freeze-dried HF-1 and HF-3 cells

Sample	Area of PHB signal	Amount of sample used (g)	Amount of PHB in sample (g)	% of PHB in sample
Std. PHB	7121432	0.0053	0.0053	100%
HF-1 freeze-dried cells	1.18197×10^7	0.0222	$(1.18197\times10^7/7121432) \times0.0053 = 0.008797$	39.62%
HF-3 freeze-dried cells	1.57408×10^7	0.0318	$(1.57408\times10^7/7121432) \times0.0053 = 0.01171$	36.84%

The percentage yield of the PHB in the cells of HF-1 and HF-3 in malt and soy wastes were 39.62% and 36.84%, respectively. The percentage yield was higher in HF-1 than that in HF-3 as shown in the FT-IR analysis.

Purity of the extracted biopolymer = (calculated amount of pure PHB in sample biopolymer extracted / pure PHB standard used) × 100%

The purity of the extracted PHB from HF-1 and HF-3 were about 80% and 83%, respectively.

Both strains were more superior in the production of PHB by using hydrolyzed malt waste, which can reach a yield as high as 40%. From the GC analysis of synthetic media, the percentage yields of PHA from HF-1 and HF-3 in sucrose were 36.38% and 33.80%, respectively, but were 28.67% and 27.58% when maltose was used. P (3HA) copolymer may be produced by feeding bacterial cultures with a variety of carbon substrates. Thus the extracted biopolymers from different carbon substrates were subjected to GC analysis. The results obtained indicate that the only homopolymer of PHB was produced in HF-1 and HF-3.

3.1.3.3 Differential Scanning Calorimetry Thermograms of Extracted Biopolymer

The melting temperature (Tm) of the biopolymer extracted from HF-1 and HF-3 was determined from DSC thermograms. The T_m value of the P (3HB) homopolymer was about 170°C; the T_m values of biopolymer extracted from HF-1 and HF-3 were 165°C (Fig. 24a) and 157°C, respectively (Fig. 24b). The T_m of the samples were lower than that of the standard PHB. The small peak beside the major peak may be due to the formation of 2 crystals with similar melting temperatures.

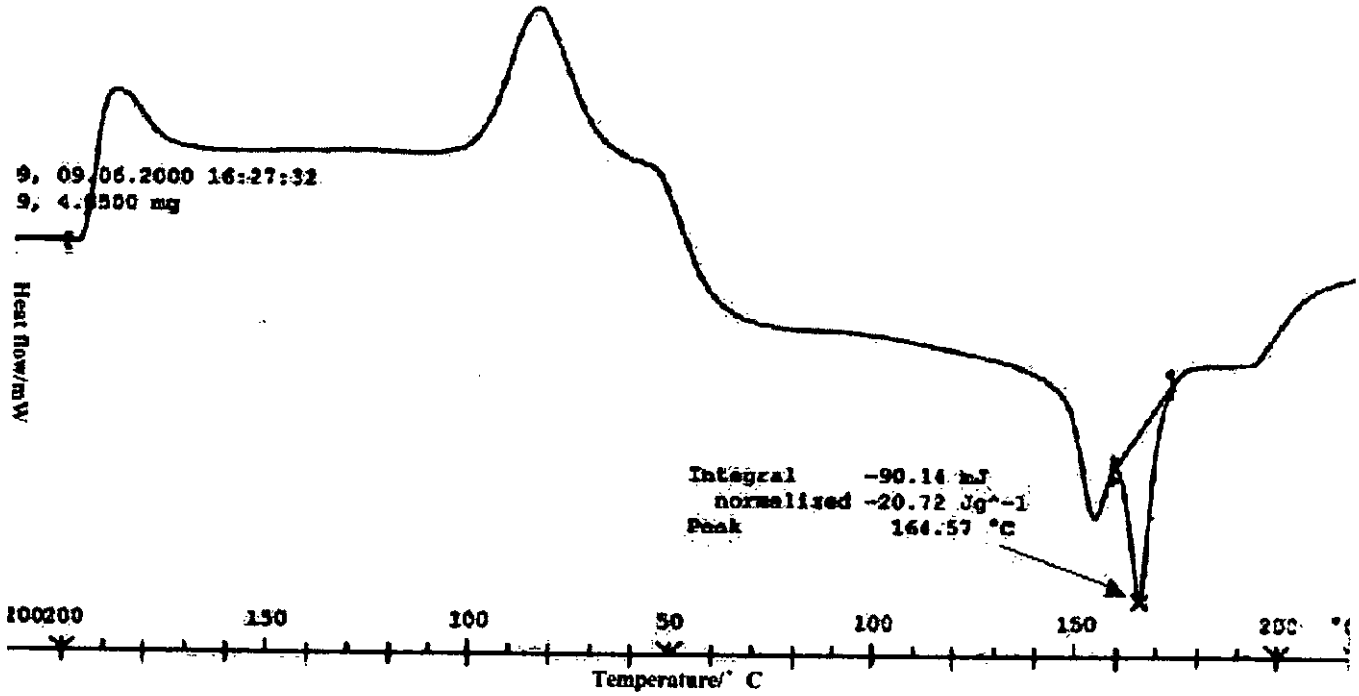


Fig. 24 (a)

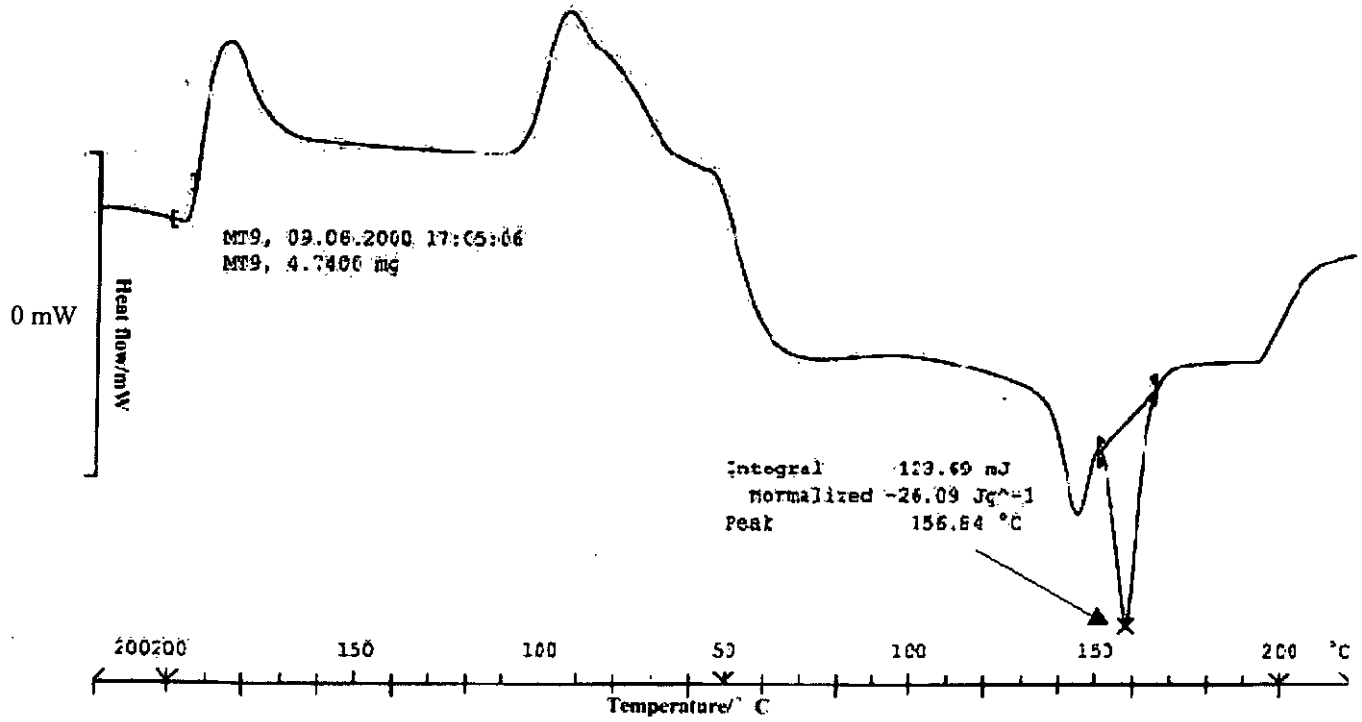


Fig. 24 (b)

Fig. 24: DSC thermograms of extracted biopolymer from (a) HF-1 and (b) HF-3

The arrows are pointed to the T_m of the test biopolymer illustrated on the figure. The temperature (°C) is expressed on the horizontal axis.

3.1.4) Isolation of PHA Inclusion bodies and SDS-PAGE Analysis

PHA inclusion bodies were isolated from fermented cells as previously described. PHA Inclusion body-associated proteins were also analyzed by SDS-PAGE for the comparison of the strain HF-1, HF-3 and *B. megaterium*. Fig. 25 shows the results of SDS-PAGE of proteins released from purified PHA inclusion bodies. There were at least 20 such proteins present in various quantities. The two most abundant proteins have a molecular mass of approximately 41 kDa and are found in all three strains. The 20 kDa protein band is more significant in HF-1 and *Bacillus megaterium*. The 14 kDa protein is lysozyme. The pattern of the visualized protein bands after Coomassie blue staining from HF-1 was similar to that from *B. megaterium* excepts for a few bands.

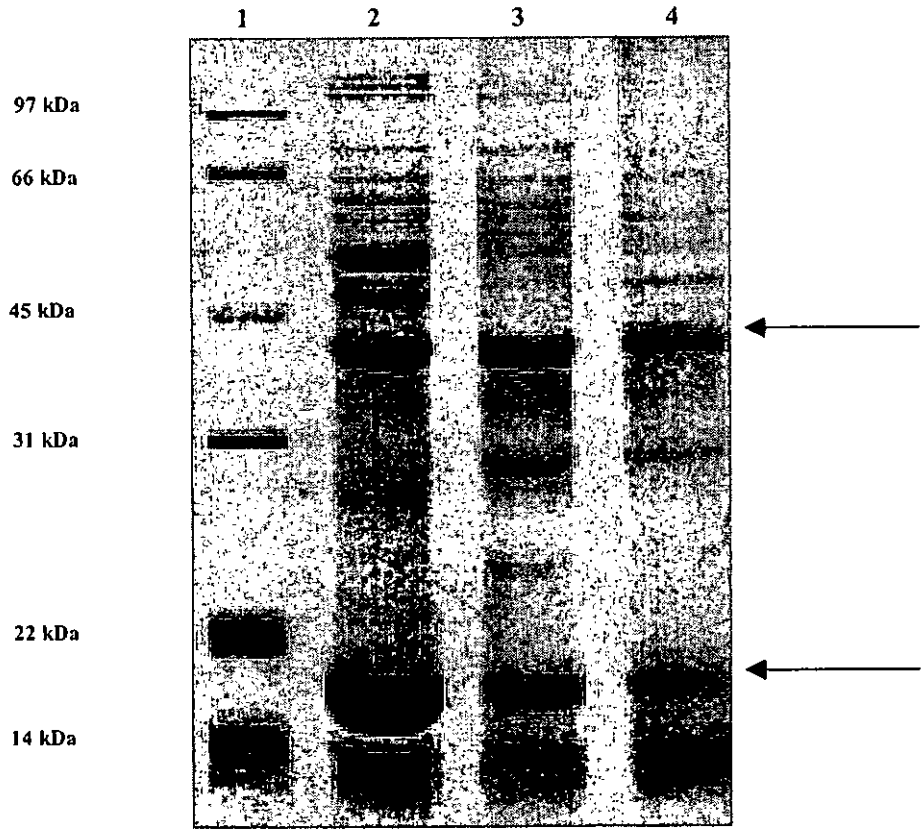


Fig. 25: PHA inclusion body-associated proteins in Coomassie blue staining (mini gel)

The results of SDS-polyacrylamide mini gel electrophoresis of proteins released from purified PHA inclusion bodies are shown. Lane 1 contains molecular weight markers; lane 2 contains proteins from inclusion bodies of B. megaterium 11561; lane 3 contains proteins from inclusion bodies of HF-1; lane 4 contains proteins from inclusion bodies of HF-3. The two arrows point to two abundant proteins.

There are too many proteins in the samples and the separation by the mini-protein gel was not efficient. Therefore, a standard gel was used instead. Figure 26 shows the protein pattern of the inclusion bodies. Similar to the mini-gel, there were two dominant bands that have the molecular weight of 20 and 41 kDa.

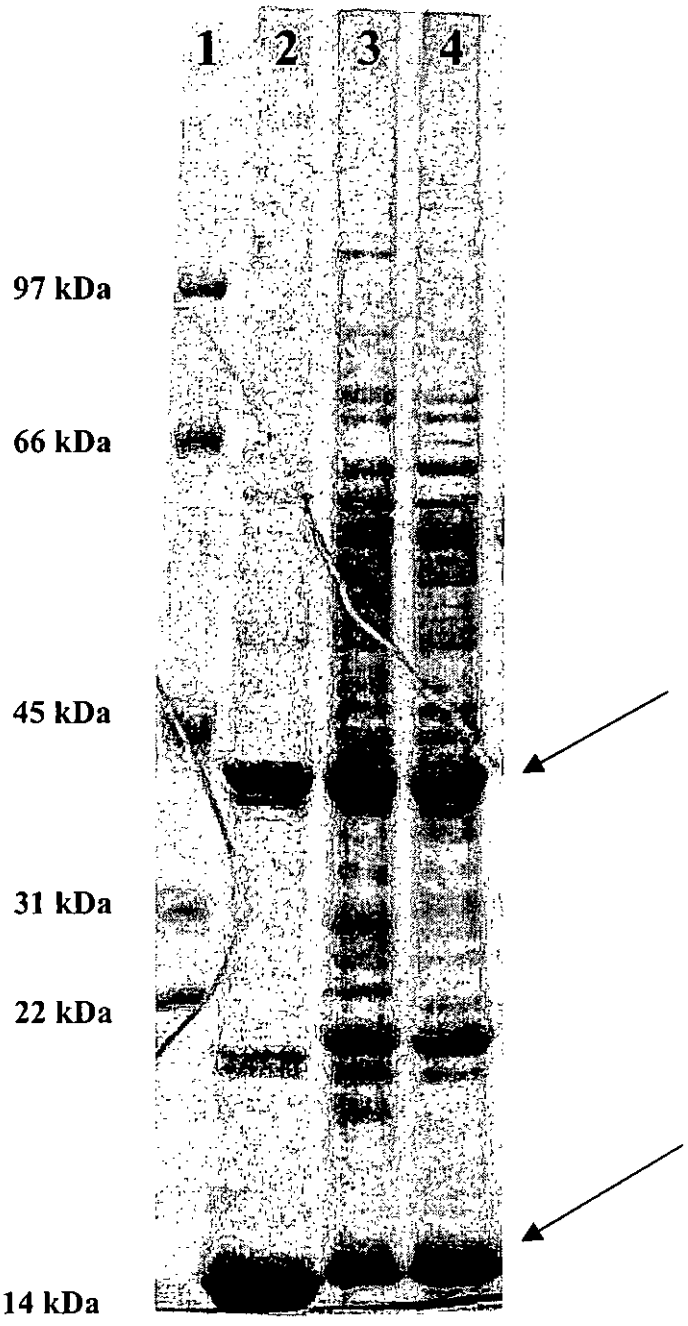


Fig. 26: PHA inclusion body-associated proteins in Coomassie blue staining (standard gel)

The results of SDS-polyacrylamide standard gel electrophoresis of proteins released from purified PHA inclusion bodies are shown. Lane 1 contains molecular weight markers; lane 2 contains proteins from inclusion bodies of B. megaterium 11561; lane 3 contains proteins from inclusion bodies of HF-1; lane 4 contains proteins from inclusion bodies of HF-3. The two arrows point to two abundant proteins.

