STUDIES ON THE MOLECULAR MECHANISM OF POLYHYDROXYALKANOATE BIOSYNTHESIS IN *PSEUDOMONAS* SPECIES

A Thesis
Submitted to the

University of Mysore

for the award of the degree of

Doctor of Philosophy

in

Biotechnology

by

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Under the supervision of

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Scientist

DEPARTMENT OF PLANT CELL BIOTECHNOLOGY
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MYSORE - 570020, INDIA

February 2008

Dedicated

To 'Neeliyara' family

Who have always been there for me

Reeta Davis
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DECLARATION

I hereby declare that the thesis entitled "**STUDIES ON THE MOLECULAR MECHANISM OF POLYHYDROXYALKANOATE BIOSYNTHESIS IN *PSEUDOMONAS* SPECIES"** submitted to the **University of Mysore,** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY,** is the result of the research work carried out by me under the guidance of **Dr. ARUN CHADRASHEKAR**, Scientist, Central Food Technological Research Institute, Mysore- 570 020, India, during the period 2003-2008.

I further declare that the results presented in this thesis have not been submitted for the award of any other degree.

Date:

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Place: Mysore

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February, 2008

CERTIFICATE

This is to certify that the thesis entitled "**STUDIES ON THE MOLECULAR MECHANISM OF POLYHYDROXYALKANOATE BIOSYNTHESIS IN *PSEUDOMONAS* SPECIES"** submitted by **Ms. Reeta Davis** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** to the **UNIVERSITY OF MYSORE** is the result of research work carried out by her under my guidance in the Department of Plant Cell Biotechnology, CFTRI during the period 2003-2008.

ARUN CHANDRASHEKAR
(Research Guide)

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ABBREVIATIONS

AA	Amino acids
AP	Alkaline phosphate
APS	Ammonium Persulphate
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine Serum Albumin
CDW	Cellular dry weight
CoA	Coenzyme A
°C	Degree Celsius
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic acid
FTIR	Fourier Transform Infrared Spectroscopy
g	Grams
GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectrophotometer
h	Hours
HA	Hydroxyalkanoate
HB	Hydroxybutyrate
HD	Hydroxydecanoate
HDD	Hydroxydodecanoate
HHX	Hydroxyhexanoate
HO	Hydroxyoctanoate
HV	Hydroxyvalerate
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
LB	Luria- Bertani (medium)
mcl	Medium chain length
min	Minutes
mM	Millimole(s)
mRNA	Messenger RNA
NBT	Nitroblue tetrazolium
nm	Nanometers
NMR	Nuclear Magnetic Resonance
OD	Optical density
ONPG	*o*-nitro-phenyl-β-D-galacto pyranose
PAGE	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PHA	Polyhydroxyalkanoate

Continued

PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
PLA	Polylactic acid
PMSF	Phenyl methyl sulfonyl fluoride
RBS	Ribosome binding site
RNA	Ribonucleic acid
rpm	Revolutions per minutes
rRNA	Ribosomal RNA
scl	Short chain length
SD	Shine Dalgarno
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-Tetramethyl ethylene daiamine
Tris	Tris (hydroxymethyl) amino methane
U	Unit
UV	Ultra violet
v/v	Volume by volume
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µl	Micro liter

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Final synopsis of the thesis

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SYNOPSIS

Polyhydroxyalkanoates (PHA) represent a class of carbon and energy storage polymers produced by numerous bacteria in the presence of excess carbon and under nutrient limitation. These polymers have been the focus of extensive research and commercial interest because of their biodegradability and similarity to thermoplastics. PHAs are grouped into two classes depending on the carbon chain length of the repeat-unit monomers. The short chain length (scl) PHAs contains monomer repeat units of 3-5 carbon atoms and is crystalline. The medium chain length (mcl) PHAs on the other hand is composed of monomers having 6-14 carbons and is amorphous.

A survey of literature indicated the involvement of different pathways in various bacteria for the biosynthesis of PHA. The pathway present in *Ralstonia eutropha* mainly produces polyhydroxybutyrate (PHB) from glucose using a three enzyme system: β-ketothiolase, NADPH dependent acetoacetyl CoA reductase and the PHA synthase. *Bacillus* sp. also utilizes a similar pathway for PHB production. Hydroxyvalerate (HV) is mainly incorporated into the polymer produced by these bacteria when an isoform of the β-ketothiolase (BktB, which condenses propionyl CoA and acetyl CoA to 3-ketovaleryl-CoA) and the precursor molecules are present. *Pseudomonas* on the other hand produces PHA rich in mcl-monomers. Most *Pseudomonas* spp. have two PHA synthases, PhaC1 and PhaC2 separated by a PHA depolymerase, PhaZ. The monomers for PHA synthesis in *Pseudomonas* are supplied from fatty acid metabolism by different intermediary enzymes such as (R)-specific enoyl CoA hydratase, 3-ketoacyl CoA reductase etc. Several high and medium cost applications of mcl-PHA and its copolymers are emerging and hence the screening of PHA producing *Pseudomonas* became the primary objective of this thesis.

The survey of literature suggests that copolymers consisting of both hydroxy butyrate and hydroxy valerate or scl and mcl-PHA have better biocompatibility and physical properties. There are only a few naturally occurring bacteria which produces copolymers of both scl and mcl-PHA. But, the production and recovery of polymer from these natural producers is a tedious affair. Review of literature pointed out that *E. coli* is an appropriate host for metabolic engineering of PHA biosynthesis pathway. But there are only a few reports on successful production of copolymer in *E. coli* of desired type and amount. It was envisaged that a hybrid pathway utilizing *Pseudomonas* and *Bacillus* genes could provide a unique and fruitful way of synthesizing PHAs with HB, HV and mcl-monomers in *E. coli*.

A survey of literature suggests the presence of isoforms of PHA synthase and (R)-specific enoyl CoA hydratase in *Pseudomonas* spp. However, there are no reports on the substrate specificity of these enzymes in the native host. These enzymes were studied only after their expression in heterologous host. It was envisaged that Antisense expression and over expression of these enzymes could provide valuable information regarding its role and specificity.

The survey of literature indicated a distinct lacuna in the studies related to PHA synthase gene regulation. There did not seem to be much literature on the delineation of the *cis* acting elements of the PHA synthase gene. A reporter gene mediated study of *phaC1* promoter in the presence of different substrates has not been hitherto reported and hence became an objective of the present study.

With this background knowledge, the work plan for the present investigation has been devised. The research work followed up to meet the proposed objectives is presented in six chapters in the thesis. The references are cited at the end in alphabetical order. The title and summary of the work carried out under each chapter are presented as follows:

Chapter 1

Introduction and review of literature

The importance of plastics, its impact on environment due to its non biodegradability and demand for a better biodegradable plastic is highlighted at the beginning of this chapter. Different kinds of biodegradable polymers, their properties, application, advantages and disadvantages are discussed. This chapter also provides an overview of the current literature regarding classification, properties, application, recovery and biodegradability of PHA, and bacteria that produces and degrades it. The biosynthetic pathways involved in PHA production and the enzymes participating in these pathways have been described in detail. The different types of PHA operons found in various bacteria are discussed. Expression of PHA biosynthetic genes from various bacteria and *Pseudomonas* spp in *E. coli* and the production of polymer by these recombinants is discussed and tabulated. An account of the use of recombinants other than *E. coli* such as *Ralstonia, Pseudomonas, Aeromonas, and Salmonella* for the potential production of copolymers is provided. The transcriptional and translational level of regulation of PHA biosynthesis is discussed at the end of this

chapter with emphasis on *Pseudomonas* spp. This chapter foregrounds the unexplored areas in polyhydroxyalkanoates research, which forms the basis for the present study.

Chapter 2

Materials and Methods (General)

This chapter gives a detailed account of the instruments, chemicals, solvents, enzymes and media used for the present investigation. All the general microbiological methods, analytical techniques, and molecular biology protocols used in the present study has been described completely with sufficient technical information.

Chapter 3

Isolation of PHA producing *Pseudomonas* spp. and heterologous expression of the PHA synthase 1 gene from *P. aeruginosa*

This chapter deals with the isolation and rapid screening of PHA producing *Pseudomonas* spp. by a PCR and a colony hybridization technique. These isolates were found to produce 18-33% PHA. Hydroxy octanoate and hydroxy decanoate were the major monomers found in the PHA produced by the different isolates on most of the substrates used. Addition of acrylic acid, an inhibitor of β- oxidation resulted in increased production of PHA when cells were grown in the presence of fatty acids.

A 1680bp PCR amplicon of the *phaC1* of *P. aeruginosa* was cloned into the broad host range vector pBSPIIKS- for functional expression in *E. coli* strains BL21 and LS1298Kan::*fadB1* and also in *P. aeruginosa* under the control of *lac* promoter. *PhaC1* was also sub cloned into the *Bacillus* expression vector pCE20 for expression in *Bacillus subtilis*. The content and composition of the PHA in *E. coli* varied based on the substrate used. 2-6% increase in the content of PHA and significant change in the molar fraction of the different constituent monomers was observed, in comparison to that of the control, when PhaC1 was over expressed in *P. aeruginosa*. An increase in the hydroxy decanoate content of the PHA in these recombinants indicated that the PhaC1 enzyme is specific towards the polymerization of hydroxy decanoyl CoA. Expression of PhaC1 in *B. subtilis* resulted in the accumulation of PHA (2-8% CDW) containing PHB and mcl-monomers, revealing that the PhaC1 of *P. aeruginosa* can polymerize variety of monomers on expression in heterologous host than during expression in its native state.