3.1.5) Further Identification of HF-1 by DNA Sequencing

The genomic DNA of HF-1 and HF-3 were isolated as described in the Methodology section. However, only HF-1 gave PCR product with the right size (~1.2 kb), and there was an absence of PCR product in the case of HF-3. The similarity index of HF-3 (0.552) measured by Microbial ID, Inc. was lower than that in HF-1 (0.745), ,suggesting that it should be *Bacillus* but may not be *B. megaterium*. The absence of PCR product in the case of HF-1 further supports this inference.

The PCR product from HF-1 was subjected to DNA sequencing and compared with the sequences of *pha* genes of *B. megaterium*. The primers used in sequencing were LKH 4 and LKH 7, respectively. Each set of sequencing was done two times to minimize the sequencing error, and the results are summarized in Fig. 27.

Primer used in sequencing: LKH 4

Identities = 357/377 (94%), Gaps = 7/377 (1%)

Query gene: 1 ctcntctactaattgtgttactacttgangatattgttcttgagcttgttttactaaaga 60

B. megaterium: 2677 ctcttctactaattgtgttactacttgatgatattgttcttgcgcttgctttactaaaga 2736

Query gene: 61 atagcttgatttgctttggttaagagaaagctcttgtaatttatttaatgcttcatncgt 120

B. megaterium: 2737 atagcttgatttgctttggttaaaaaaaagctcttgtaatttatttaatgcttcatgcgt 2796

Query gene: 121 gcggtttgtccattcttcataagaatcggcaacagcgtttccagctgttttacgtaagtt 180

B. megaterium: 2797 gcggtttgtccattcttcataagaatcggcaacagcgtttccagctgttttacgtaagtt 2856

Query gene: 181 ttcaactgtttttgttgaagg-cttctaattcagctttccattgtttgtctgttgcttg 239

B. megaterium: 2857 ttcaactgtttttgttgaaggtcttccaattcagctttccattgtttgtctgttgcttg 2916

Query gene: 240 aagctg-ccaactgcttttgttacaaattcttgctg-tgctcaagtgcttttaacg-cca 296

B. megaterium: 2917 aagttgttcaactgcttttgttacaaattcttgctgttgctcaagtgcttttaacgtcca 2976

Query gene: 297 ttgctcaatttgnttgnttccg-ctgcaatg-tttggaaccccttttgtccattg-tccca 353

B. megaterium: 2977 ttgctcaatttgtttgtttccgtctgcaatgttttgtaaccccttttgtccattgttccca 3036

Query gene: 354 cattgcatcaattactg 370

B. megaterium: 3037 cattgcatcaattactg 3053

Fig. 27 (a)

Primer used in sequencing: LKH 7

Identities = 370/388 (95%), Gaps = 5/388 (1%)

```
Query gene:     1  caagagaaaaagataacacctntaattcaaacaacttagaaaaatcgattagcggtnacc 60
                   ||||  ||||||||||||||||  ||||||||||||||||||||||||||||||||||  |
B. megaterium: 3677 caagtgaaaaagataacacctctaattcaaacaacttagaaaaatcgattagcggtgcac 3618
Query gene:    61  cnaaaaacttgatggttccttttcttctttttaagtttaagagggtggaatctacatggtt 120
                   | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B. megaterium: 3617 caaaaaacttgatggttccttttcttctttttaagtttaagagggtggaatctacatggtt 3558

Query gene:   121  acaagctcattcagcaactaatgagctttggattcacatcagttgatcaaggaantgtct 180
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||  ||||  |||||
B. megaterium: 3557 acaagctcattcagcaactaatgagctttggattcacatcagttgatcagggaaatgtct 3498

Query gene:   181  accgcacgctgagacagcttgaaaagggacaacttgattacatcgcaatgggatacgtaa 240
                   ||||||||||||||||||||||||  ||||||||||||||||||||||||||||||  |
B. megaterium: 3497 accgcacgctgagacagcttgaaaa-agacaacttgattacatcgcaatgggatacgtca 3439

Query gene:   241  gctgaaggacctgcacgccggatttattcattaacagatgcc-gtgaacaatatttaagc 299
                   ||||||||||||||||||||||||||||||||||||||||||||||||||  |  ||||||||||||||
B. megaterium: 3438 gctgaaggacctgcacgccggatttattcattaacagatgccggcgaacaatatttaagt 3379

Query gene:   300  atgt-ggctaattcacttgaacagtatcaaaacatgttagattcatttttt-acatgtat 357
                   ||||  ||||||||||||||||||||||||||||||||||||||||||||||||  ||||||||
B. megaterium: 3378 atgtgggctaattcacttgaacagtatcaaaacatgttagattcatttttcacatgtat 3319
```

```
Query gene:     358  accg-catgttggtccctttttagctctt 384
                     ||||  ||||||| ||||||||||||||||
B. megaterium: 3318  accgacatgttgttccctttttagctctt 3291
```

Fig. 27 (b)

Fig. 27. DNA sequence alignment of the *pha* genes of *B. megaterium* ATCC11561 and PCR fragments from HF-1 (Fig. 27 a) and HF-3 (Fig. 27 b).

The sequence of *B. megaterium* ATCC11561 was retrieved from the Gene Bank with the accession number AF 109909. The sequences were aligned using the computer program BLAST (National Center for Biotechnology Information).

The result of the percentage of identity of the two sets were 94% and 95% identical to that of the *Bacillus megaterium* 11561.

3.2) Cloning and Expression of *B. megaterium* PHA Gene in *B. subtilis* 1A304 (ϕ105 MU331)

3.2.1) Sub-cloning of the *B. megaterium* PHA Gene into the plasmid pSG703

The genomic DNA of the *B. megaterium* 11561 was prepared as shown in the methodology section. The genomic DNA was then checked by agarose electrophoresis. The genomic DNA and the 1 kb DNA ladder (Promega) are shown in Fig. 28. The genomic DNA was used as a template for the PCR amplification of the PHA gene.

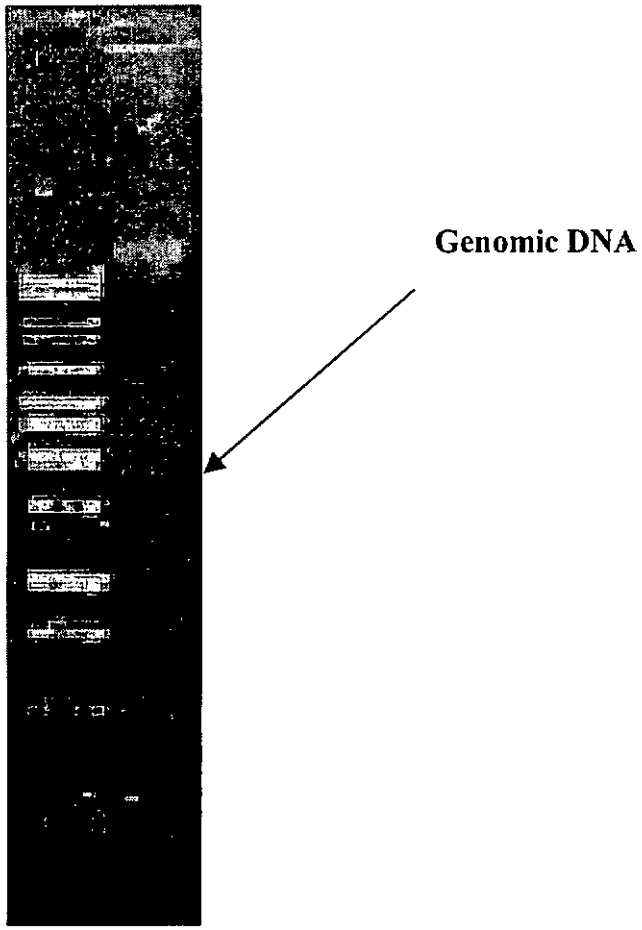


Fig. 28: Agarose gel electrophoresis genomic DNA of *B. megaterium*

PCR amplification of the PHA gene was performed using the Expand High Fidelity PCR System. Primer LKH 1 designed according to the region before the *pha* R gene and primer LKH 2 designed according to the region behind the *pha* C gene were synthesized according to the published *B. megaterium* 11561 *pha* gene sequence (Gene Bank accession no. AF109909). Primer LKH 3 was designed according to the region before the *pha* P gene. The *pha* RBC genes (3058 bp) and the *pha* PQRBC (4612 bp) PCR products were amplified. The amplified PCR products are shown in Fig. 29.

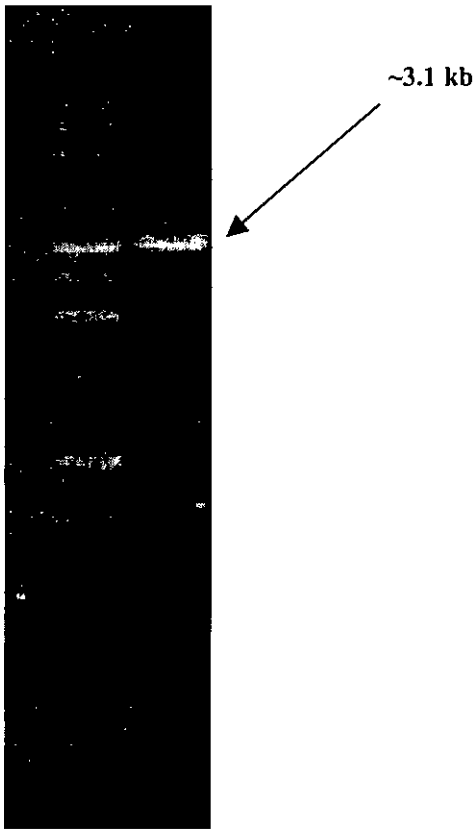


Fig. 29 (a)

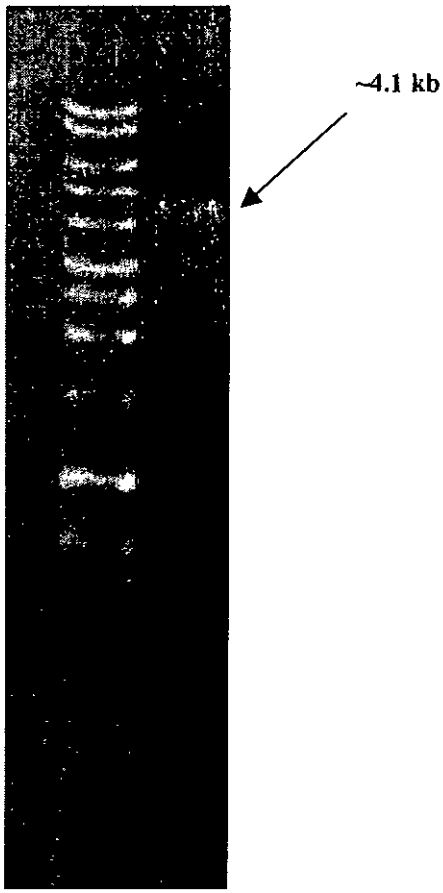


Fig. 29 (b)

Fig 29: PCR product of (a) *pha* RBC amplified by the primer *LKH* 1 and *LKH* 2 (b) *pha* PQRBC amplified by the primer *LKH* 3 *and LKH* 2.

The PCR product in 29 (a) is about 3.1 kb and the PCR product in 29 (b) is about 4.1 kb

The PCR products were subsequently purified by phenol/chloroform extraction and digested by restriction enzymes. They were subcloned into plasmid pSG703 (5.3 kb) after agarose gel purification. The PCR products were subcloned into pSG703 successfully and showed in Fig. 30.

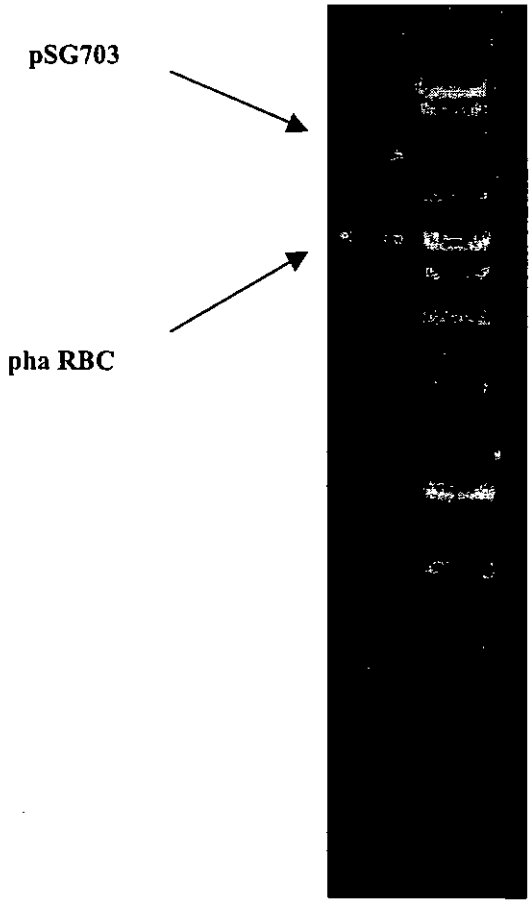


Fig. 30 (a)

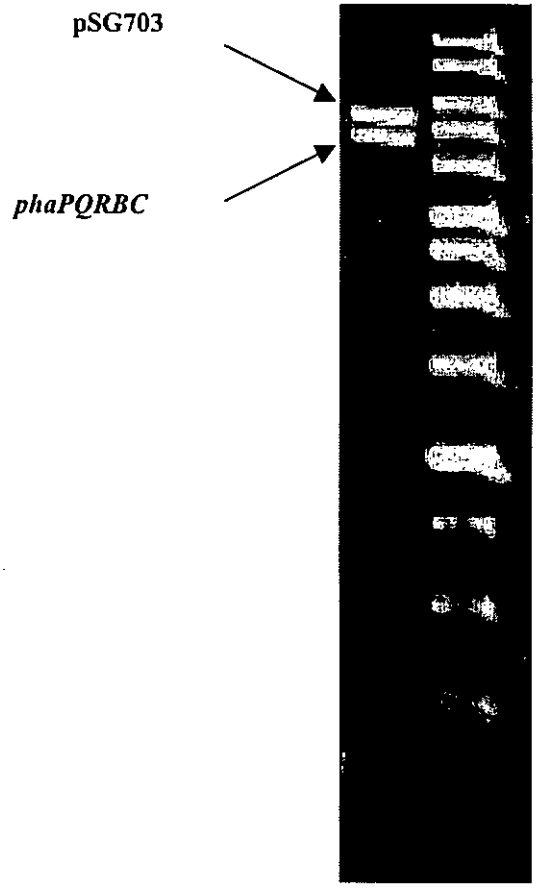


Fig. 30 (b)

Fig. 30: Gel electrophoresis of the constructed plasmid pSG703/*pha*RBC **and** pSG703/*pha*PQRBC **after restriction digestion**

Fig. 30 (a) is digested plasmid pSG703/*pha*RBC, the 5.3 kb band is vector pSG703 and the smaller about 3.1 kb is the *pha genes* RBC

Fig. 30 (b) is digested plasmid pSG703/*pha*PQRBC, the 5.3 kb band is vector pSG703 and the smaller about 4.6 kb is the *pha genes* PQRBC

The two plasmids constructed were digested by restriction enzymes for size checking. Both plasmids after digestion showed two bands, one was vector pSG703 (5.3 kb) and the other was the insert (~3.1 kb for *pha* RBC and ~4.6 kb for *pha* PQRBC).