Chapter 4

Biosynthesis of polyhydroxyalkanoates copolymer in *E. coli* using genes from *Pseudomonas* and *Bacillus*

Four (R)-specific enoyl CoA hydratases (PhaJ) interconnect the β-oxidation pathway with PHA biosynthesis in *P. aeruginosa.* In this chapter cloning of the two genes *phaJ1* and *phaJ4* which codes for (R)-specific enoyl CoA hydratases 1 & 4 is presented. These genes were amplified by PCR from genomic DNA of *Pseudomonas* sp. and cloned into pBPC1 plasmid individually in order to analyze their effect of co-expression with *phaC1* gene on the accumulation and composition of PHA. The resultant plasmids, pBPC1J1 and pBPC1J4, containing *phaJ1* and *phaJ4* upstream of *phaC1* gene, were introduced into *E. coli* BL21 and into a *fadB* mutant of *E. coli.* The content of PHA produced in the *fadB* mutant (18-30%) was higher than that of *E. coli* BL21 (15-25%). PHA produced by the *fadB* mutant was richer in butyrate (40-50%) than that produced by *E. coli* BL21 (.0.5-2.5%) as shown by the use of GC and ^{1}HNMR. The PHA extracted from *E. coli* bearing pBPC1J4 produced more butyrate than those bearing pBPC1J1. The difference in the composition of mcl-monomer in the polymer depended on the differences in isoforms of the hydratase enzyme and the substrate in which the recombinant bacteria were grown. The yield of PHA with glucose and fatty acid as substrate varied from 20-25% and was attained within 24 h in a fermentor while 48 h was required by shake flask culture.

In order to improve the yield and quality of PHA, plasmid bearing the above genes was introduced into *E. coli* JC7623*,* harboring integrated β-ketothiolase (*phaA*) and NADPH dependent-acetoacetyl CoA reductase (*phaB*) genes from a *Bacillus* sp. also driven by a *lacZ* promoter. The recombinant *E. coli* (JC7623ABC1J1) grown on various fatty acids along with glucose was found to produce 28-34% cellular dry weight of PHA. Gas chromatography and ^{1}H Nuclear Magnetic Resonance analysis of the polymer confirmed the ability of the strain to produce PHB-co-Hydroxy valerate (HV)-co-mcl-PHA copolymers. The ratio of scl to mcl-PHA varied from 78:22 to 18:82. Addition of acrylic acid, an inhibitor of β-oxidation resulted in improved production (3-11% increase) of PHA copolymer. The combined use of enzymes from *Bacillus* sp. and *Pseudomonas* sp. for the production of scl-co-mcl PHA in *E. coli* is a novel approach and is reported for the first time.

Chapter 5

Role of (R)-specific Enoyl CoA hydratases of *Pseudomonas* sp in the production of polyhydroxyalkanoates

The use of antisense technique and hyper-expression to delineate the role of two enzymes, PhaJ1 and PhaJ4 forms the basis of this chapter. *phaJ1* and *phaJ4* genes were cloned in the sense as well as anti sense direction with respect to *lac* promoter in a broad host range vector. *P. aeruginosa* transformants bearing these construct have shown significant variation in the quantity and monomer composition of the PHA with respect to that of the control. It has been found that *P. aeruginosa* recombinant, with *phaJ1* antisense construct, fed with different fatty acids, produced PHA with less hydroxy octanoate (7% reduction) with a proportionate increase in other fractions. Recombinants bearing *phaJ4* antisense construct were found to contain less hydroxy decanoate (8% decrease) and more or less equal amount of hydroxy octanoate, compared to that of the control. PhaJ1 and PhaJ4 have been hyper expressed individually, in *P. aeruginosa* and co expressed, with PHA synthase1 (PhaC1) of *P. aeruginosa*. *Pseudomonas* produced PHA with more hydroxy octanoate and decanoate (6-17 % increase), respectively, when PhaJ1 and PhaJ4 were hyper-expressed individually or along with PhaC1. PhaJ1 and PhaJ4 are found to be involved mainly in the production of hydroxy octanoyl CoA and hydroxy decanoyl CoA, respectively, in *P. aeruginosa.* Experiment on heterologous expression has revealed that these hydratases could contribute PHB monomers in addition to the mcl-PHA.

Chapter 6

Regulation of polyhydroxyalkanoates synthase gene expression in *P. aeruginosa*

The full length promoter (-416) and two deletion fragments (-300 and -200) of *phaC1 g*ene of *P. aeruginosa* were amplified by PCR using primers designed based on the sequences available in the data bank. These fragments were fused with β-galactosidase gene in the pMP220 vector (a promoter less vector with β-galactosidase reporter gene), transformed into *P. aeruginosa* and the activity of the reporter gene was assayed under different growth conditions. The full promoter and the two deletion constructs induced expression of β-gal in the presence of hexanoic acid, octanoic acid, nonanoic acid, decanoic acid and glucose under conditions of nitrogen limitation. Decanoic acid was the strongest inducer while hexanoic acid was the

weakest. The full length promoter was able to drive higher levels of expression of the reporter gene than did the -300 and -200 promoter fragments.

P. aeruginosa was transformed with constructs expressing antisense RNA to the promoter and the corresponding SD sequence. Changes were observed in the composition of the PHA produced by recombinants bearing the promoter antisense constructs. These changes depended on the substrate on which the recombinants were grown. Recombinant *P. aeruginosa* grown in the presence of decanoate or glucose and bearing the antisense construct to the *phaC1* promoter accumulated polyhydroxyalkanoates with less hydroxy decanoate than that of the control bacteria. Recombinants *P. aeruginosa* in the presence of decanoate or octanoate and bearing the antisense constructs to the *phaC2* promoter accumulated PHA with less hydroxy octanoate in comparison to that of the control. These results indicated that both the *phaC1* and *phaC2* genes are differentially regulated in the presence of different carbon substrates and that PhaC1 and PhaC2 of *P. aeruginosa* preferentially polymerize hydroxy decanoate and hydroxy octanoate, respectively. Differential regulation of the expression of PHA synthase gene under different environmental conditions that forms part of the present investigation lays the foundation for further research in this area.

Chapter 7

Conclusions and Future perspectives

The major findings of this investigation and the future prospects are briefly highlighted in this chapter.

1.0 INTRODUCTION

Plastics are polymers covering a range of synthetic or semi synthetic products. A vast majority of plastics are composed of polymers of Carbon and Hydrogen alone or with Oxygen, Nitrogen, Chlorine or Sulfur in the backbone. Common thermoplastics range from 20,000 to 500,000 in molecular weight, while thermosets are assumed to have infinite molecular weight. Each polymer chain has several 1000's of repeating molecular units known as 'repeat units' or 'monomers'. Many desirable properties such as stability, malleability, non-toxicity, barrier properties etc., make plastic an integral part of day-to-day life.

Plastics have helped shape the world around us. They are an essential part of almost every industry and are being incorporated into every commodity, right from heart valves to baby bottles. The world's annual consumption of plastic materials has increased from around 5 million tones in the 1950s to nearly 100 million tones today. (http://www.wasteonline.org.uk) This increase may be explained by the greater number of uses for plastics in Eastern Europe, Asia, and Latin America. The per capita consumption of plastics in The United States of America, European countries and India are 80, 60 and 2 kgs respectively (Kalia *et al*., 2000). Major application of plastics is in packaging. In India it accounts for nearly 52 per cent of plastic consumption and is poised to become the third largest consumer of plastics by 2010, with the industry growing at a rapid pace. Currently, the country consumes around five million tones of plastic products which are expected to reach 12.5 million tones by 2010 (The Hindu Business line, Saturday, January 21, 2006).

There are about 50 different groups of plastics, with hundreds of different varieties, of which majority are recyclable. The American Society of Plastics Industry has sorted the main types of plastic into 7 groups, Polyethylene terephthalate (PET), High-density polyethylene (HDPE), Polyvinyl chloride (PVC), Low density polyethylene (LDPE), Polypropylene (PP), Polystyrene (PS), and any other plastics. The considerable growth in plastic use is due to its beneficial properties. These include versatility and ability to be tailored to meet very specific technical needs, lighter weight than competing materials, reducing fuel consumption during transportation, good safety and hygiene properties for food packaging, water and gas

barrier properties and mechanical properties, etc., which lead to durability and resistance to degradation. The structure of plastics may be chemically manipulated to obtain a wide range of strength and shapes (Khanna and Srivastava, 2005; Madison and Huisman, 1999) and perhaps this vantage with plastics sets them apart from their contemporaries. Plastics are relatively inexpensive to produce (Rs. 60-80 per kg). They are manufactured from petroleum; a non–renewable resource and are formed either by simple addition or by condensation reaction of monomers to form large polymers. Their composition and extensive molecular size appears to provide resistance to degradation (Atlas, 1993; Lee, 1996).

However, the same features that makes plastics so versatile, also presents a major concern. One of the most pressing environmental problems in industrialized nations is that of the constantly growing mountain of waste (Thompson *et al*., 2004). Conventional plastics made from oil do not degrade easily contributing to the growing trash heaps across the world. Conventional plastics not only take many decades to be decomposed in nature, but also produce toxins during the process of degradation (Suriyamongkol *et al*., 2007). Plastics make up around 7% of the average household dustbin. Therefore, plastic recycling has become a very important part of environment preservation (Lee, 1996; Derraik, 2002; Gross and Kalra, 2002). According to a 2001 Environment Agency report, 80% of post-consumer plastic waste is sent to landfill, 8% incinerated and only 7% is recycled. Some plastics are recyclable, for instance, polyethylene plastics may be washed, melted and reformed as many as five times with little or no loss in their physical properties, while in most cases recycling limits further application (Johnstone, 1990). Plastic recycling is tedious and time consuming and the presence of wide variety of additives like pigments, coatings fillers limits the use of recycled material (Khanna and Srivastava, 2005).

Incineration is an alternate to recycling, but a very hazardous one, since harmful chemicals like hydrogen chloride, hydrogen cyanide, etc., are also released during the process (Atlas, 1993) and does not allow reuse of the petroleum carbon source. Photodegradable plastics, which are broken down by UV light, have been suggested as an alternate to conventional plastics (Kalia *et al*., 2000). However, landfills lack sun light and these plastics remain non-degraded (Reddy *et al*., 2003).

The plastic industry is therefore pressurized to seek out for environmentally friendly plastics, while retaining the desired physical and chemical properties as that of the conventional synthetic plastics. Understanding the need, scientists have ventured out to find solutions back into the nature, which they are constantly manipulating. At last, the thermo stable natural polyesters: biopolymers and bioplastics have been developed. For the past 25-30 years much consideration has been given to the production of biodegradable plastics (Volova *et al*., 2007).

Biopolymers and bioplastics go by different names. They are often referred to as bio-based plastics and polymers, or as biodegradable plastics or polymers. Biopolymers are polymers which are present in, or created and catabolized by living organisms (Jendrossek and Handrick, 2002; Kim and Rhee, 2003; Akar *et al*., 2006) and do not cause toxic effects in the host and have many advantages over petrochemical plastics (Steinbuchel and Fuchtenbusch, 1998; Angelova and Hunkeler, 1999; Zinn *et al*., 2001; Williams and Martin, 2002; Reddy *et al*., 2003; Chen and Wu, 2005; Steinbüchel, 2005). These include polymers from renewable resources that can be polymerized to create bioplastics, such as starch, cellulose and pullulan. Bioplastics are plastics manufactured using biopolymers and are biodegradable. There are two main types of biopolymers: those that come from living organisms; and, those which need to be polymerized but come from renewable resources. Both types are used in the production of bioplastics (www.greenplastics.com).

- Biopolymers from living organisms: These biopolymers are synthesized by living organisms and are present either as intracellular components or secreted out depending on their specified functions. These include carbohydrates and proteins, which can be used in the production of plastic for commercial purposes. Eg. Cellophane.
- Polymerizable Molecules: These molecules come from renewable natural resources, and can be polymerized for use in the manufacture of biodegradable plastics. Eg. Lactic acid and Triglycerides.

First generation biodegradable plastic products were conventional plastic with starch causing slow disintegration of the product in the nature. Second generation

products are emerging based on completely biodegradable polymers derived from synthetic or natural materials or blends of both (Fang *et al*., 2005). Some of the biodegradable plastic materials under development include polyhydroxyalkanoates (PHAs), polylactic acids (PLAs), polyglycolic acid, and the co-polymers and/or blends of these (Lee, 1996; Scott, 2000; Muller *et al*., 2001).

Polylactic acid or polylactide (PLA) is a biodegradable thermoplastic and an aliphatic polyester derived from renewable resources like starch. Bacterial fermentation is used for the production of lactic acid, which is then oligomerized and catalytically dimerized in processing the monomer for ring-opening polymerization (Tsuji, 2002). PLA shows properties of crystallinity around 37%, glass transition temperature between 50-80°C and melting temperature between 173-178°C. It is currently used in a number of biomedical applications, such as sutures, dialysis media and drug delivery devices and can also be employed in the preparation of bioplastics, useful for producing loose-fill packaging, compost bags, food packaging and disposable tableware (Ikada and Tsuji, 2000). 'Lacea' is a type of PLA manufactured by Mitsui Chemicals, Japan, from fermented starch, derived from a variety of renewable resources, such as corn, beet, cane, and tapioca. Lacea is comparable to polyethylene in terms of transparency and similar to polystyrene or polyethylene in terms of processability.As of September 2006, PURAC in the Netherlands, was the primary company producing PLA for medical applications. Other companies involved in PLA manufacturing include Nature work (USA), Toyota (Japan), Hycail (The Netherlands), Galactic (Belgium) and several Chinese manufacturers. However, PLA is still more expensive than conventional plastics and its degradation rate is much slower than the rate of waste accumulation.

Polyglycolide or polyglycolic acid (PGA) is a biodegradable, thermoplastic polymer and the simplest linear aliphatic polyester known since 1954. It can be prepared from glycolic acid as a starter by means of condensation or ring opening polymerization. Currently polyglycolide and its copolymers poly(lactic-*co*-glycolic acid) with lactic acid, poly (glycolide-*co*-caprolactone) with ε-caprolactone and poly (glycolide-*co*-trimethylene carbonate) with trimethylene carbonate) are widely used as a material for the synthesis of absorbable sutures and are currently being evaluated in the biomedical field. Polyglycolide has a glass transition temperature between 35-

40°C and its melting point is reported to be in the range of 225-230° C. PGA had found little use because of its ease of degradation when compared to other synthetic polymers. In 1962 this polymer was used to develop the first synthetic absorbable suture which was marketed under the trade name of Dexon by the Davis and Geck subsidiary of the American Cyanamid Corporation. Implantable medical devices including anastomosis rings, pins, rods, plates and screws have been produced with PGA.

Polyanhydrides are emerging as a new class of biodegradable polymers for drug delivery. These are biocompatible and have excellent controlled release characteristics. They may be prepared from readily available low cost resources (dicarboxylic acid) in a single step synthesis, with no need for purification step. The main disadvantages of this polymer are short term degradation and release periods, and the need for specialized storage conditions (Kumar *et al.,* 2002).

Aliphatic polyesters ('bionolle') such as polybutylene succinate and polybutylene succinate adipate are degraded by different microbes and is now increasingly applied in numerous packaging, health and agricultural needs (Ando *et al*., 1998).

Biodegradable Aliphatic-Aromatic Polyesters: "Ecoflex" is a new and innovative class of biodegradable plastics similar to low density polyethylene has applications in agriculture and food industry (Yamamoto *et al*., 2002).

Polyhydroxyalkanoates (PHAs): The most widely produced microbial bioplastics are PHAs and their derivatives (Madison and Huisman, 1999; Witholt and Kessler, 2002). PHAs are homo or hetero polyesters of hydroxyalkanoates that are synthesized by many Gram-positive and Gram-negative bacteria from at least 75 different genera as intracellular carbon and energy storage compounds and accumulated as granules in cell cytoplasm (Rehm and Steinbuchel, 1999) (Figure 1.1).These polymers accumulated intra-cellularly to a level as high as 90% of the cell's dry weight under conditions of nutrient stress, as inclusion bodies in the presence of high carbon source and under conditions of limited nutrients such as nitrogen, phosphorous, sulphur, oxygen and magnesium (Anderson and Dawes, 1990; Steinbuchel and Fuchtenbusch, 1998; Madison and Huisman, 1999). These have attracted increasing attention for both commercial as well as research applications due

to their similar physical and material properties as conventional plastics, complete biodegradability, biocompatibility as well as a best suited model compound for metabolic engineering (Lee, 1996; Aldor and Keasling, 2003).

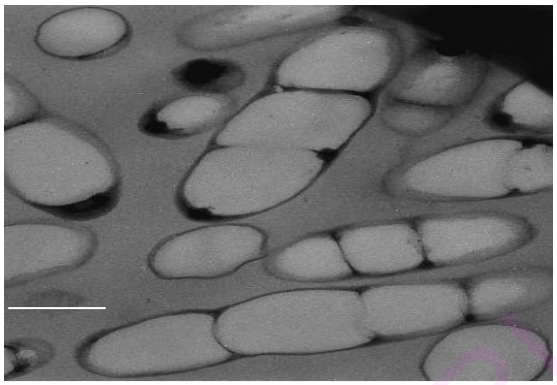


Figure 1.1 Native poly (3HB) granules in *Ralstonia eutropha* (Transmission electron micrograph, Bar represents 0.5µm.) adapted from Sudesh *et al*., (2000)

PHAs may be broadly sub divided into short chain length PHAs (PHA-scl) consisting of 3-5 carbon monomers as that produced by *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus)* (Steinbuchel and Lutke-Eversloh*,* 2003) and medium chain length PHAs (PHA-mcl) consisting of 6-14 carbon monomers of 3-hydroxy alkanoates units as produced by *Pseudomonas oleovorans*. *Pseudomonas* species can produce PHA-mcl from wide variety of organic substrates such as acetate, propionate and other unrelated substrates such as gluconate and glucose. The co-polymer composition of PHA-mcl depends mainly on carbon source, cultivation conditions and metabolic routes leading to the formation of PHA. It has been suggested that intermediates of *de novo* fatty acid biosynthesis and fatty acid β-oxidation are channeled to PHA synthesis through (D)-3- hydroxy acyl CoA derivatives (Qi *et al.,* 1997).

A number of bacteria like *R. eutropha*, *Alcaligens latus, Bacillus* sp*., Beijerinckia, Halobacterium, Methylotrophes, Pseudomonas, Rhizobium, Rhodospirillum, Streptomyces* (Kim and Lenz, 2001) and recombinant *Escherichia coli* have been employed for the production of PHA. Of these *R. eutropha* has been the most intensively investigated. They can produce poly (3-hydroxy butyrate), poly (3-hydroxy butyrate-co-hydroxy valerate) and poly (3-hydroxy valerate) according to different organic substrates. Cyanobacteria like *Nostoc muscorum and Spirulina*

platensis were also found to accumulate 10-20% of PHA under different growth conditions (Panda *et al*., 2005; Jau *et al*., 2005). Further research revealed that PHB is not only found in eubacteria, but also in eukaryotic cells, eg., yeast, peanut, spinach, sheep (intestine), cat muscle and many more (Reusch, 1995).