3.2.2) Transformation of the Two Plasmids into *B. subtilis* 1A304 (ϕ105 MU331)

The circular plasmid pSG703/*pha* RBC and pSG703/*pha* PQRBC (2 μg) were transformed into *B. subtilis* 1A304 (ϕ105 MU331), respectively. In each set of transformations, five colonies were selected and plated on both chloramphenicol (5 μg/ml) and erythromycin (10 μg/ml) plates for selection and identification. All the plates were incubated at 37°C and all the selected clones were grown on plates. This suggested that the plasmids were transformed into *B. subtilis* successfully.

3.2.3) Expression of pSG703/*pha* RBC and pSG703/*pha* PQRBC

The selected strain was first grown in BHY for 16 h with 280 rpm shaking in 37°C (Growth phase).The cells were centrifuged down and washed with sterile ddH_2O and resuspended in Medium C.

A) Fermentation without Heat Shock

The culture was incubated at 37°C with 280 rpm shaking (Production phase). The change in A_{600} of the *B. megaterium* and recombinant *B. subtilis* 1A304 (ϕ105 MU331) in production medium are shown in Fig 31.

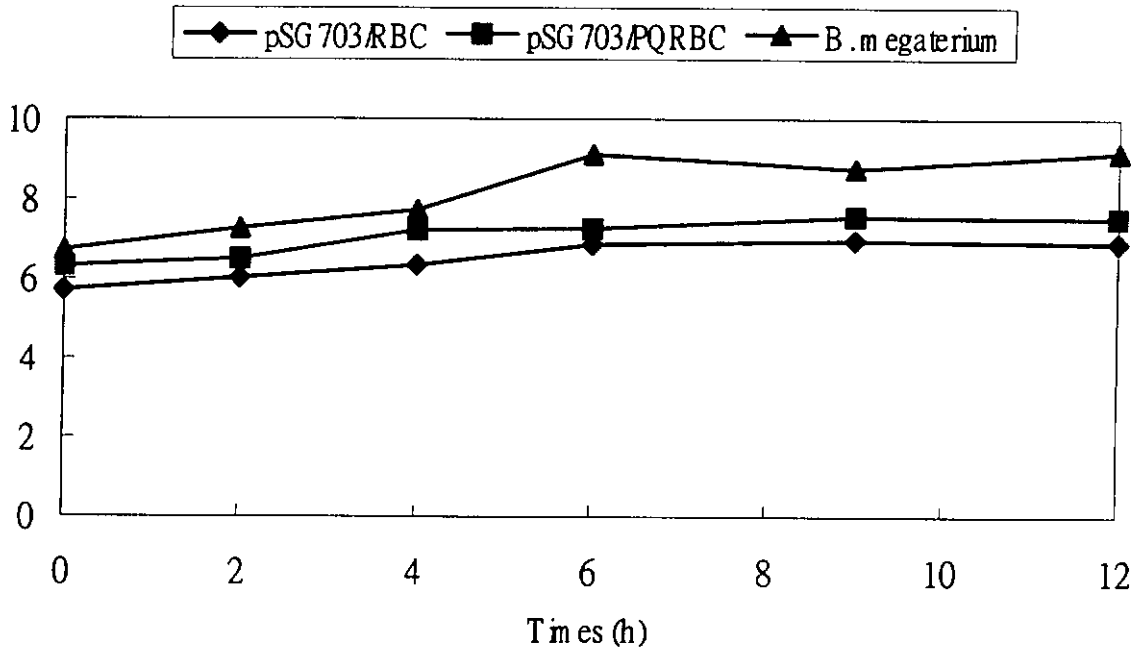


Fig. 31: Time profile of the absorbance at 600 nm of the 2 cloned strains and B. megaterium in production medium during flask culture

B. megaterium grew faster than the *B. subtilis* 1A304 (ϕ105 MU331) harboring *pha*PQRBC and *pha*RBC in both the growth and the production media respectively. The cells after fermentation were freeze-dried and subjected to GC analysis. The results are listed in Table 5. The time point for both 6 h and 12h of 1A304 (ϕ105 MU331)/*pha*PQRBC was determined for twice, but the time point of *B. megaterium* was determined for once.

Table 5: PHB yield (w/w) in *Bacillus* 1A304 (ϕ105 MU331) without heat shock induction

Time (h)	*B. megaterium*	1A304 (ϕ105 MU331)/*pha*PQRBC)	1A304 (ϕ105 MU331)/*pha*RBC)	1A304 (ϕ105 MU331)
6	4.78%	0.52% ± 0.02	0% ± 0	0% ± 0
12	16.66%	5.80% ± 0.495	0% ± 0	0% ± 0

The results show that there was no PHA accumulation in both the control *B. subtilis* without *pha* gene inside and the recombinant *B. subtilis* with chromosomal integration of *pha* genes *pha* RBC. However, the *B. subtilis* with *pha* genes (*pha* PQRBC) had the PHA accumulation after fermentation but with a yield less than the native strain *B. megaterium* ATCC 11561.

B) Fermentation with Heat Shock

The effect of heat shock on the PHA accumulation was studied. The culture was incubated at 37°C with 280 rpm shaking (Production phase) and heat shocked at the second hour. The A_{600} values of the bacterial strains in the production medium are shown in Fig. 32.

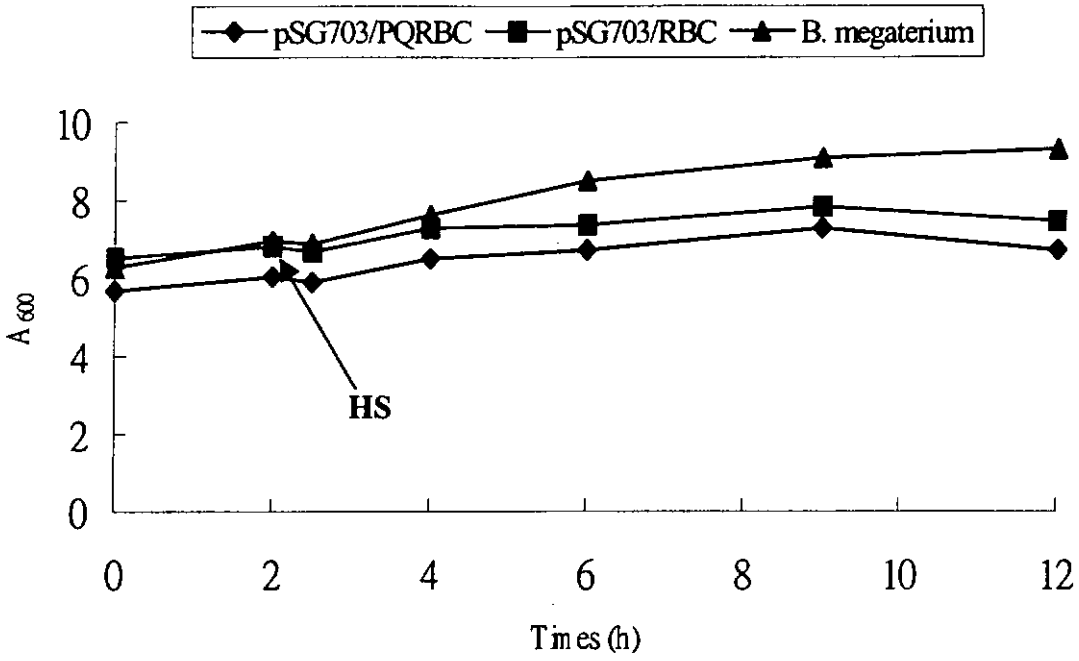


Fig. 32: Effect of heat shock (HS) on the time profile of the absorbance at 600 nm of the 2 recombinant strains and B. megaterium in production medium during flask culture

Better growth of *B. megaterium* and similar change in A_{600} of *B. subtilis* 1A304 (ϕ105 MU331) harboring *pha*PQRBC and *pha*RBC were observed in the production medium. It also showed that there was no marked drop in A_{600} after heat shock. The GC data are listed in Table 6. The time point 6 h of 1A304 (ϕ105 MU331)/*pha*PQRBC and the time point of *B. megaterium* was determined for once only and the time point 12 h of 1A304 (ϕ105 MU331)/*pha*PQRBC and both time points of 6 and 12 h of 1A304 (ϕ105 MU331) /*pha*RBC were determined for twice.

Table 6: GC analysis of the PHB percentage yield (w/w) in *Bacillus* 1A304 (ϕ105 MU331) with heat shock induction

Time (h)	*B. megaterium**	1A304 (ϕ105 MU331)/*pha*PQRBC	1A304 (ϕ105 MU331) /*pha*RBC
6	5.47%	0.39%	0% ± 0
12	15.32%	0.442% ± 0.04	0% ± 0

* indicates that the bacteria were not subjected to heat shock

There was no PHA accumulation in the recombinant strain *B. subtilis* 1A304 (ϕ105 MU331) with chromosomal integration of *pha* genes *pha* RBC. However, the *B. subtilis* 1A304 (ϕ105 MU331) with *pha* genes (*pha* PQRBC) had the PHA accumulation after fermentation with a yield lower than the same strain without heat shock. It also had lower yield than that of *B. megaterium*.

3.3) Cloning and Expression of *B. megaterium* PHA Gene in *B. subtilis* 168

3.3.1) Cloning of *B. megaterium* PHA Gene in Vector pYCL18 & pGK13

The plasmid pGEM10 (10864 bp) was used in this part of the experiment as the template for the following procedures:

a) PCR reaction to put *pha* PQRBC into vector pYCL18;

b) Isolation of the *pha* QRBC genes by restriction digestion.

Fig. 33 shows the *pha* QRBC genes isolated from *Eco*RI restriction digestion of pGEM10 and the plasmid pGK13 (5 kb). The size of the isolated *pha* QRBC genes was about 5264 bp, which was similar in size to the vector pGK13. Fig. 34 shows the PCR product obtained by using primers LKH 4 and LKH 2. It included *pha* PQRBC but the designed restriction sites were *Bam*HI and *Xba*I and the size of the PCR product was about 4.3 kb.

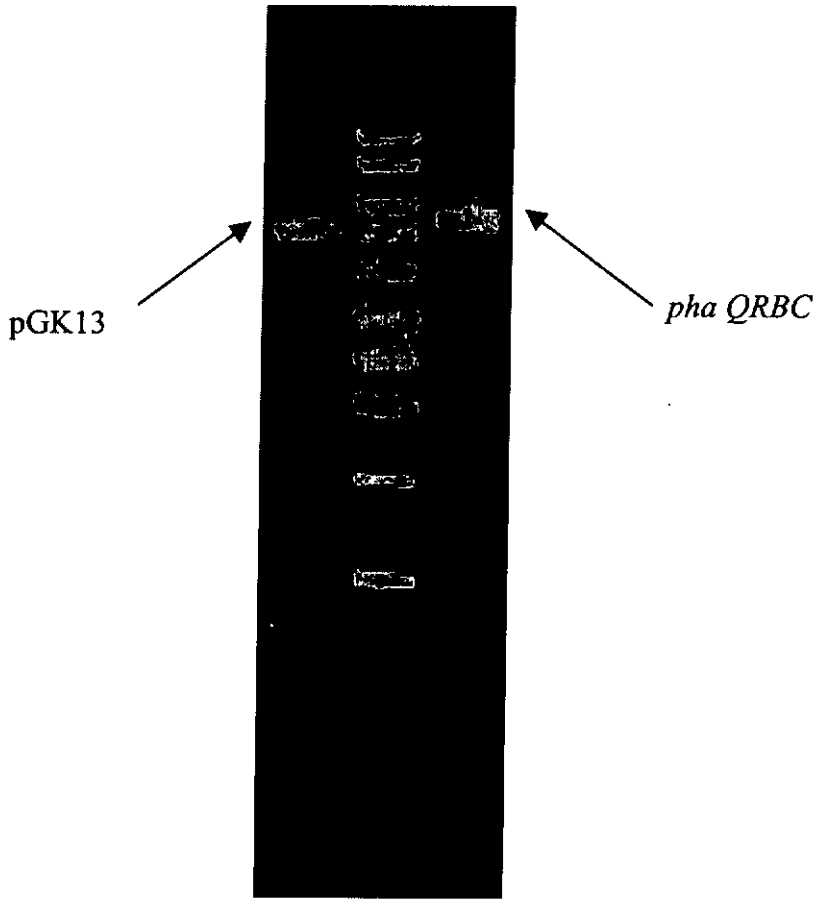


Fig. 33: *Eco*RI digested plasmid pGK13 & *pha* QRBC from *Eco*RI digested pGEM10

The 5264 bp band is *pha genes* QRBC of *B. megaterium* ATCC 11561 that sub-cloned into plasmid pGEM10. It was released from *Eco*RI digestion and purified from agarose gel. The smaller plasmid by about 5 kb is the *Eco*RI digested vector pGK13.

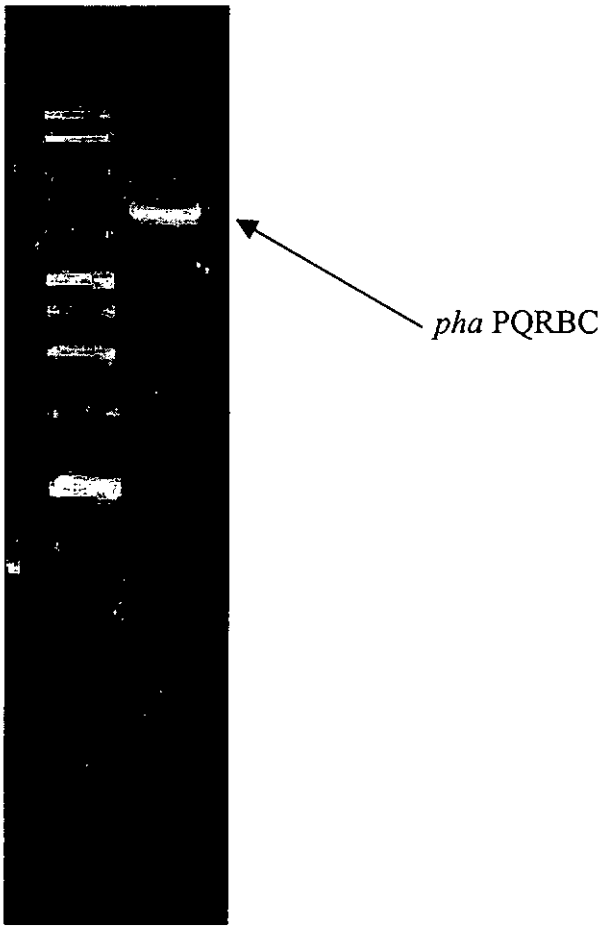


Fig.34: PCR product of (a) *pha* PQRBC amplified by the primer *LKH* 4 and *LKH* 2

The 4.3 kb band in the figure is the PCR product corresponding to the *pha* genes PQRBC of *B. megaterium* ATCC 11561

The PCR products *pha* PQRBC and the *Eco*RI digested fragment (*pha* QRBC) were subsequently purified by phenol/chloroform extraction and digested by restriction enzymes that were located on the primers. Then *pha* PQRBC was inserted into *Eco*RI/*Xba*I digested vector pYCL18 (~6.7 kb) after agarose gel purification. The PCR products were cloned into pYCL18 successfully as shown in Fig. 35, which

shows the *Eco*RI/*Xba*I digestion of the cloned plasmid.