The outstanding finding that PHA can consist of various monomers and cloning of its biosynthetic genes leads to the development of microbial plastic factories to produce PHAs with desirable structure and material properties. In spite of all these advantages, the obstacle for the use of bioplastics is the very high cost of manufacturing and unavailability of proper scale-up technology and facilities. Many approaches like external substrate manipulation, inhibitor addition, recombinant gene expression, host cell genome manipulation, protein engineering, proper scale up studies and studies on regulation of PHA biosynthesis would determine whether it would be possible for mankind to make useful environmentally friendly polymers in an efficient manner.

1.1 OVERVIEW OF POLYHYDROXYALKANOATES (PHAs)

PHAs were first investigated by Lemoigne, who isolated the reserve polymer in *Bacillus megaterium* in early 19th century which was later on identified as 3-polyhydroxy butyrate. The functional role of PHA was first proposed by Macrae and Wilkinson in the year 1958. The identification of hydroxyalkanoate (HA) units other than 3HB units as constituents of microbial polyesters by Wallen and Rodwedder in 1974 had a major impact on research and development in the microbial polyesters. Investigations into the characterization of various HA units in microbial PHA began earnest during 1980s when a number of bacteria were found to produce different homopolymers and copolymers of (R)-3HAs ranging from four to 14 carbon atoms. Approximately 300 different types of Gram negative and Gram positive bacteria synthesize and accumulate PHAs as granular inclusions in the cytoplasm. A list of some such bacteria capable of accumulating PHA and the carbon sources used is complied in Table 1.1.

The PHAs are accumulated to a level as high as 90% of the cell dry weight as intra-cellular carbon and energy storage materials or as a sink for the redundant

reducing power under limiting nutrients such as N, P, O, S or Mg (Steinbuchel and Valentin, 1995). The molecular mass is generally of the order of 50,000 to 1,000,000 Daltons. The number and size of granules in the cells appears to be strain specific. For example *Pseudomonas oleovorans* contains one or two large granules whereas *R. eutropha* contains 8-12 granules per cell.

Table 1.1 Accumulation of PHA in various microorganisms (Kim and Lenz, 1999)

Genus	PHA %CDW	Substrate from which PHA is produced
Axobacter	73	Glucose
Azospirillum	57	3-hydroxybutyrate
Azotobacter	46	Sucrose
Bacillus	25	Glucose
Beggiatoa	57	Acetate
Beijerinckia	38	Glucose
Caulobacter	36	Glucose/ Glutamate
Chromatium	20	Acetate
Chromobacterium	37	Glucose
Clostridium	13	Glucose
Halobacterium	38	Glucose
Leptothrix	67	Pyruvate
Methylocystis	70	Methane
Pseudomonas	67	Methanol
Ralstonia	96	Glucose
Rhizobium	57	Methanol
Rhodobacter	60	Acetate
Rhodospirillum,	47	Acetate
Spirillum	40	Lactate
Streptomyces	4	Glucose
Syntrophomonas	30	Crotonate
Thiocaspa	36	Acetate
Thiocystis	83	Acetate

CDW : Cellular Dry Weight

1.2 STRUCTURE OF PHA

PHAs are linear polyesters composed of 3-hydroxy fatty acid monomers as shown in the following figure:

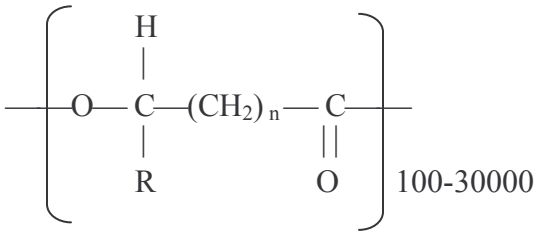


Figure 1.2 Chemical structure of PHA

n=1	R=Hydrogen	Poly (3-hydroxy propionate) (PHP)
	R=Methyl	Poly (3-hydroxy butyrate) (PHB)
	R=Ethyl	Poly (3-hydroxy valerate) (PHV)
	R=Propyl	Poly (3-hydroxy hexanoate) (PHHX)
	R=Pentyl	Poly (3-hydroxy octanoate) (PHO)
	R=Nonyl	Poly (3-hydroxy dodecanoate) (PHDD)
n=2	R=Hydrogen	Poly (4-hydroxy butyrate)
n=3	R=Hydrogen	Poly (5-hydroxy valerate)

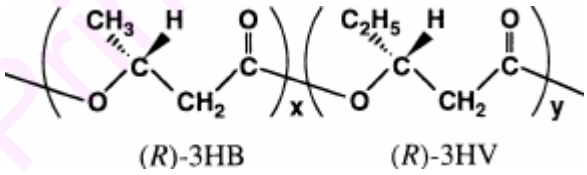


Figure 1.3 Ester bond in a copolymer

The carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer (Figure 1.3). The monomer HA units in these microbial polyesters are all in the D (-) configuration because of the stereo-specificity of the biosynthetic enzymes. The different PHAs vary at the C3-or β-position (Merrick, 2002). The monomeric constitution of the PHA is decided by the different precursor substrates used and the polymer composition changes as the (R)-dependent group varies (Lee, 1996). Some PHAs are homopolymers of a hydroxyalkanoate, whilst others are copolymers of two or more HAs. Saturated, unsaturated,

halogenated, branched and aromatic side chains in (R)-3HA monomeric units have been found in the sequence of microbial PHA (Kim and Lenz, 2001; Kessler *et al*., 2001). In addition some bacteria produce copolymers containing monomeric units without side chains such as 3-hydroxypropionate and 4-hydroxybutyrate. At present approximately 150 different hydroxy alkanoates have been detected as constituents of the biosynthetic PHA (Rehm and Steinbuchel, 1999; Suriyamongkol *et al*., 2007).

1.3 CHARACTERISTICS OF PHA

- PHAs exist as discrete granular inclusions localized in cell cytoplasm (Figure 1.1) and can be observed as refractile bodies of 0.2 to 0.5μm diameter (Madison and Huisman, 1999). Sabirova *et al*., (2006) reported a PHA-hyper producer mutant of marine bacterium *Alcanivorax borkumensis* deposit the PHA in the extra cellular environment by an unknown mechanism.
- PHAs are non toxic and biocompatible (Zinn *et al*., 2001).
- PHAs are water insoluble and soluble in solvents like chloroform.
- The shape of the PHA granule is usually spherical and varies in size according to the type of organism and substrate used (Sudesh *et al*., 2000).
- The molecular weights of polymers are in the range of 2×10^5 to 3×10^6 Daltons depending on the microorganism and growth condition (Lee, 1996).
- PHAs exhibit thermo plasticity and/or elastomeric features besides other interesting physical and mechanical properties (Ramachander *et al*., 2002).
- These are mobile amorphous polymers and can be stained with Sudan black B, Oxazine dye Nile Blue A etc. indicating that they are of lipid nature (Sudesh *et al*., 2000).
- PHA appears as electron dense bodies and are optically active (Anderson and Dawes, 1990).
- PHAs are well known for their biodegradability. Many organisms like *Aspergillus fumigatus, Comamonas* spp. *Pseudomonas fluorescence, P. lemoignei* etc., excrete extra cellular PHA depolymerase and PHA hydrolases to degrade the PHAs into water-soluble monomers and oligomers (Jendrossek, 2002; Jendrossek and Handrick, 2003).

1.4 CLASSIFICATION OF PHA

PHAs can be broadly divided into two groups (Rehm and Steinbuchel, 1999) depending on the number of carbon atoms in the monomeric units. These are as described below:

1. **Short chain length** PHAs (scl-PHA) having carbon atom in the range of 3-5 in the constituting monomeric unit of the polymer and predominantly occurs in *C. necator.* Poly (R)-3-hydroxybutyrate), (PHB) is the most common and well studied polymer among this group.
2. **Medium chain length** PHAs (mcl-PHA) consisting of 6-14 carbon monomers and mainly produced by *Pseudomonas* spp.

1.4.1 Properties of poly(R)-3-hydroxybutyrate homopolymer

PHB is the most common biological polyester produced by various microorganisms in nature (Anderson and Dawes, 1990). The molecular weight of PHB produced from wild type bacteria ranges from $1x10^4$ to $1x10^6$ g mol^{-1}. PHB isolated from bacteria reveals 55-80% crystallinity while the molecules within the bacteria are amorphous. The density of the crystalline polymer is 1.26 cm^{-3} and has a glass transition temperature of ~4 °C and a melting temperature of ~180 °C (Sudesh *et al*., 2000). The mechanical properties of Young's modulus (3.5 GP) and the tensile strength (43 M Pa) of PHB are close to that of polypropylene (PP) while the extension to break (5%) is remarkably lower. Therefore PHB is regarded as material with poor properties of stiffness and brittleness. The physical properties of PHB may be improved by increasing the molecular weight or incorporating other hydroxyalkanoate units to form PHA copolymers.

1.4.2 Copolymers of hydroxy butyrate with hydroxyalkanoates

Random copolymers containing PHB as a constituent and hydroxy alkanoates with a chain length ranging from three to 14 carbon atoms have been produced from various carbon substrates by variety of bacteria. A random copolymer of 3HB and 3HV has been produced in *R. eutropha* by feeding pentatonic acid and butyric acid (Doi *et al*., 1988 a). Shimamura *et al*., 1994 reported the production of a copolymer

of 3HB and 3HHx from olive oil by *A. caviae.* Random co-polyester of 3HB and 3HP was produced by *R. eutropha* when hydroxy propionic acid was used as the carbon substrate. A random copolyester of 3HB and 4HB was produced by *R. eutropha* (Doi *et al*., 1988 b), *A. latus* (Hiramitsu *et al*., 1993), or *C. acidovorans* (Saito and Doi, 1994) when 4-hydroxybutyric acid, 1,4-butanediol, or gamma butyrolactone was used as the carbon source. Recently it was found that *Pseudomonas* sp.61-3 accumulated a novel random copolymer of 3HB and medium chain length hydroxyalkanoate of carbon numbers ranging 6 to 12 from glucose and alkanoates (Kato *et al*., 1996). The properties of above mentioned random copolymers are given in the following table (Table 1.2).

Table 1.2 Properties of different copolymers of hydroxy butyrate with other hydroxyalkanoates*

PHA	Melting temperature (°C)	Glass transition temperature (°C)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)
Poly(3HB)	~180	~4	3.5	40	5
Poly(3HB-co-4HB)	130	-48		26	444
Poly(3HB-co-HV)	145	-1	0.8	20	50
Poly(3HB-co-HP)	44	-19	-	-	-
Poly(3HB-co-HHx)	52	-4	-	20	850
Poly(3HB-co-mcl)	133	-8	0.2	17	680

*Consolidated from (Doi *et al*., 1988a; 1988b; Hiramitsu *et al*., 1993; Saito and Doi, 1994; Kato *et al*., 1996)

1.4.3 Properties of medium chain length PHA

PHAs of medium chain length 3-HA with 6 to 14 carbon atoms can be produced by a number of prokaryotes including many fluorescent Pseudomonads (Huisman *et al*., 1989; Timm and Steinbuchel, 1990). After the discovery of mcl-PHA in *P. oleovorans* (de Smet *et al*., 1983) about 100 different monomers have been shown to be incorporated into mcl-PHA (Steinbuchel and Valentine, 1995). With only a few exception mcl-PHAs are obtained as copolymers containing from two to six different types of 3HA units rather than homopolymer. *P. putida* and *P. oleovorans* have been the most intensively investigated microorganisms of those synthesizing mcl-PHA. These bacteria use fatty acid β-oxidation route for PHA

synthesis. *P. aeruginosa* and other Pseudomonads have similar pathway in the utilization of substrates such as alkanes and fatty acids but are also able to synthesize mcl-PHA from non-related substrates such as gluconate or glucose using fatty acid biosynthetic pathway (Huijberts *et al*., 1995; Rehm *et al*., 1998).

The structure and properties of random copolymers of poly (R)-3-hydroxy octanoate have been investigated most extensively in the poly (mcl-3HA). Poly (3HO) has a glass transition temperature of -35°C (Brandl *et al*., 1988) and a melting temperature of 61°C (Gross *et al*., 1989). The degree of crystallinity is approximately 30%, Young's modulus ranged from 2.5 to 9 MPa and tensile strength at break ranged from 6 to 10 MPa and ultimate elongation varied from 300-450%. The low rates of crystallization, limits their applicability in many processing techniques.

1.5 RECOVERY AND ANALYSIS OF PHA

1.5.1 Recovery of PHA from biomass

The recovery of PHA contributes significantly to the production cost of the polymer. A variety of methods are available for extraction of PHA from cells. The cells after incubation will be harvested, washed, dried and disrupted to recover the polymer. The commonly used method involves the use of different solvents like chloroform, methanol, methylene chloride, propylene carbonate and dichloroethane. This method requires large quantities of these volatile solvents which are not only cost prohibiting but also hazardous to the environment (Lee, 1996; Yasotha *et al*., 2006).

Another popular method is the use of sodium hypochlorite lyses of the cell (Berger *et al.*, 1989). Even though this method is effective in the digestion of non-PHA cellular materials, it causes severe degradation of PHA (Lee, 1996). The use of chloroform together with hypochlorite significantly reduces the reduction in the molecular weight of PHA (Hahn, 1994). However, this also leads to a higher cost and environmental hazard. One separation method both gentle (100% biological) and selective, is through enzymatic digestion. Bacterial cells are treated with a cocktail of enzymes (including proteases, nucleases and lysozymes) and detergents to remove proteins, nucleic acids, and cell walls, leaving the PHA intact (Byrom, 1987). A protease enzyme called alcalase and anionic detergent sodium dodecyl sulphate

(SDS) is commonly used. De Koning and Witholt, (1997) found that simultaneous use of alcalase and SDS at optimum pH and temperature reduces time consumption. Lysozyme treatment is also effective in breaking the peptidoglycan layer surrounding the PHA granule (Eggink and Northolt, 1999). The proteases (trypsin, chymotrypsin, papain and bromelain) and the β-glycosidases (cellulase) and lysozyme were the enzymes studied for the recovery of PHA from *R. eutropha* (Kapritchkoff *et al.,* 2006). Yasotha *et al*., (2006) optimised an enzymatic digestion method for release of PHA from *P. putida* using alcalase, lysozyme, SDS and EDTA.

Few bacteria like *A. vinelandi* and recombinant *E. coli* become fragile after accumulation of PHA. *A. vinelandi* cells can easily be lysed by treating with 1N ammonia water for 10 min (Page and Cornish, 1993). Successful attempts have been made to lyse *E. coli* using phage lysis genes (Fidler and Dennis, 1992; Yu *et al*., 2000). Similarly E-mediated lyses (gene E of phage ΦX 174) has been successful in a genetically modified natural PHA producer *R. eutropha* (Schroll *et al*., 1998)**.**

1.5.2 Methods for quantification of PHA and compositional analysis

Gravimetry is the most common analytical technique used for PHA estimation, consisting of PHB extraction from lyophilized biomass with chloroform followed by precipitation with diethyl ether or acetone. Further Law and Slepecky, (1969) developed a spectrophotometric (235nm) method for estimation of PHA after converting it into crotonoic acid with concentrated sulphuric acid. These methods are not accurate and require larger amount of samples. Fourier Transmission Infra Red spectroscopy (FTIR) can be used to detect the chloroform extracted PHA or PHA within the lyophilized cells (Mishra *et al*., 2000). FTIR and Raman spectroscopy are very useful simple techniques to analyze the PHA based on the functional groups present in the monomer and to study the thermal behavior of the polymer (Satoa *et al*., 2005).

Gas chromatographic (GC) analysis of PHA offers quantitative information about the total amount and concentration of monomers present in the PHA. When combined with Mass Spectroscopy, information about the mass and identity of the monomer involved is added. This method involves simultaneous extraction and methanolysis of PHA, in mild acid or alkaline conditions, to form hydroxyalkanoate

methyl esters which are then analyzed by GC (Brandl *et al*., 1988; Braunegg, 1978). This method is rapid, sensitive, and reproducible and can handle small quantities of samples. Specialized detectors like atomic emission detector gives relevant information about the monomers which contains halogens.

High performance Liquid Chromatography (HPLC) is another method of separation used for the analysis of PHA, especially poly-(3HB-co-3HV). Initially HPLC was only used for the quantification of PHB after converting into crotonoic acid and the acid was chromatographed on an ion exchange column followed by ultraviolet measurement. Later many more modified methods have been developed. An enzymatic separation method (Parry *et al*., 1980) could detect combined amount of R-3-hydroxybutyric acid and (R)-3-hydroxy valeric acid. Karr *et al*., (1983) and Hesselmann *et al*., (1999) were able to separate 3-hydroxybutyric acid, 3- hydroxy valeric acid and 3-hydroxyhexanoic acid using HPLC method. This method doesn't require lyophilization or derivatization thus reducing the total analysis time.

Nuclear Magnetic Resonance spectroscopy (NMR) is a very powerful tool to determine the exact location of double bonds in the monomer and also its cis/trans configuration. The presence of 3-hydroxybutyric acid was shown by a doublet in the H-NMR spectrum, 3-hydroxy fatty acids with a longer carbon chain by a triplet and a quartet (De Rijk *et al*., 2002). The determination of PHA inside intact cells by two dimensional fluorescence spectroscopy and flow cytometry has also been proposed recently (Degelau *et al*., 1995; Gorenflo *et al.*, 1999). Cells stained with Nile blue, show a clear fluorescence maximum between 570 and 605 nm when excited between 540 and 560 nm. A good correlation between fluorescence intensity and PHB concentration was be obtained by this method. However, differentiation of PHA composition was not possible with this method. An approach for rapid differentiation between scl and mcl PHA producers has been developed by Wu *et al.*, (2003) by combining Nile red staining and fluorescence spectroscopy.

1.6 BIODEGRADABILITY OF PHA

PHA is unique in its biodegradability in various environments compared to other biodegradable polymers. PHA is insoluble in water and doesn't degrade in the normal conditions of storage (Mergaert *et al*., 1993). It degrades in the environment

by the action of microbial enzymes (Lee, 1996). Bacteria can colonize the surface of the polymer and degrade it into monomeric units. These monomeric units can be absorbed as carbon and energy source for its growth later. Degradation depends on many factors like surface area, pH, temperature, moisture, microbial activity in the environment and the availability of other nutrients. The end products of PHA degradation in aerobic environments are carbon dioxide and water, while methane is also produced in anaerobic conditions. A number of microorganisms such as bacteria and fungi in soil, sludge and sea water excrete extra cellular PHA-depolymerase enzymes to hydrolyze solid PHA into water-soluble oligomers and monomers, and subsequently utilize the resulting products as nutrients within the cells. Such P(3HB)-degrading microorganisms have been isolated from various environments such as soil, sea water, lake water, air, hot springs, compost and sludge (Kobayashi *et al*., 1999). P(HB-co-HV) completely degrades after 6, 75 and 350 weeks in anaerobic sewage, soil and sea water respectively (Lee, 1996). A list of micro organisms that are able to degrade PHA is given in Table.1.3.