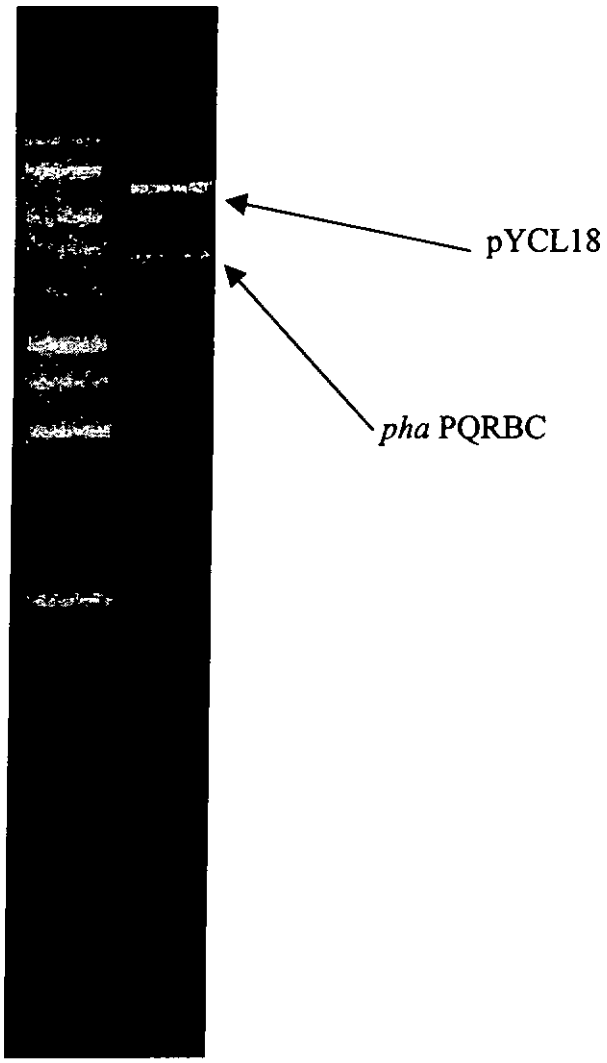


Fig. 35: Gel electrophoresis of the constructed plasmid pYCL18/*pha* PQRBC after *Eco*RI restriction digestion

The figure shows digested plasmid pYCL18/*pha* PQRBC: the 6.7 kb band is vector pYCL18 and the lower band with the size about 4.3 kb is the *pha genes* PQRBC

The *Eco*RI digested *pha* QRBC genes were successfully cloned in another vector pGK13, the cloned plasmid was digested by *Eco*RI and is shown in Fig. 36. Two bands with similar sizes were observed on gel, corresponding to *pha* QRBC

(5264 bp) and pGK13 (5 kb).

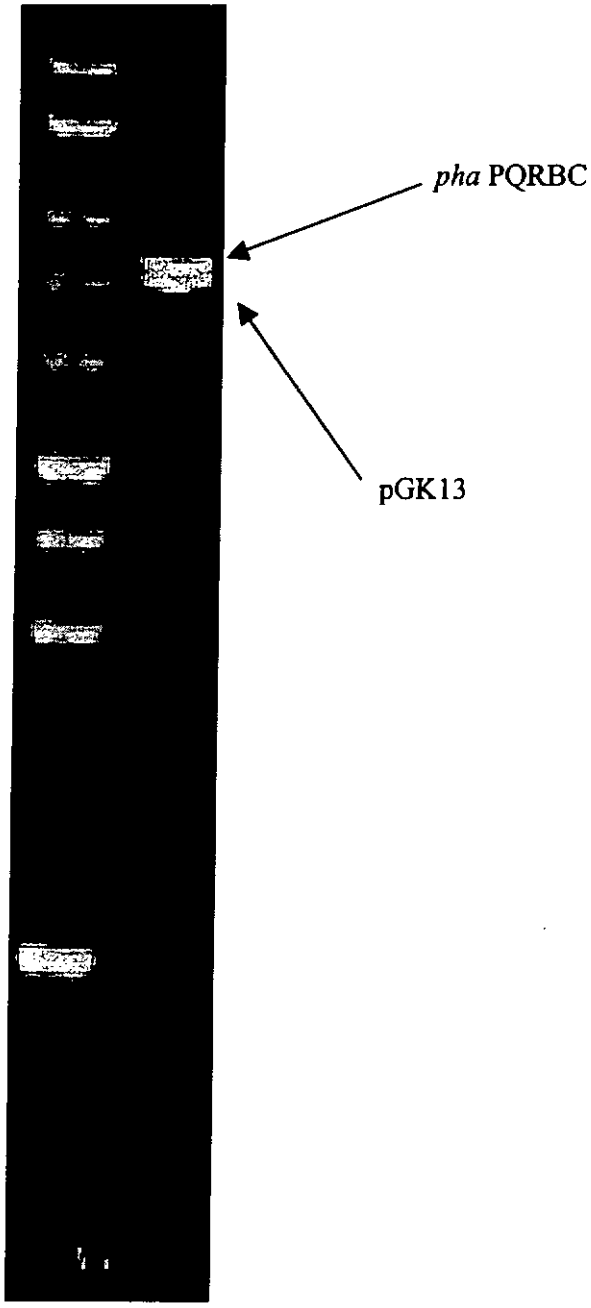


Fig. 36: Gel electrophoresis of the constructed plasmid pGK13/*pha* QRBC after *Eco*RI restriction digestion

The figure shows digested plasmid pGK13/*pha* QRBC: the 5284 bp band is *pha genes* QRBC and the lower band with the size 5 kb is the vector pGK13

3.3.2) Transformation of Cloned Plasmid in *B. subtilis* 168

The circular plasmid pGK13/*pha* QRBC and pYCL18/*pha* PQRBC (2 μg) were used to transform into *B. subtilis* 168 separately. The selection method was the same as the experiment in the transformation of *B. subtilis* 1A304 (ϕ105 MU331). Moreover, for each set of transformations, 2 colonies were selected and subjected to a mini preparation of plasmids. Their sizes were checked by restriction digestion to confirm the successful cloning of pGK13/*pha* QRBC and pYCL18/*pha* PQRBC.

3.3.3) Expression of pGK13/*pha* QRBC and pYCL18/*pha* PQRBC

The expression strategy was the same as the *B. subtilus* 1A304 (ϕ105 MU331) except that the fermentation was with heat shock. The GC spectra of the *B. subtilis* 168 harboring plasmid pGK13/*pha* QRBC, pYCL18/*pha* PQRBC, and the native strain *B. megaterium* after fermentation are shown in Fig. 37. It indicated that PHB and PHV were absent in both recombinant strains (Fig. 37b,37c), but the PHB was present in strain *B. megaterium* (Fig. 37a).

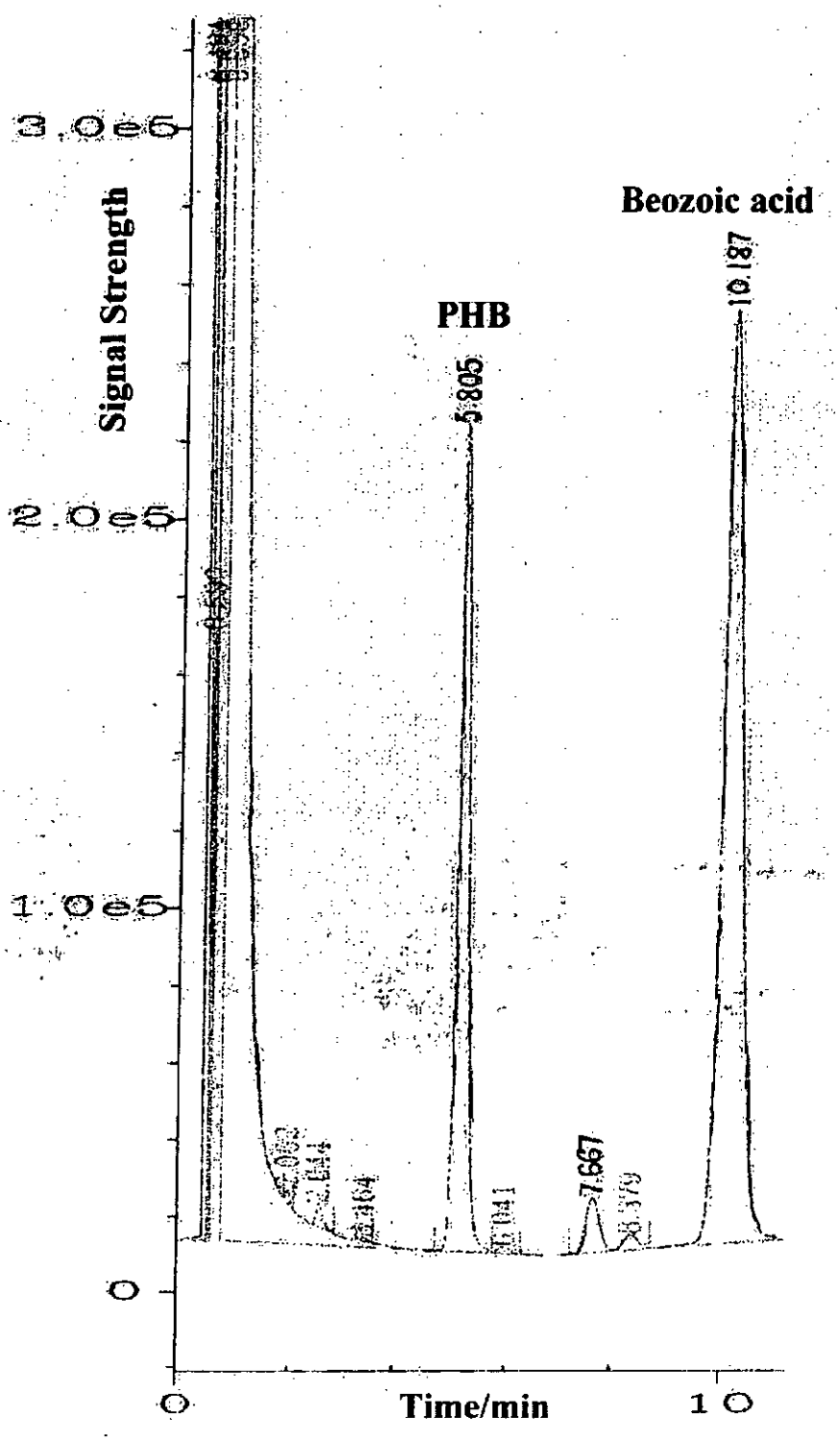


Fig. 37 (a)

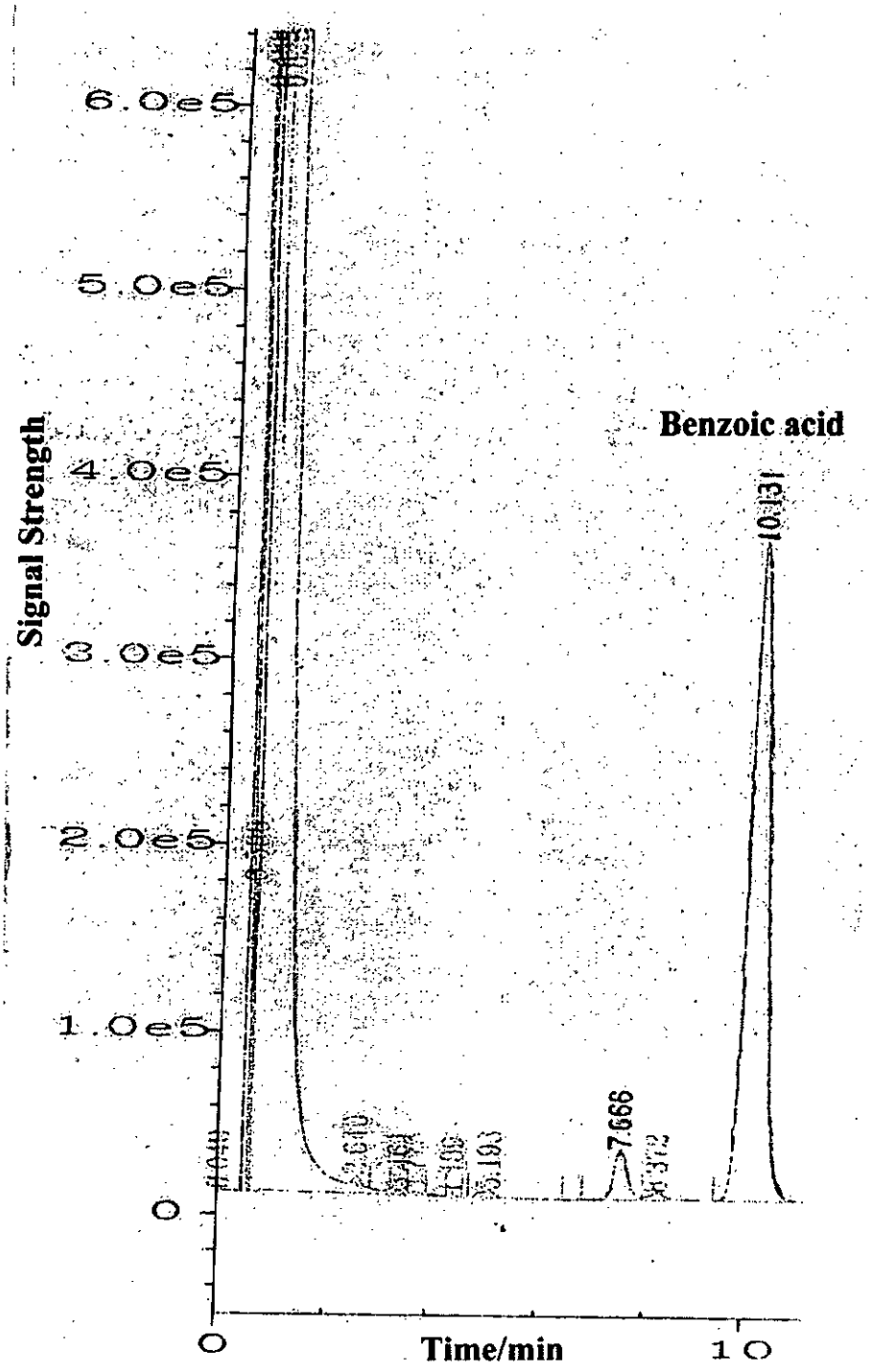

Signal Strength
6.0e5
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
0
Benzoic acid
10.131
7.666
0
Time/min
10

Fig. 37 (b)

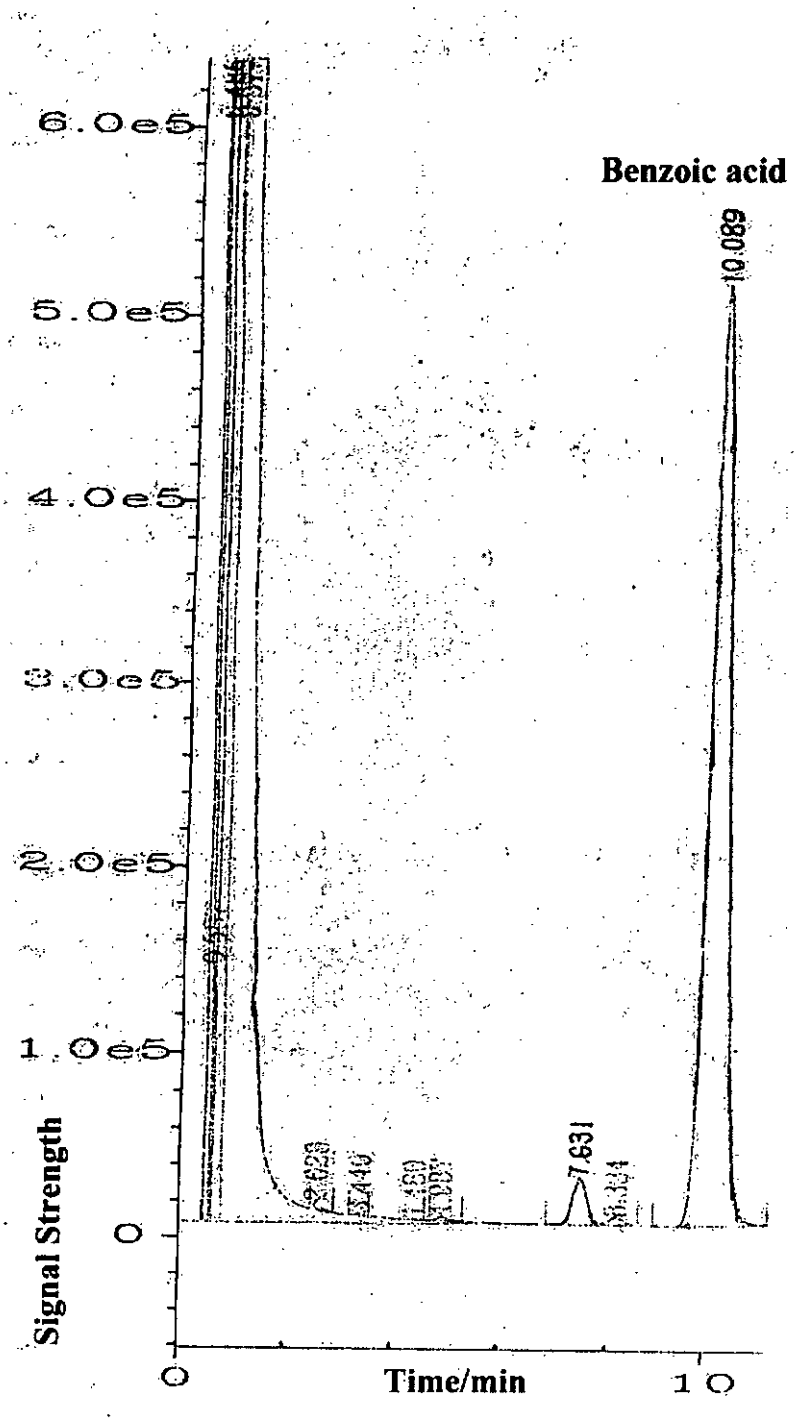


Fig. 37 (c)

Fig. 37: GC spectra of freeze-dried cells after 2-stage fermentation (a) *B. megaterium* (b) *B. subtilis* 168 (pGK13/*pha* PQRBC) (c) *B. subtilis* 168 (pYCL18/*pha* QRBC)

(a) PHB characteristic peak is observed on spectrium when *B. megaterium* ATCC 11561 was used. (b) PHA accumulation was absent in *B. subtilis* 168 with plasmid pGK13/*pha* PQRBC

after the 2-stage fermentation. (c) PHA accumulation was absent in *B. subtilis* 168 with pYCL18/*pha* QRBC after fermentation.

3.4) Cloning & Expression of *phaC1AB* Gene in *E. coli*

3.4.1) Cloning of *phaC1* Gene from *Pseudomonas* to *phaAB* Gene in *Ralstonia eutrophus*

The plasmid pGEM-HBQ06 (5941 bp) was used as the template DNA in PCR. The pGEM-HBQ06 had the whole *pha*C1 gene (3057 bp) from *Pseudomonas* and the PCR product from primers LKH 4 and LKH 5, and the restriction sites located in the primers were *Hin*dIII and *Eco*RI, respectively.

The *pha* AB genes were isolated by *Pst*I digestion of the plasmid pJM9131 (Fig. 38), and the isolated fragment of *pha* AB was about 2.28 kb.

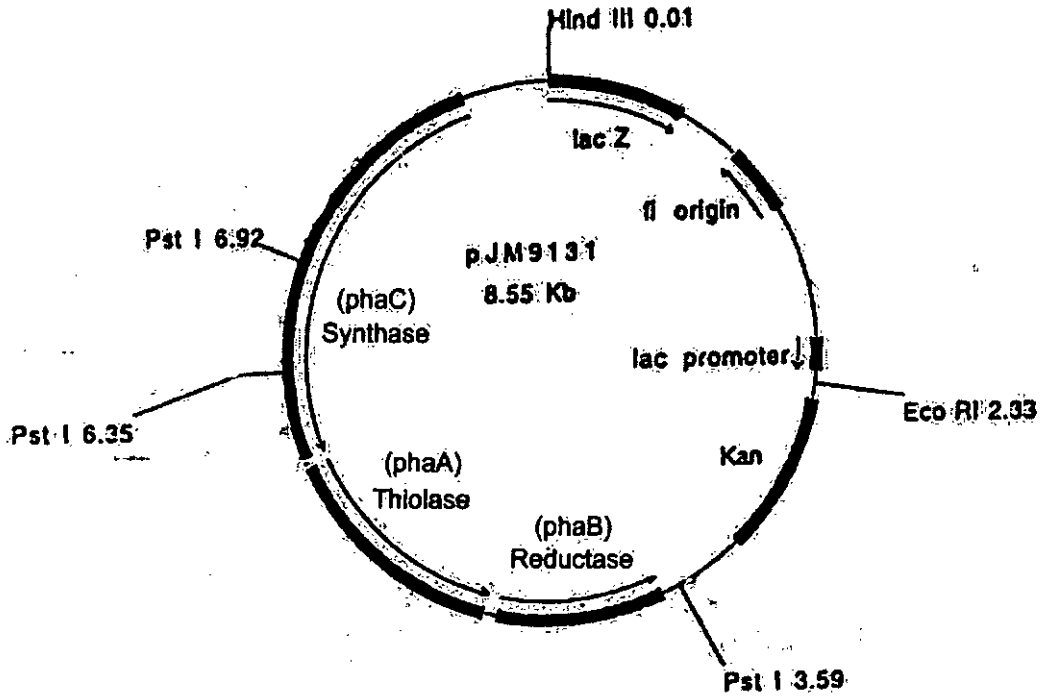


Fig. 38: The gene map of plasmid pJM9131

The *pha* AB genes were then cloned into the *Pst*I digested plasmid pKS- (2.96 kb). The size of the cloned plasmid was about 5.3 kb and the *Pst*I digested plasmid is shown in Fig. 39.

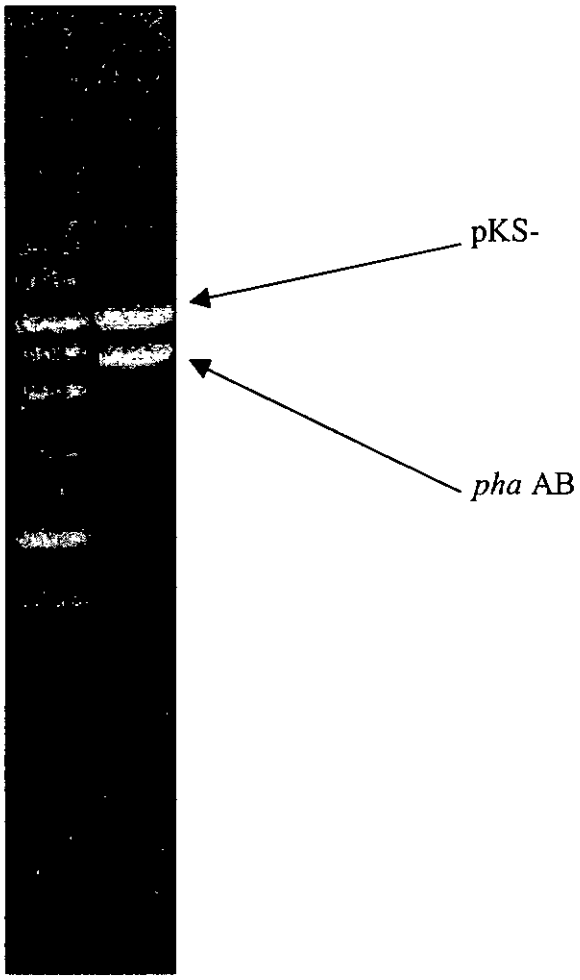


Fig. 39: Gel electrophoresis of the constructed plasmid pKS-/*pha* AB after *Pst*I restriction digestion

The figure shows *Pst*I digested plasmid pKS-/*pha* AB. The 3 kb band is vector pKS- and the lower band about 2.28 kb is the *pha* AB genes of *R.. eutrophus*

The plasmid (5.3 kb) was then ligated to PCR product *pha*C1 gene (3028 bp), and the restriction sites used were *Hin*dIII and *Eco*RI. The cloned plasmid digested by *Eco*RI and *Hin*dIII are shown in Fig. 40.

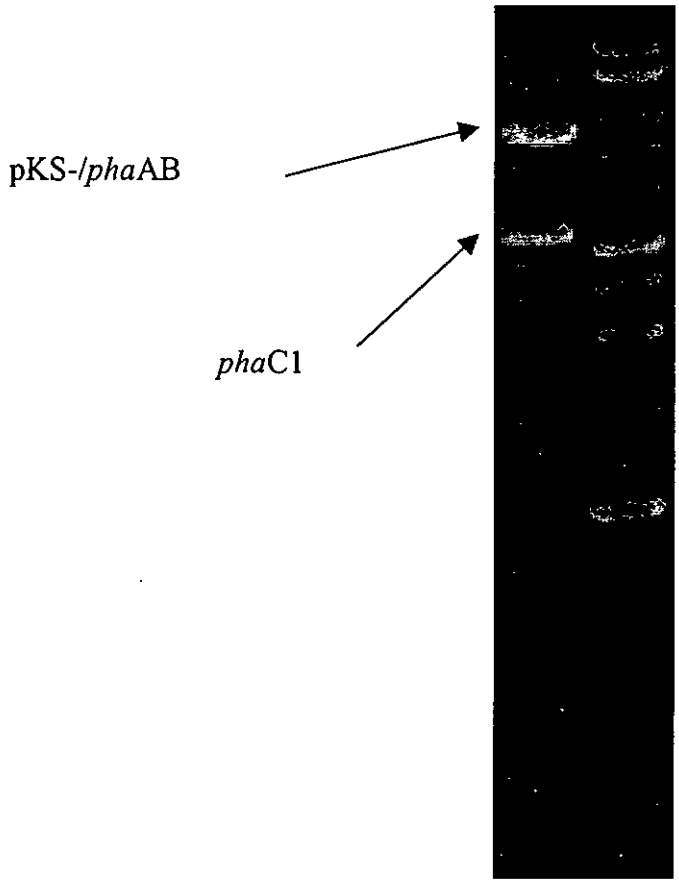


Fig. 40: Gel electrophoresis of the constructed plasmid pKS-/*pha* C1AB after *Hin*dIII/*Eco*RI restriction digestion

The figure shows *Hin*dIII/*Eco*RI digested plasmid pKS-/*pha* C1AB. The 5.3 kb band is pKS-/ *pha* AB and the lower band about 3028 bp is the *pha* C1 gene of *Pseudomonas*.

The whole *pha*C1AB (5337 bp) genes were isolated from pKS- /*pha*C1AB by restriction enzymes HindIII and XbaI. The whole gene was then ligated to vector pUC19 (2686 bp) and the cloned plasmid was checked by HindIII/XbaI digestion (Fig.

41).

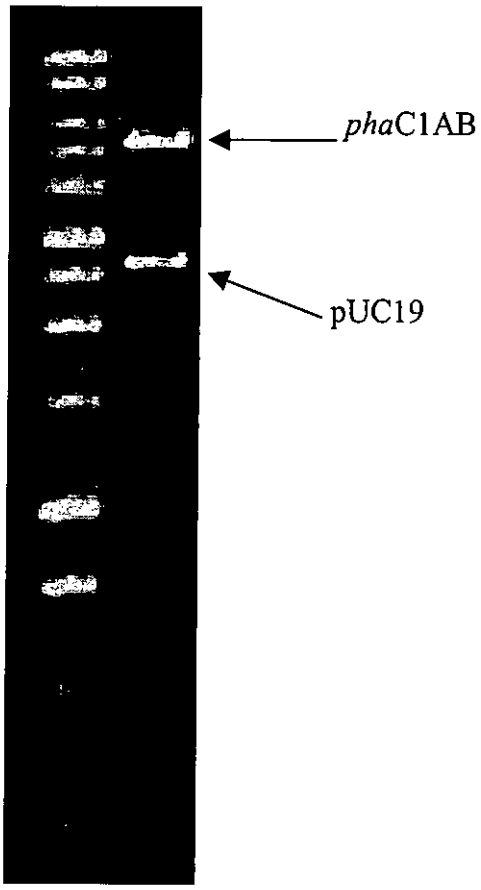


Fig. 41: Gel electrophoresis of the constructed plasmid pUC19/*pha*C1AB after *Hin*dIII/*Xba*I restriction digestion

The figure shows *Hin*dIII/*Xba*I digested plasmid pUC19/*pha* C1AB. The 5337 bp band is *pha* C1AB genes and the lower band about 2686 bp is the vector pUC19.

3.4.2) Transformation of pKS-/*pha*C1AB and pUC19/*pha*C1AB into *E. coli* XL1-Blue and LS1298

The plasmids were transformed into the *E. coli* XL1-Blue cells and the fatty

acid degradation mutant strain LS1298, respectively. The transformed clones were selected by the use of ampicillin. The transformation was successfully done.

3.4.3) Expression of Cloned Plasmid in *E. coli*

LB was used as a medium and 0.5 mM IPTG was used as the culture which reached A_{600} near to 1 in order to trigger the lac promoter. However, the cells sampled at different fermentation times showed that there was an absence of PHA accumulation in GC analysis. The GC spectra are shown in Fig. 42.