Table 1.3 PHA degrading microorganisms isolated from various environment

Strain	Source	Class of polymer
Alcaligenes faecalis	Activated sludge	PHB
Acidovorax faecalis	Soil	P(HB-HV)
Commamonas sp.	Fresh water	PHB
Ralstonia pickettii	Soil	P(HB-HV)
Pseudomonas stutzeri	Lake water	mcl -PHA
Pseudomonas lemoignei	Activated sludge	P(HB-HV)
Pseudomonas fluorescens	Activated sludge	PHB and mcl-PHA
Aspergillus fumigatus	Soil	PHB

PHB is degraded by specific hydrolyzing enzyme, the extracellular and intracellular PHB depolymerases. Extracellular depolymerases are able to degrade partially crystallized (denatured) PHB, whereas intracellular depolymerases act on amorphous (native) PHB. Extracellular PHB depolymerases are typically comprised of three functional domains: a catalytic (320–400 aa), a linker (50–100 aa), and a

substrate binding (40–60 aa) domain. The catalytic domain contains a lipase-like catalytic triad (serine, asparticacid, and histidine residues), as well as a pentapeptide signature sequence [Gly-Xaa-Ser-Xaa-Gly] (where Xaa denotes any amino acid residue), known as the lipase box (Jendrossek and Handrick, 2003).The extracellular PHA depolymerases have been purified and studied from *Ralstonia pickettii* T1 (Tanio *et al*., 1982; Feng *et al*., 2004), *Pseudomonas lemoignei* (Lusty and Doudoroff, 1966), *Acidovorax* sp. *TP4* (Kobayashi *et al*., 1999; Feng *et al*., 2004), *Commamonas acidovorans* (Kasuya *et al*., 1997) and *Penicillium funiculosum* (Hisano *et al*., 2005).

1.7 APPLICATIONS OF PHA

Very interesting applications of PHA are due to its biocompatibility and uniform chirality. The degradation products of PHB, D(-)3-hydroxybutyrate is a common intermediate metabolite present in higher animals. Therefore implantation of PHA in mammalian tissue is safe. A few of the possible applications of PHA suggested and worked out by scientists (Lee, 1996; Ojumu *et al*., 2004; Zinn *et al*., 2001; Bucci *et al*., 2005; Banki *et al*., 2005) are listed below.

- Packaging films, bags and containers for food and agriculture commodities
- Cosmetic containers-shampoo bottles and cups
- Chiral precursor for the chemical synthesis of optically active compounds
- Biodegradable carrier for long-term dosage of drugs, medicine, insecticides, herbicides, or fertilizers
- PHB can be used for the purification of recombinant protein using self cleaving affinity tags and specific PHB binding protein
- Disposable items such as razor, utensils, diapers etc
- Surgical pins, sutures, staples, and swabs
- Wound dressing
- Bone replacements and plates as it has the ability to stimulate bone growth and healing due to its piezoelectric properties
- Blood vessel replacement
- 4HB has potential therapeutic value and used as intravenous anesthetic agent, sleeping drug and in the treatment of alcohol withdrawal syndrome

1.8 METABOLIC PATHWAY OF PHA BIOSYNTHESIS

During limited nutrient conditions, the intermediate molecules of the major metabolic pathways will be converted into reserve food materials in the form of PHA by involving series of enzymes. Based on the type of monomer incorporated into PHA, various metabolic pathways have been shown to be involved in the generation of these reserve PHA granular inclusions in the cytoplasm. The general pathway of PHA synthesis is shown below (Figure 1.4).

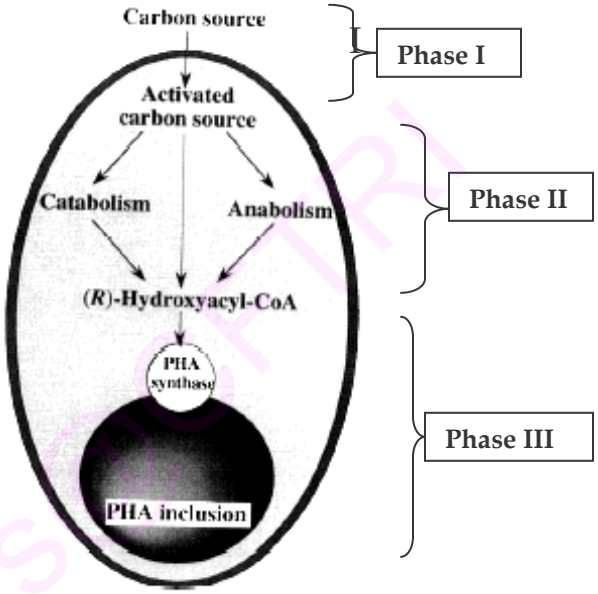


Figure 1.4 General scheme for the metabolic pathways of PHA synthesis from different carbon sources within a bacterial cell (Adapted from Sudesh *et al*., 2000)

The whole pathway can be distinguished into four major phases:

Phase I: This phase comprises the entry of a carbon source from the environment into a bacterial cell. This entry is facilitated by a transport system located on the cytoplasmic membrane or by simple diffusion mechanism. Thus, membrane associated proteins play important role in this phase.

Phase II: This phase includes anabolic or catabolic or both reactions which convert the carbon source into a hydroxy acyl coenzyme A thioester (immediate precursor of PHA).

Phase III: This is the final phase, which requires granule-associated proteins and related with the formation of ester bond by the PHA synthase enzyme and thus the polymer.

Phase IV: This phase comprises intracellular degradation of PHA. This phase is very slow during the formation of the granule, and will be rapid once the external carbon source exhausted and the degradative enzymes become active.

Three types of major metabolic pathway for PHA biosynthesis exist in nature. This classification is based on the type of major pathway, which is providing the precursor molecule for PHA synthesis. These pathways are summarized in figure1.5:-

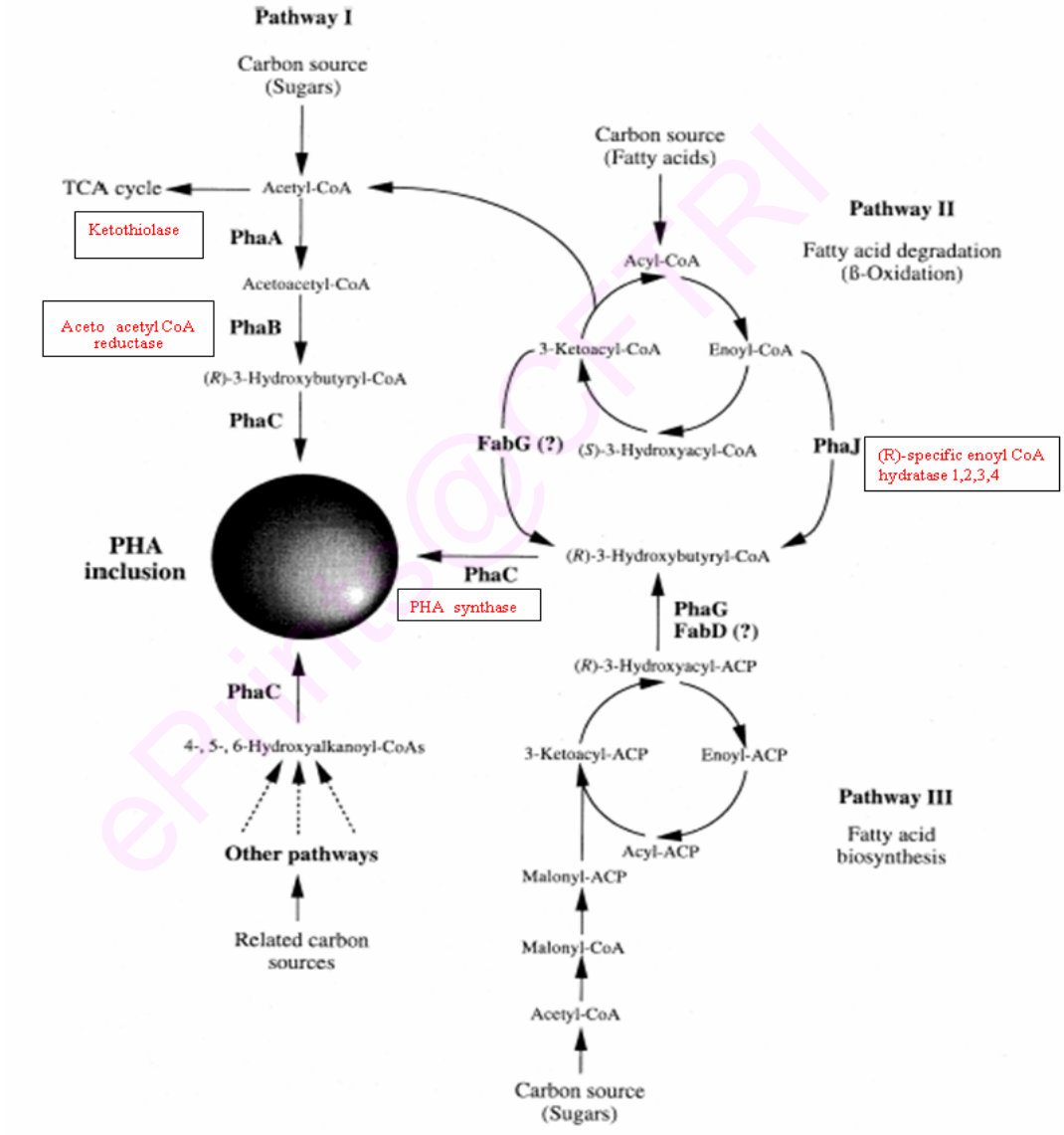


Figure 1.5 Various metabolic pathways that supply hydroxyalkanoate monomer for PHA biosynthesis (Sudesh *et al*., 2000)

The enzymes indicated in the boxes forms part of the work reported in chapters 4 and 5 of this thesis.