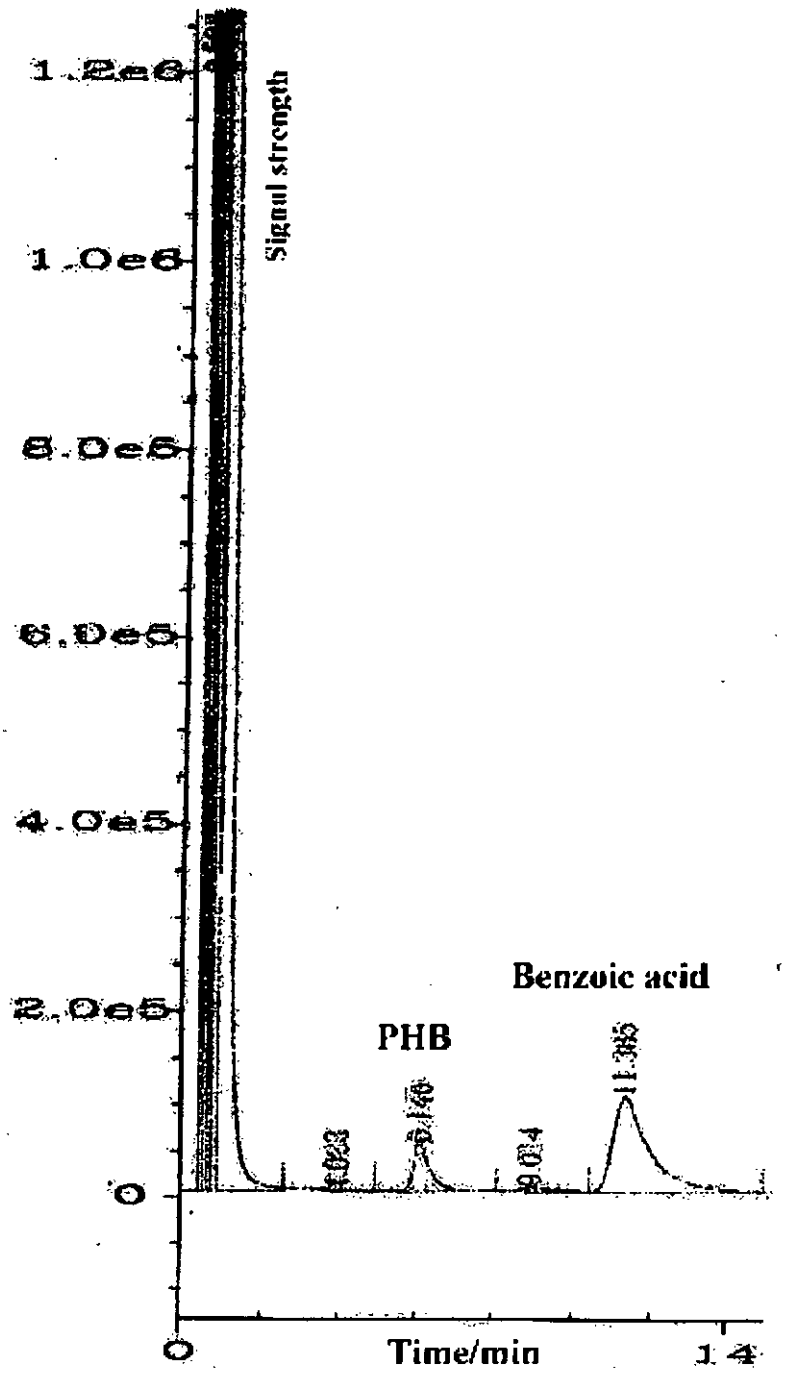

1.2e6
1.0e6
8.0e5
6.0e5
4.0e5
2.0e5
0
Signal strength
Benzoic acid
PHB
11.385
0
Time/min
14

Fig. 42 (a)

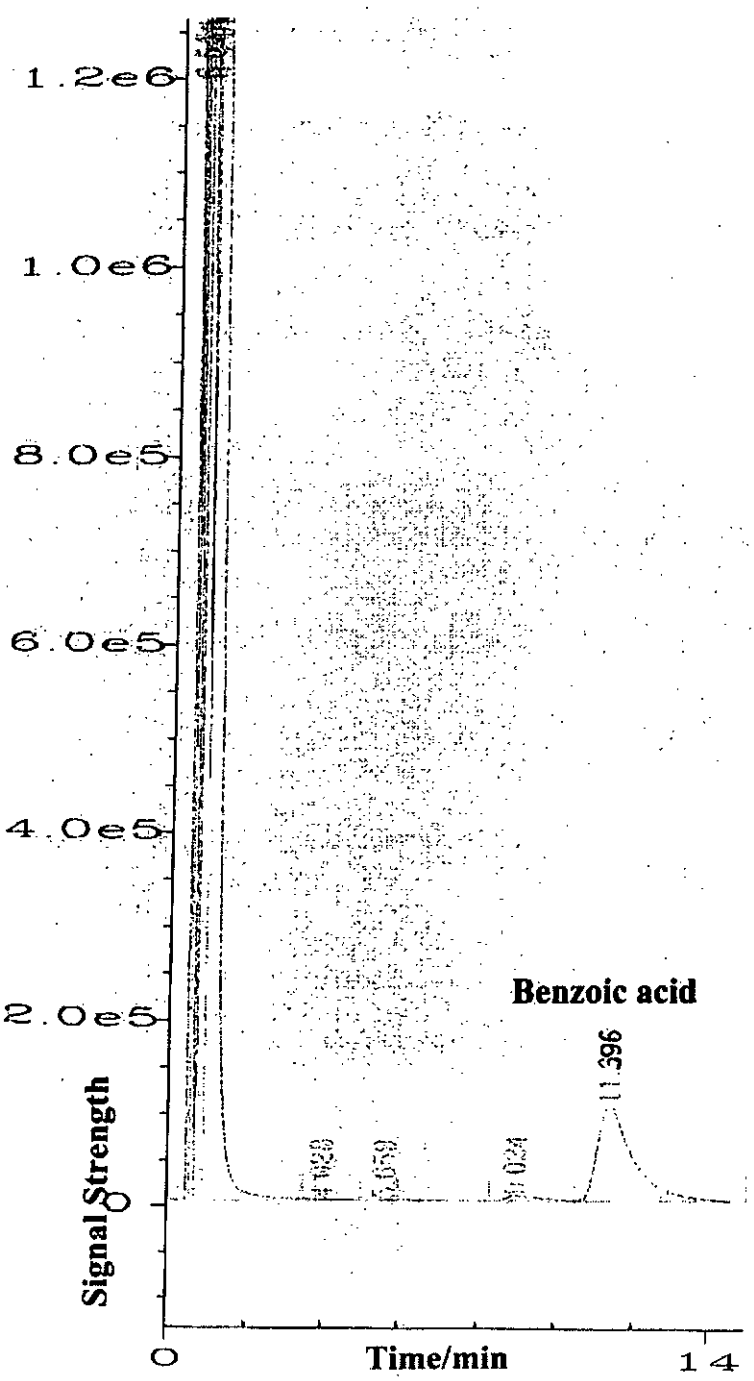


Fig. 42 (b)

Fig. 42: The GC spectra of freeze-dried *E. coli* cells with plasmid (a) pUC19/*pha* CAB (b) pUC19/*pha* C1AB after flask culture with 0.5 mM IPTG induction

The PHB characteristic peak is observed on spectra when *E. coli* XL1-Blue harboring plasmid pUC19/*pha* CAB was used (Fig. 42 a) in flask fermentation at 37°C with 0.5 mM IPTG induction at A_{600} equal to 1. There is an absence of PHA accumulation in *E. coli* XL1-Blue with plasmid pUC19/*pha* C1AB after the flask fermentation (Fig. 42 b).

The Fig. 42 (a) shows the spectrum of *E. coli* XL1-Blue with plasmid pUC19/*pha* CAB. The results showed the PHB characteristic peak when compared to the purchased standard PHB. However, there was an absence of the PHB characteristic peak in *E. coli* XL1-Blue cells with plasmid pUC19/*pha* C1AB (Fig. 42b).

The percentage yield was calculated by the GC spectra of the *E. coli* cells with different plasmids. The percentage yield of the cells with plasmid pUC19/*pha* C1AB and control plasmid pUC19/*pha* CAB are listed in Table 7. The time points of *E. coli* with plasmid pUC/*pha*C1AB were determined for twice.

Table 7: PHB yield (w/w) in *E. coli* with plasmid pUC/*pha*CAB and pUC/*pha*C1AB

	Time after IPTG Induction (h)	
	6	20
pUC/pha CAB	27.62% ± 5.39	24.66% ± 4.26
pUC/pha C1AB	0% ± 0	0% ± 0

3.5) Sub-cloning & Expression of the *phaCAB* Gene in different *E. coli*

3.5.1) Construction of recombinant E. coli strains

Using a 5.2 kb *EcoRI* / *HindIII* restriction fragment comprising the entire *pha* operon obtained from plasmid pJM9131 as an insert, and plasmid pUC19 possessing

lac promoter. The cloning was confirmed by restriction digestions and agarose gel electrophoresis.

In Fig. 43, there were two bands appeared. The size of the top one is around 5.2 kb and the size of the lower one is around 2.68 kb: one is the linear pUC19 vector with size of 2.68kb and the other is the *phb*CAB genes with size of 5.2kb. The two bands present in Fig. 43 show that the newly cloned plasmid was successfully transformed into the XL1-Blue and HMS174 and also demonstrate that the construction of the recombinant *E. coli* with plasmid pUC19 containing the entire *phb* operon has been done successfully.

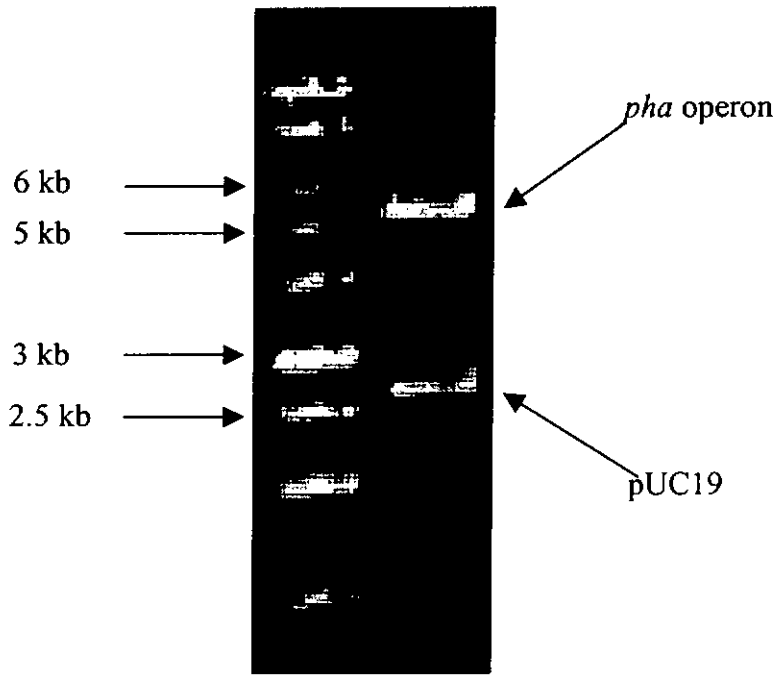


Fig. 43: Agarose gel of DNA electrophoresis after double restriction digestions showing the insert 5.2 kb pha operon and 2.68 kb pUC19 vector.

3.5.2) Comparison of PHA production in different recombinant *E. coli*

Comparing the growth pattern the two *E. coli* strains, the *E. coli* HMS174 harboring plasmid pUC19/PHA had better growth than the *E. coli* XL1-Blue harboring plasmid pUC19/PHA. The absorbance of both strains during flask cultivation at 600 nm was shown in Fig.44.

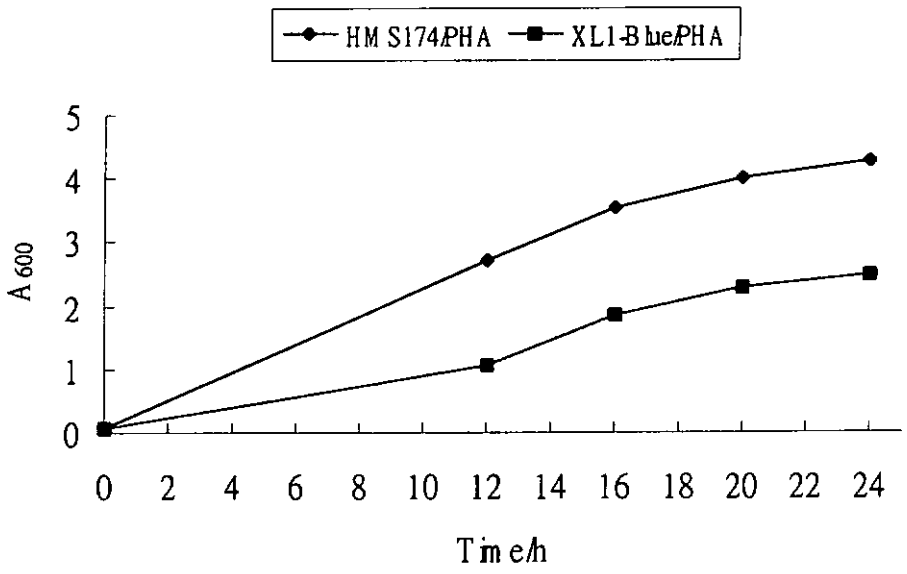


Fig. 44: Time profile of the absorbance at 600 nm of the *E. coli* strains during the flask cultivation

The composition and percentage yield of biopolymer within the cells were analyzed by the GC and the GC spectra showed that the biopolymer accumulated was P(HB-HV). The percentage yield of copolymer of dry cell weight at different times was shown in Fig. 45. It shows that the majority of biopolymer accumulated in cells was PHB and its yield was much higher in *E. coli* HMS174/PHA than that in *E. coli* XL1-Blue/PHA and also the higher PHV yield was obtained in it.

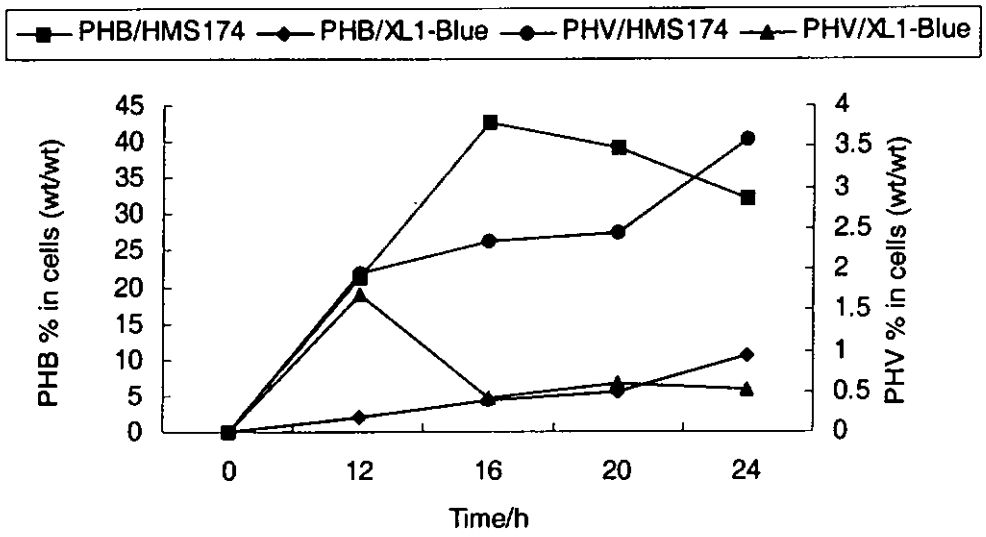


Fig. 45: Percentages of PHB and PHV in dry cell weight of *E. coli* strains during the flask cultivation

3.5.3) Fed-batch Fermentation in a computer-controlled 15 L fermenter

In according to the well growth of the *E. coli* HMS174/PHA in the presence of propionate and its high yield of P(HB-HV) accumulation in flask fermentation., *E. coli* HMS174/PHA was chosen as the target strain in fermenter cultivation. The A_{600} and its CDW were recorded at different time points throughout the fermentation: the A_{600} and cell dry weight could reach as high as 26.7 and 10.27 g/L respectively at 59 h cultivation in fed-batch culture and the results are shown in Fig. 46.

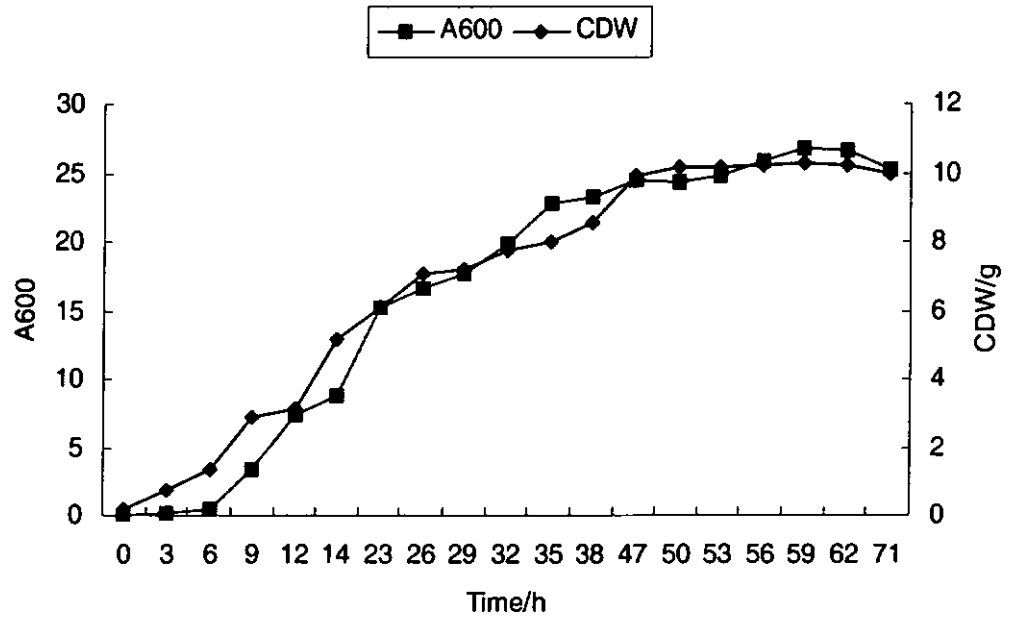


Fig. 46: Time profile of the absorbance at 600 nm and cell dry weight of the *E. coli* HMS174 with plasmid pUC19/PHA during the 15-L fermenter cultivation

The PHA content was analyzed by GC and the percentage yield of PHB and PHV in cells was shown in Fig. 47.

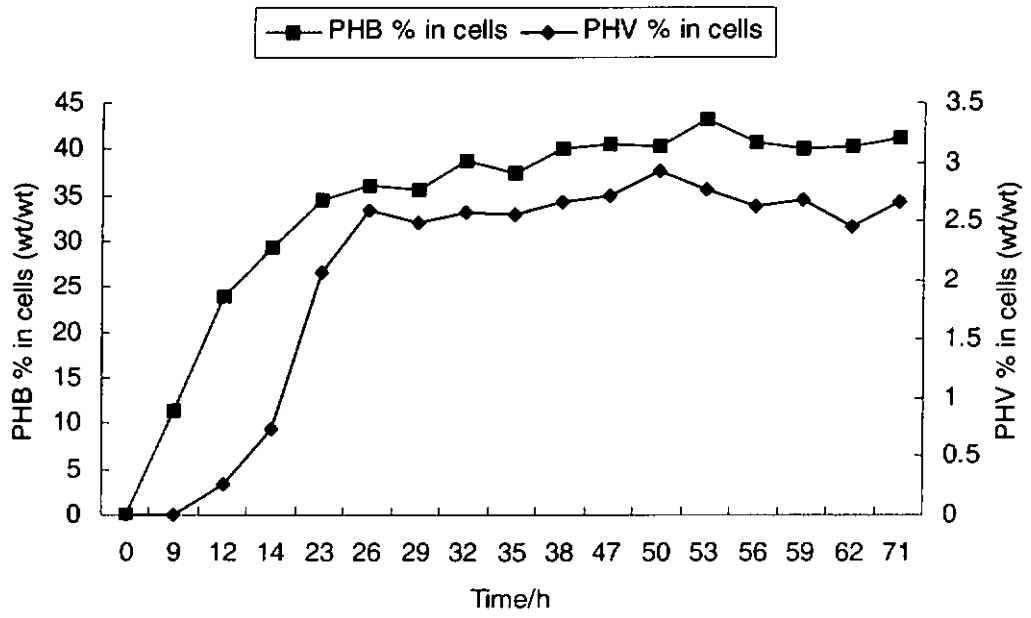


Fig. 47: Percentages of PHB and PHV in dry cell weight of *E. coli* HMS174 with plasmid pUC19/PHA during the 15-L fermenter cultivation

The cell concentration increased to 10 g/L CDW and the PHB content reached as high as 43.2%. However, the PHV content was maintained at about 2% of cell dry weight.

3.5.4) Physical properties of the extracted biopolymer from fermenter

The composition of the hydroxyalkanoates units in extracted products were determined by analyzing the nuclear magnetic resonance (NMR) spectra. ^{1}H-NMR spectrum (Fig. 48) shows the presence of 5 groups of characteristic signals of the P(HB-HV). The signals with different chemical shifts were attributed to the different groups coupled to different numbers of proton as labeled in the figures.

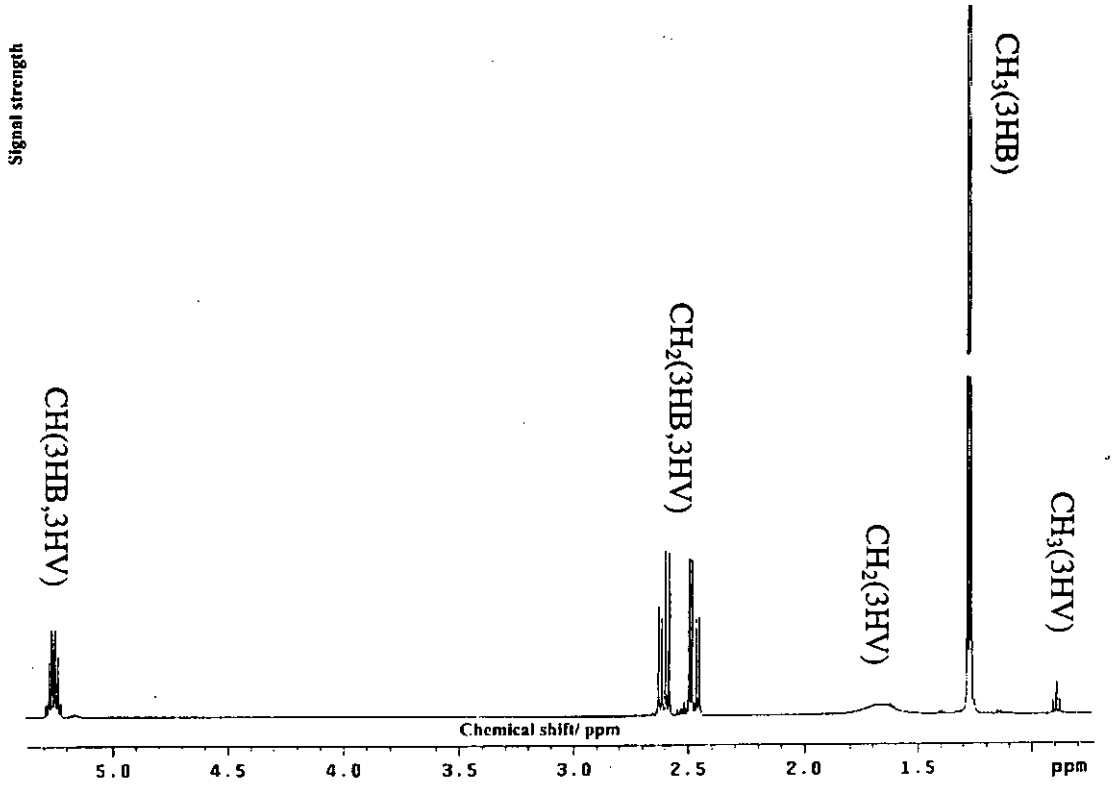


Fig. 48: ^{1}H NMR spectrum of biopolymer produced by *E. coli* HMS174 with plasmid pUC19/PHA in fermentation

Chapter 4: Discussion

4.1) The PHA-producing Strain Isolated from Activated Sludge

4.1.1) Isolated strains identification

Two PHA-producing microbial strains (HF-1 and HF-3) were successfully isolated from the activated sludge. Because of their heat resistance, morphology and cell staining, they were suspected to be members of the *Bacillus* strains. *Bacillus* is Gram-positive and has the superior properties to grow efficiently on inexpensive feedstock. Its products are generally regarded as safe.

The isolated strains were identified by Microbial ID, Inc. and they were both identified to be possibly *B. megaterium*. However, the low similarity index (0.552) of HF-3 indicated that it should not be *B. megaterium*, but should be *Bacillus* instead. The subspecies HF-3 is not certain and further analysis should be done.

4.1.1.1) Isolated strains DNA sequences analysis

The genomic DNA of HF-1 and HF-3 was isolated and further identified by DNA sequencing as described in section 3.1.5. However, only HF-1 produced a PCR product with the right size (~1.2 kb) by using primer *LKH* 4 and 7 that were designed

according the *pha* gene sequences of *B. megaterium*. The presence of the PCR product in HF-1 but not in HF-3 further supports the assumption that HF-1 is more likely to be *B.* megaterium. Moreover, the percentage identity of DNA sequences of the PCR fragment from HF-1 were found to be 94% and 95% in two separate regions (Fig. 27) when compared to the corresponding sequences of *pha* gene in the *Bacillus megaterium* ATCC11561. It suggests that HF-1 may be the strain *B. megaterium* and the slight difference may be because it belongs to a different subspecies.

4.1.2) Fermentation of the isolated PHA-producing strains

Because of the efficient utilization of inexpensive carbon sources by *Bacillus* strains, food wastes have been used for the production of PHA. The use of inexpensive carbon sources could lower the PHA production cost.

The absorbance of HF-1 and HF-3 during the flask culture in malt and soy wastes (Fig. 18) were high, indicating the efficient utilization of the food wastes. The wavenumber in FT-IR spectra are similar to that of the previous studies [44,45,46] and the sharp absorption band is assigned to the stretching vibration for the ester carbonyl of PHA. The changes in PHA content (Fig. 19) generally follow a sigmoid curve pattern. The drop in the PHA content at the final stage is due to the utilization of PHA by the cells as a carbon source when the carbon sources in the medium become limited.

In a previous study [12], copolymer was observed from the fermentation of glucose and fructose as carbon source by activated sludge as well as malt and soy wastes. Thus, apart from malt and soy wastes, both HF-1 and HF-3 were selected to ferment several types of carbon sources, including sucrose, fructose and maltose. Both HF-1 and HF-3 were poor in utilizing fructose as the carbon source, but were good in utilizing sucrose and maltose (Fig.20).

Fig. 19 shows that the highest PHA content in cells occurred at 8 h-10 h when hydrolyzed food wastes were used as the medium. Similar results shown in Fig. 21 indicate that the highest PHA content in HF-3 cells occurred at 10-11 h when a synthetic medium was used. The copolymer cannot be clearly identified from the FT-IR spectra, and there was only a little variation in the wavenumber when compared to the purchased control PHB and PHB-co-PHV since the similar or equal stretching vibration for the ester carbonyl of PHA [31].

4.1.3) NMR and GC analysis of produced biopolymer

Similar pattern of ^{1}H-NMR spectrum and chemical shift of biopolymer extracted from HF-1 and HF-3 in were obtained (Fig. 22) and it was equal to that of purchased standard PHB. ^{1}H-NMR spectrum shows the presence of three groups of characteristic signals of the homopolymer PHB. The signal at about 1.26 ppm was

attributed to the methyl group coupled to one proton, the signal at about 2.52 ppm was attributed to a methylene group and a multiplet at 5.25 ppm was attributed to a methyne group. The signal at about 7.27 ppm was due to the chloroform [40]. The spectra shows the present of CH-, CH_2- and CH_3- groups in the molecule, but no –COOH and –OH groups. So the compound should be a polymer but not β-hydroxybutyrate. Therefore, HF-1 and HF-3 might not produce copolymer when the isolated strains were cultivated in medium with different selected carbon sources as well as the food wastes.

The extracted biopolymers from freeze-dried cells cultivated in different carbon substrates were subjected to GC analysis. The purity of the extracted biopolymer and the percentage yield of PHA in the freeze-dried cells (w/w) could be calculated from the GC spectra. The recovery yield and purity of PHA are strongly dependent on PHA content and hence production cost. It was shown that there was a PHB peak at retention time around 4.77 min but no PHV peak at retention time around 6.56 min when compared to the standard (Fluka). The purity of extracted PHB of HF-1 and HF-3 was about 80% and 83% respectively. The purity of PHB from HF-1 and HF-3 was not very high. The reason may be that after polymer recovery, some cell residues cannot be removed by simple filtration and thus are weighed together with the extracted polymer.

Both strains were superior in the production of PHB when hydrolyzed malt waste was used as a medium, the yield of PHB can reach as high as 40% of their cell dry weight. From the GC analysis of synthetic media, the percentage yields of PHA from HF-1 and HF-3 cells were 36.38% and 33.80% respectively in sucrose, but were 28.67% and 27.58% in maltose. The results obtained indicate that only a homopolymer of PHB was produced in HF-1 and HF-3.

4.1.4) DSC analysis of extracted biopolymer

Mechanical properties and physical properties of a new environmentally compatible material have received much attention since the stiffness of PHB was not suitable for the replacement of petroleum plastic. The T_m value of the P (3HB) homopolymer was about 170°C, which was near the T_m of polypropylene [47], but the biopolymers extracted from HF-1 and HF-3 were 165°C and 157°C, respectively (Fig. 24). The T_m of the samples were lower than that of the standard PHB. This may be due to the impurities in the samples, such as cell debris left after PHA extraction, that may affect the physical properties of the products made by the biopolymer with impurities. Therefore the extraction method needs to be improved. Two nearby peaks were observed in DSC spectra (Fig. 24), which may be because different forms of the PHB exist in the sample (e.g. -α and -β forms of PHB), and therefore two peaks that

each represented a slightly different T_m, could be observed. The value of elongation to break PHB was much lower than that of polypropylene [35,36]; the brittle property of PHB is limited its use on daily applications, so novel copolymer of PHA with superior mechanical properties and lower production cost is important to the popularity of the PHA usage.

4.1.5) PHA inclusion body-associated proteins characterization

Recently, Cannon *et al.* [24] reported that the PHA inclusion body-associated proteins of *B. megaterium* were related to its *pha* gene expression and the most abundant proteins were 20 kDa and 41 kDa in *B. megaterium*. Fig. 25 shows the results of SDS-PAGE of proteins released from purified PHA inclusion bodies. There were at least 20 such proteins present in various quantities. The two most abundant proteins have molecular mass of approximately 41 kDa and were found in all three strains. The 20 kDa protein band was more prominent in HF-1 and *Bacillus megaterium*. Thus it suggests that HF-1 is closely related to *B. megaterium*. However, the electrophoretic pattern of proteins in the gel from HF-3 was quite different to that of the other two, except those representative protein bands from *pha* gene cluster. This suggests that it might be the *Bacillus* species but a different subspecies.