1.8.1 Biosynthesis of scl –PHA: Pathway I

Mainly related with the formation of small chain length PHA molecules where the precursor molecule is acetyl CoA, provided by glycolytic pathway. This is the most common pathway found in a wide range of organisms. The pathway is well studied in *R. eutropha.* Mainly three enzymes are involved in this pathway. β-ketothiolase (PhaA) condenses two acetyl CoA moieties into acetoacetyl CoA which in turn reduces to hydroxy butyryl CoA by the enzyme NADPH-dependent acetoacetyl CoA reductase (PhaB) and PHA synthase (PhaC) polymerizes hydroxybutyryl CoA into polyhydroxy butyrate. A modified pathway was described in *Rhodospirillum rubrum* (Moskowitz and Merrick, 1969), where acetoacetyl CoA is first reduced to L-3-hydroxybutyryl CoA by an NADH-dependent acetoacetyl CoA reductase, which is subsequently converted into D-3-hydroxy butyryl CoA by stereo specific enoyl-CoA hydratases.

The synthesis of PHV also follows the same pathway, except for variation in substrate. Acetyl CoA and propionyl CoA, condense to form (3)-ketovaleryl CoA, which after reduction to (3)-hydroxyvaleryl CoA (Figure1.6) is incorporated into PHA by PHA synthase. In *R. eutropha,* it has been reported that the first step in the pathway of PHA synthesis is catalyzed by β-ketothiolase coded by the gene *bktB*, a gene not located in the PHA biosynthesis operon (Slater *et al*., 1998). The biosynthesis of PHV or P(HB-co-HV) requires the formation and occurrence of propionyl CoA in the cells. Propionyl CoA can be derived from propionic acid, via propionyl CoA synthase enzyme or from fatty acid, via β-oxidation of odd chain fatty acids or from various aromatic amino acids via transcarboxylation reactions or from succinyl CoA via methylmalonyl CoA pathway (Steinbuchel and Lutke-Eversloh, 2003). Aldor *et al*., (2002) have constructed a pathway for the synthesis of PHV in *Salmonella enterica* (Figure 1.11).

In *Bacillus cereus* an unique mechanism has been postulated to be involved in the biosynthesis of 4HB and copolymers of 3HB, 4HV and 4HB. In this process succinic semialdehyde dehydrogenase could reduce the succinyl-CoA produced to succinic semialdehyde, which in turn could be reduced to 4HB by a 4-hydroxybutyrate dehydrogenase and activated to 4-hydroxybutyryl-CoA by a Co-enzyme A transferase (Valappil *et al*., 2007a). The (R)-3-hydroxyvaleryl-CoA and/or

(R)-4-hydroxybutyryl-CoA, together with (R)-3-hydroxybutyryl-CoA could then be polymerized to form P(3HB-co-3HV-co-4HB)/P(3HB-co-3HV)/P(3HB-co-4HB) by the PHA synthase. *B. cereus* 14579 contains a 3-ketoacyl-CoA thiolase enzyme (Genbank accession number AAP11875), capable of condensing the Propionyl-CoA with acetyl-CoA to yield 3-ketovaleryl-CoA. In this study a similar 3-ketoacyl-CoA thiolase from a *Bacillus* species (Gene bank accession number EU239690) was employed to achieve terpolymer production in recombinant *E. coli* (Chapter 4, Section 4.2).

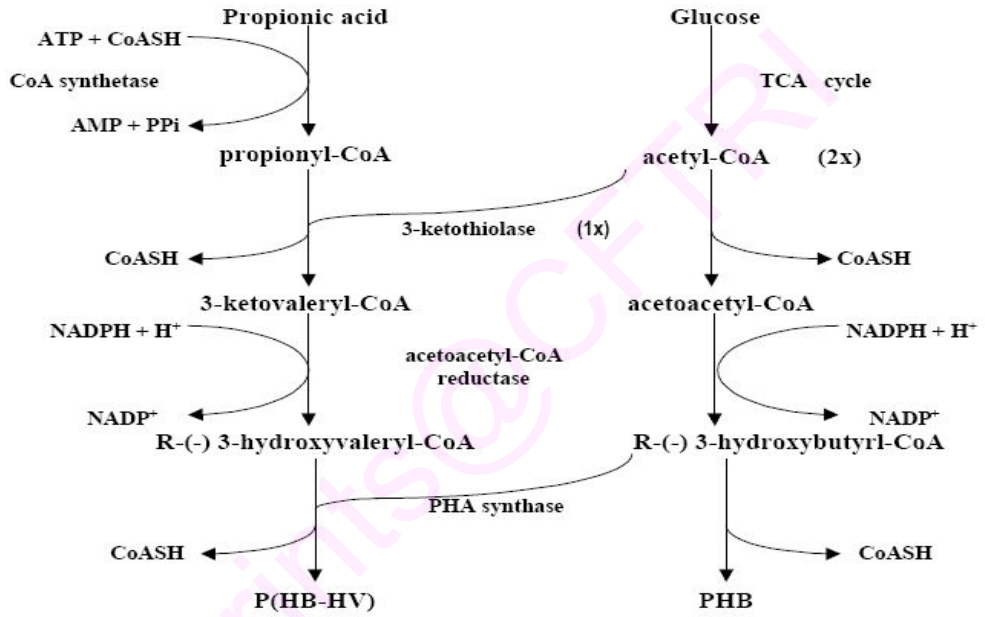


Figure 1.6 Pathway leading to the synthesis of P (HB-co-HV) (Ojumu *et al*., 2004)

1.8.1.1 TCA cycle

TCA cycle is not directly involved in PHA biosynthesis, but various intermediates formed in the pathway may be converted in to substrates for PHA sythase. Acetyl CoA is the prime molecule for scl-PHA biosynthesis. Succinyl CoA is another molecule which connects the TCA cycle with PHA pathway through methylmalonyl CoA. Other intermediates like oxaloacetate and citric acid can also be channeled towards the scl-PHA biosynthesis pathway. It has been shown that PHB synthesis is stimulated by both high intracellular concentrations of NAD(P)H and high ratios of NAD(P)H:NAD(P) (Lee *et al*., 1995), which is an essential reducing power for scl-PHA biosynthesis. Furthermore, citrate synthase activity is significantly

inhibited by NADH and NADPH, indicating that PHB accumulation is enhanced by facilitating the metabolic flux of acetyl-CoA to the PHB synthetic pathway. This suggests that citrate synthase is a potentially important control point in the PHB synthesis process based on its ability to control the availability of CoA (Henderson and Jones, 1997), which regulates the activity of 3-ketothiolase.

1.8.1.2 Methylmalonyl CoA pathway

This pathway is found in certain bacteria, which are able to produce P(HV). In addition to the methyl citric acid cycle, the co-enzyme B12-dependant methyl malonyl-CoA pathway is another important pathway for production of PHA copolymer production from propionic acid. Wild-type bacteria are known to synthesize co-polymer of poly (3HB-*co*-3HV) with very high content of 3HV from unrelated carbon substrates like glucose. In *Nocardia* and *Rhodococcus* glucose is degraded via the 2-keto-3-deoxy-6-phosphogluconate pathway to pyruvate and through reverse reactions of citric acid cycle results in the accumulation of succinyl CoA. The succinyl CoA is then converted in to propionyl CoA via methylmalonyl CoA pathway for PHV synthesis (Haywood *et al*., 1991; Anderson *et al*., 1995). The conversion of succinyl CoA to methylmalonyl CoA is catalyzed by the enzyme methylmalonyl CoA mutase (Figure 1.11) and the methylmalonyl CoA is epimerized to its (S)-form in the next step by an epimerase. The decarboxylation of methylmalonyl CoA to propionyl CoA is catalyzed by propionyl CoA decarboxyalse. The valerate specific β-ketothiolase can take up this molecule for transform into 3-hydroxyvaleryl CoA for PHV synthesis (Figure 1.6).