4.2) Expression of *B. megaterium* PHA Gene in *B. subtilis* 1A304 (ϕ105 MU331)

Recombinant *E. coli* is currently used in the industrial production of PHA. There are many advantages such as better understanding in both genetics and biochemistry and short generation time. But endotoxins could be produced in *E. coli*. *Bacillus* is potentially a very attractive host for gene expression and secretion. It can easily grow to very high cell density using inexpensive carbon and nitrogen sources and is capable of secreting large amounts of enzymes. There is a great deal of information on its transcriptional and translational regulation and it appears to have no major codon bias. Furthermore, because of its lack of pathogenicity and its safe use as a production organism, recombinant *B. subtilis* was chosen as the host in this project.

4.2.1) Expression of pSG703/*pha* RBC and pSG703/*pha* PQRBC

B. megaterium was the first discovered PHA-producing bacterium [32] and its *pha* genes have recently been sequenced by Cannon *et al.* [24]. The *pha* RBC genes were under the same promoter (Fig. 4), so two sets of cloning (pSG703/*pha* RBC and pSG703/*pha* PQRBC) were done in order to analyze whether the expression of *pha* PQ genes are essential to the PHA accumulation. The *pha* genes PQRBC and RBC were isolated from the genomic DNA of *B. megaterium* ATCC 11561 and cloned into the vector pSG703 separately (Fig. 29). The plasmid was amplified in *E. coli* and

finally transformed into *B. subtilus* 1A304 (ϕ105 MU331).

4.2.1.1) Expression of *B. subtilis* 1A304 (ϕ105 MU331) without heat shock

After the expression of the genes in *B. subtilis* 1A304 (ϕ105 MU331) (Fig. 30 and Table 5), the A_{600} value of the strain was high in the growth medium BHY, and there was little fluctuation of the A_{600} value in the production medium. It might be due to the lack of nitrogen in the production medium for the proteins and amino acid synthesis that is essential for cell division.

PHA yield is one of the major factors in the economical production of PHA. The higher the cell density, the higher the amount of PHA produced. The expression in the recombinant strain without heat shock is controlled by the native promoters of the *pha* PQRBC genes. The promoters are triggered by the nitrogen-limiting condition, as is the case for many other PHA-producing bacteria. Table 5 shows the results of the gene expression. There was no PHA accumulation in both the control *B. subtilis* 1A304 (ϕ105 MU331) without *pha* genes and the recombinant strain *B. subtilis* 1A304 (ϕ105 MU331) with chromosomal integration of *pha* genes *pha* RBC. However, the *B. subtilis* 1A304 (ϕ105 MU331) carrying the *pha* genes (*pha* PQRBC) showed PHA accumulation, which was the first report for the expression of the *pha* genes of *B. megaterium* in *B. subtilis*. It suggested that the *pha* P gene or Q gene or

both were essential for the PHA production. Although inclusion body-associated low molecular proteins (phasins) have been described in many bacteria [48], the DNA sequence database search from the gene bank (National Center for Biotechnology Information) indicated that there was no sequence homology that could suggest the function of these two genes. Low molecular weight, PHA inclusion body-abundant proteins might play an important role in PHA-producing cells, since they are involved in determining inclusion body size and shape in the case of PHA-producing *A. eutrophus* [49].

4.2.1.2) Expression of *B. subtilis* 1A304 (ϕ105 MU331) with heat shock

Heat shock process in *B. subtilis* 1A304 (ϕ105 MU331) could trigger its strong phage promoter. Table 4 shows that there was no PHA accumulation in the recombinant strain *B. subtilis* 1A304 (ϕ105 MU331) with *pha* RBC genes. Moreover, the *B. subtilis* 1A304 (ϕ105 MU331) with *pha* genes (*pha* PQRBC) had the PHA accumulation after fermentation with the yield lower than the same strain without heat shock. The A_{600} measured before and after the heat shock were similar (Fig. 32) which means that the heat shock process did not cause a large amount of cell death. Thus the lower yield was not related to the death of the cells after heat shock. It might be related to the gene arrangement of the *pha* genes of *B. megaterium*. The gene

arrangement of *pha* PQRBC is shown in Fig. 4. The 5' to 3' direction of the *pha* P and Q genes were opposite to the phage promoter of *B. subtilis* 1A304 (ϕ105 MU331). Therefore, the RNA polymerase bound to phage promoter and native promoter of *pha* P gene respectively and then was run along in the opposite direction during the transcription as the phage promoter was triggered by heat shock. It might affect the *pha* genes expression.

4.3) Expression of the *B. megaterium* PHA Gene in *B. subtilis* 168

The results obtained from heat shock fermentation suggested that the heat shock process might impose adverse effects on the expression of the *pha* genes. Because the *pha* genes were integrated into the chromosomal DNA and resulted in the single copy number of the *pha* genes in the recombinant *B. subtilis*, the yield of PHA from the fermentation without heat shock (Table 5) was less than that of *B. megaterium*. The *pha* genes were inserted into the plasmids pGK13 and pYCL18 and finally transformed into *B.* subtilis 168. The *pha* genes were not integrated into the chromosomal DNA, resulting in the multi-copy of the genes. The *pha* RBC genes were cloned into pSG703 and no PHA accumulation was observed. Therefore *pha* QRBC genes were cloned into pGK13 to see whether PHA could be produced by

these four genes only. However, there was no PHA in both recombinant strains (Fig. 35b), but PHB was present in strain *B. megaterium* (Fig. 35a) in expression. The size of the plasmid pYCL18/*pha* PQRBC in *B. subtilis* 168 was nearly 12 kb and may cause the plasmid pYCL18/*pha* PQRBC to become unstable in the *B. subtilis* 168. The size of plasmid pGK13/*pha* QRBC was also larger than 10 kb, and the absence of expression may be due to the instability of plasmid in pGK13/*pha* QRBC or the presence of the *pha* P gene is essential in the expression. There was an absence of the plasmid instability in case of the *B. subtilus* 1A304 (ϕ105 MU331) since the genes were integrated in its chromosomal DNA. In addition, the absence of expression may be due to the error during PCR fragment since the error rate increases as fragment length increases.

4.4) Expression of the *pha*C1AB Genes in *E. coli*

Among the PHA family, PHB is the most common member. It belongs to the short chain length PHA (scl PHA) with its monomers containing 4-5 carbon atoms. *R. eutrophus* is the typical bacterium to produce scl PHA. Other PHA containing monomers consisting of 6-16 carbon atoms are known as medium chain length PHA (mcl PHA) and *Pseudomonas oleovorans* is the typical bacteria to produce them [50].

The mechanical properties of the biopolymer were important for its as replacement of petroleum plastics. Steinbuchel *et al.* [51] reported that *pha* C1 from *P. oleovorans* could be functionally expressed in *E. coli* LS1298. The *Pseudomonas pseudoalcaligenes* HBQ06 PHA synthase 1 *(pha*C1*)* gene (Gene Bank accession number AF336848) was cloned into the vector pKS- and pUC19 with *pha* AB genes of *R. eutrophus*. The target of the subcloning was whether it could produce scl and mcl PHA copolymer. Its mechanical properties were analysed to see if the copolymer could be produced. The *pha*C gene was first deleted from *pha* CAB genes of *R. eutrophus* by restriction enzyme and the PCR product of *pha* C1 gene was cloned into it. Two different vector pUC19 and pKS- in both *E. coli* XL1-Blue and LS1298 were used and finally subjected to expression. However, there was also no PHA accumulation. It might due to the some differences in biosynthetic pathways for the PHA accumulation in different *Pseudomonas* subspecies and the specific conditions or chemicals needed in PHA accumulation.

4.5) Expression of the *pha*CAB Genes in *E. coli* XL1-Blue and HMS174

Comparing the absorbance (Fig. 44) of the two *E. coli* strains, the *E. coli* HMS174 harboring plasmid pUC19/PHA had better growth than the *E. coli* XL1-Blue harboring plasmid pUC19/PHA. A high level of propionate is toxic to the cells. The

better cell growth of the recombinant *E. coli* HMS174/PHA than the *E. coli* XL1-Blue/PHA represents the better resistance to the growth inhibition effect of the propionic acid and hence the *E. coli* HMS174/PHA was chosen to be the target, which subjected to the high-cell density fed-batch fermentation in the fermenter.

Fig. 45 shows that the majority of biopolymer accumulated in cells was PHB and its yield was much higher from *E. coli* HMS174/PHA than from *E. coli* XL1-Blue/PHA. A higher PHV yield was also obtained with *E. coli* HMS174/PHA. Two reasons might account for this. First, the pUC19 might be a better expression vector for *pha* operon in this case, and therefore the higher amount of PHA was accumulated. Second, the PHA synthesis in different *E. coli* strains was different because the metabolite may be different in *E. coli* HMS174 and *E. coli* XL1-Blue.

^{1}H-NMR spectrum (Fig. 48) showed the present of CH-, CH_2- and CH_3- groups in the molecule as labeled in the figure, but no –COOH and –OH groups. So the compound should be a polymer, not a monomer. The magnitudes of the CH_3- and CH_2- groups of PHV in the figure were lower than that of the PHB, representing the lower percentage of PHV content in the biopolymer when compared to PHB content.

Chapter 5: Conclusion

The project can be divided into four parts: The PHA-producing strain isolated from activated sludge; Cloning and expression of *B. megaterium* PHA gene in *B. subtilis;* Cloning & expression of *pha*C1AB gene in *E. coli*; and cloning and expression of *R. eutrophus* PHA gene in *E. coli* XL1-Blue & HMS174.

Two PHA-producing strains (HF-1 and HF-3) were successfully isolated from the activated sludge. The isolated strains were identified by Microbial ID, Inc. and they were both identified to be possibly *B. megaterium* and *Brevibacillus laterosporus*. However, the low similarity index of HF-3 indicated that it should be *Bacillus* but its subspecies could not be identified. Both strains achieved superior production of PHB when hydrolyzed malt waste was used as a medium, the PHB yield can reach as high as 40% of their cell dry weight. The percentage yields of PHA from HF-1 and HF-3 in sucrose were 36.38% and 33.80% respectively, but were 28.67% and 27.58% when maltose was used. The biopolymer produced was subjected to GC and ^{1}H-NMR analyses and the results indicate that only PHB homopolymer was produced in HF-1 and HF-3. From the GC analysis of the cells after fermentation using synthetic and food wastes as substrates, only PHB homopolymer was accumulated in the cells. The T_m value of the P (3HB) homopolymer was about 170°C, which is near the T_m of polypropylene, but the biopolymers extracted from HF-1 and

HF-3 were 165°C and 157°C respectively, which may be due to impurities.

From the results of SDS-PAGE of proteins released from purified PHA inclusion bodies of HF-1, HF-3 and *B. megaterium*, the two most abundant proteins have a molecular mass of approximately 41 kDa and 20 kDa are found in all three strains after SDS-PAGE analysis. However, the other protein bands pattern of HF-3 was quite different to the other two strains and only HF-3 didn't give PCR product by using primer *LKH* 4 and 7, which were designed according the *pha* gene sequences of *B. megaterium*. This further supports the assumption that HF-3 is more unlikely to be *B. megaterium*. The DNA sequences of two selected regions of HF-1 on *pha* gene were 94% and 95% identical to the corresponding sequences of *pha* gene in the *Bacillus megaterium* ATCC11561. It suggests that HF-1 may be a strain of *B. megaterium*, and the slight difference may be due to different subspecies.

Construction of recombinant bacteria was only partially successful in this project. Although the construction of recombinant *B. subtilis* 168 with *pha* genes from *B. megaterium* and recombinant *E. coli* with *pha* C1 gene from *Pseudomonas pseudoalcaligenes* HBQ06 and *pha* AB genes from *R. eutrophus* were successful, the gene expression for the PHA production failed. The failure might be because the recombinant plasmids constructed (> 10 kb) were not very stable to replicate in the transformed cells as its size was too large. However, the cloning of *pha* PQRBC genes

from *B. megaterium* into *B. subtilis* 1A304 (ϕ105 MU331) was successful. The *pha* genes is stable in the absence of selective pressure because the prophage is covalently inserted, in a single copy, into the host chromosome. The *B. subtilis* 1A304 (ϕ105 MU331) carrying the *pha* genes (*pha* PQRBC) was subjected to fermentation and showed PHA accumulation, which was the first report of the expression of the *pha* genes of *B. megaterium* in *B. subtilis*. The biopolymer was subjected for FT-IR and GC analysis and identified to be PHB homopolymer. However, the yield of PHB was similar to that in native strain *B. megaterium* for about 6%, which might be due to the weak native promoter was used in the expression. The recombinant *B. subtilis* was expressed with heat shock process since the heat shock process in *B. subtilis* 1A304 (ϕ105 MU331) could trigger its strong phage promoter, but it had the PHA accumulation after fermentation with the yield lower than the same strain without heat shock. This might be due to the different gene arrangement in *pha* PQRBC. RNA polymerase bound to the phage promoter and native promoter, respectively and then was run along in the opposite direction during the transcription.

Construction of recombinant *E. coli* harboring *pha* operon from *R. eutrophus* was successful in this project. Both strains were successfully expressed in the production of PHA but *E. coli* HMS174 was superior for the production of Poly-(3-hydroxybutyrate-co-3-Hydroxyvalerate) [P(HB-HV)], the levels of which can

reach as high as 45% in shake flask culture and could reach relatively high cell density. The cell dry weight and PHA content of recombinant HMS174 could reach as high as 10.27 g/L and 43% (wt/wt) respectively in fed-batch fermenter culture.

For further study, the two abundant proteins in HF-1 and HF-3 can be western blotted and subjected to protein sequencing. The DNA sequences that coded for the two most abundant proteins on purified inclusion bodies can be cloned by the use of degenerate oligonucleotides probes based on their N-terminal amino acids sequences. The restriction fragments indicated by probes, when separately hybridized in Southern blotting to various restriction enzyme digests of HF-1 and HF-3 genomic DNA, can be purified and cloned into the vector for expression. Moreover, various conditions, in which PHA are produced with the bacterial strains isolated from activated sludge, can carried out. The mechanical properties of extracted biopolymer from HF-1 and 3 can be studied.

Steinbuchel *et al.* [51] reported that pha C1 from *P. oleovorans* and *Pseudomonas aeruginosa* could be functionally expressed in *E. coli* LS1298 and mcl PHA could be produced. The *pha* CAB genes of *R. eutrophus* and pha C1 gene of *P. oleovorans* or *Pseudomonas aeruginosa* can hence be subcloned into pUC19 and subjected for the expression.

Recently, the production of C3/C4/C6/C8 copolymer in native *R. eutrophus* was first discovered by Green, [52] Whether the copolymer could be produced in our recombinant *E. coli* with *pha* genes from *R. eutrophus* by medium or simple molecular basis modification is an interesting topic that we can study.

Develop the self-disruptive cell. e.g, the lysin genes such as holin or endolysin gene from *B. amyloliquefaciens* bacteriophage can be utilized. The lysin genes can be cloned into *E. coli-Bacillus* shuttle vector (e.g. pX[53]) and amplified in it. The vector has xylose-promoter. Then the genes can integrated into genome of PHA-producing *Bacillus* strain (such as *B. megaterium* or rec. *B. subtilis*) by homologous recombinant at *amy*E locus.

Chapter 6: References

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