1.8.2 Biosynthesis of mcl-PHA: Pathway II & III

These two pathways are involved in the generation of medium chain length PHA where the precursor is derived from *de novo* fatty acid biosynthesis and β-oxidation pathways. Most *Pseudomonas* such as *P. aeruginosa, P. oleovorans* and *P. putida* synthesize mcl-PHA from carbohydrate as well as fatty acids. They are generally unable to synthesize poly (3HB) and other scl-PHA. There are few other bacteria which are able to incorporate mcl-PHA into PHB such as *Aeromonas caviae* which synthesizes co- polyester consisting of 3HB and 3HHx. *P. aeruginosa* follows

mainly pathway III (Figure 1.5) for PHA synthesis in which the 3-hydroxy acyl monomers are derived from the *de novo* fatty acid biosynthesis pathway (Huijberts *et al*., 1995). Along with this, there is some evidence for the existence of pathway II also. In this study we have focused on the role of enzymes involved in these pathways and the use of these enzymes for recombinant production of PHA. Various enzymes involved in this pathway and their function are described below**.**

1.9 ENZYMOLOGY OF PHA BIOSYNTHESIS

Enzymes involved in PHA biosynthesis fall into three classes based on their function. The first enzyme, PHA synthase, the key enzyme in PHA biosynthesis, polymerizes (R)-hydroxyacyl-coenzymes [(R)-HA CoAs]. The variety of monomer constituents in PHAs is due to the wide substrate specificity of PHA synthase. The second group of enzyme is those, which generate (R)-HA CoAs from intermediates of metabolic pathways, such as β-ketothiolase, 3-ketoacyl- CoA reductase and enoyl-CoA hydratase. The third group of enzymes is those involved in regulation of PHA synthesis, and accessory enzymes involved in stabilization of PHA granules in bacteria, such as phasin (PhaP).

1.9.1 PHA synthase (phaC)

PHA synthases catalyze the stereo-selective conversion of (R)-3-hydroxy acyl CoA substrate into PHA polymer with the concomitant release of CoA. They are broad substrates specificity enzymes using CoA thioesters of (R)-hydroxy alkanoic acids with hydroxyl group at position 3, 4, 5 or 6 and acyl groups varying in carbon chain lengths as natural substrates. In PHA accumulating cells, the PHA synthases are bound to the surface of the PHA granules along with structural proteins such as phasins (phaD) and some regulatory proteins (Haywood *et al*., 1989; Pieper-Furst *et al*., 1995; Maehara *et al*., 2002). The catalytic activity of this enzyme is present in the C-terminal region (Rehm, 2003). Extensive comparison of 44 PHA synthases reveals that these enzymes from various bacteria exhibit strong similarity (21–88 % identical amino acids). With respect to amino acid sequence, six conserved blocks may be identified, whereas N terminal region is highly variable. The N- terminal region is also dispensable for a functionally active enzyme as revealed by the analysis of

truncated *R. eutropha* PHA synthase that lack 100 or even 300 amino acids (Schubert *et al*., 1991). Overall 15 amino acids are identical in all known 30 PHA synthases suggesting an important role of these residues in enzyme functions (Rehm and Steinbuchel, 2002).

Based on the primary structures deduced from the PHA synthase sequences, the sub-unit composition and the substrate specificity, four major classes of PHA synthases have been recognized (Rehm and Steinbuchel, 1999; Rehm, 2006). These classes are described in table 1.4. *Pseudomonas* spp. contains two or three PHA synthase enzymes (Qi *et al*., 1997; Matsusaki *et al*., 1998).

Table 1.4 Different classes of PHA synthases (Nomura and Taguchi, 2007)

Properties	Class I	Class II	Class III	Class IV
Subunit present	One subunit (PhaC)	One subunit (PhaC)	Two subunits (PhaC, PhaE)	Two subunits (PhaC, PhaR)
Mol. Wt. of the subunit	60- 73 kDa	60-65 kDa	40 kDa each	40 kDa & 22 kDa
Substrate	CoA thioesters of R-3-hydroxy fatty acids	CoA thioesters of R-3-hydroxy fatty acids	CoA thioesters of R-3-hydroxy fatty acids	CoA thioesters of R-3-hydroxy fatty acids
Type of polymer	scl	mcl	scl+mcl	scl
Example	*R. eutropha* +most other bacteria	*P. aeruginosa* + all *Pseudomonas* spp belonging to rRNA homology groupI	*A. vinosum Thiocapsa pfennigii*	*B. megaterium*

The secondary structure of PHA synthase consists of 49.7% variable loops, 39.9% α- helices and 10.4 % β– pleated sheet. Multiple sequence alignment of the PhaC sequence showed the presence of six conserved blocks and eight conserved aa residues (Rehm and Steinbuchel, 1999; Rehm and Steinbuchel, 2002; Rehm, 2003; Rehm, 2006). The residues cysteine 319, aspartate 480 and histidine 508 of the class1 polyester synthase from *R. eutropha* are conserved in all polyester synthases and are important in enzyme catalysis (Jia *et al*., 2001; Rehm, 2003). The substrate specificity of PHA synthases and role of isoforms of the enzymes is always a topic of discussion. The substrate specificity of these enzymes has only been determined in their native

environment or in heterologous physiological environment by genetic engineering techniques. These methods are not so accurate since there are possibilities of limitations posed by the metabolic pathway that supply the particular monomer units in that particular environment. Our study described in the following chapters is also focused in this area.

The tertiary structure of PHA synthases has not yet been resolved by X-ray diffraction analysis. The knowledge on PHA synthases and efficient screening system are now allowed the make up of modified PhaC with enhanced activity through site directed saturation mutagenesis (Takase *et al*., 2003; Tsuge *et al.,* 2004; Matsumoto *et al*., 2005), gene shuffling (Rehm *et al.,* 2002), localized semi random mutagenesis (Sheu and Lee, 2004) and *in vivo* random mutagenesis (Taguchi *et al.*, 2001; 2002).

1.9.2 Enzymes interconnecting the PHA synthesis and major metabolic pathways

1.9.2.1 β- ketothiolase (PhaA)

The gene, *phaA,* code for the ezyme β-Ketoacyl CoA thiolase, which catalyzes the first step in the PHB synthesis. It is a member of a family of enzyme involved in the thiolytic cleavage of substrates in to acyl CoA and acetyl CoA. They are found in higher eukaryotes, yeasts, prokaryotes, and are divided in to two groups based on their substrate specificity (Madison and Huisman, 1999). First group consists of thiolases with broad specificity for β-ketoacyl CoA ranging from C_4-C_{16}. This class of enzyme is mainly involved in the degradation of fatty acids. The second class of β-ketoacyl CoA thiolase is considered biosynthetic and has a narrow range of chain length specificity (C_3-C_5). Even though many genes encoding PhaA have been identified and cloned, enzymatic studies have been mostly limited to *R. eutropha* and *Zoogloea ramigera*. In *R. eutropha* there are two β-ketothiolases (enzyme A and enzyme B) that take part in PHA biosynthesis. These enzymes differ in their substrate specificity. The enzyme A convert acetyl CoA into acetoacetyl CoA. In contrast, the enzyme B (termed BktB) possessing broad substrate specificity is mainly responsible for 3HV monomer formation for P(3HB-3HV) synthesis during growth on fructose and propionate (Slater *et al*., 1998). Both enzymes exists as homotetramer in the native state (see also 1.8.1).

1.9.2.2 NADPH dependant acetoacetyl CoA reductase (PhaB)

The enzyme coded by the *phaB,* NADPH dependant acetoacetyl CoA reductase, is a (R)-3 hydroxyacyl CoA dehydrogenase and catalyzes the second step in PHB biosynthesis pathway by converting acetoacetyl CoA in to 3-hydroxybutyryl CoA. PhaB have been identified from different genera are of basically two types: NADH dependent or NADPH dependent (Haywood *et al*., 1988). Those involved in PHA biosynthesis have been mostly categorized into NADPH dependent family of enzymes, the NADH dependent PhaB from *A. vinosum* is one exception (Liebergesell and Steinbuchel, 1992). *Z. ramigera* PhaB is a homo tetramer with identical 25kD subunits and shows substrate specificities to C4-C6 (Ploux *et al*., 1988). In the PHB biosynthetic pathway the substrate specificities of these enzymes are crucial since the reaction catalyzed by the thiolase and reductase provide monomer for PHA synthase.

1.9.2.3 (R)-specific enoyl CoA hydratases (PhaJ)

PhaJ is one of the major metabolic links between fatty acid β-oxidation and PHA biosynthesis. Various (R)-specific enoyl CoA hydratases (PhaJ) have been cloned and characterized from PHA producers. PhaJ was first reported from *A. caviae* FA440 (Fukui and Doi, 1997). In this bacterium, the (R)-3-hydroxy acyl CoAs are derived from the intermediates of fatty acid β-oxidation (trans 2-enoyl-CoAs), through the (R)-specific hydration catalyzed by PhaJ (Figure1.7). There are four isoforms of PhaJ present in *P. aeruginosa* (Tsuge *et al*., 2000; 2003) which vary in their, size, specific activity and substrate specificity. *E. coli maoC*, which is homologous to *P. aeruginosa phaJ1,* has been found to be involved in PHA synthesis in recombinant *E. coli* strains (Park *et al*., 2003). Another *E. coli* protein YfcX homologous to the rat long chain enoyl CoA hydratase was found to be involved in the formation of mcl-PHA in *fadB* mutants of *E. coli* from fatty acids (Snell *et al*., 2002). Park and Lee, (2004a) have identified five new FadB homologous enzymes in *E. coli*: PaaG, PaaF, BhbD, SceH, and YdbU, by protein database search, and examined their roles in the biosynthesis of mcl-PHA in a *fadB* mutant *E. coli* strain.

Co-expression of *Aeromonas phaJ* and *phaC* genes in *fadR atoC* mutant *E. coli* LS5218 resulted in accumulation of a PHA copolymer consisting of 3-hydroxy butyrate (3HB) and 3-hydroxy hexanoate (3HHx) (Fukui *et al*., 1999). When the

Pseudomonas sp. 61-3 *phaC* gene product, which has broad substrate specificity for monomers having C_4-C_{12} atoms, was co-expressed with different *phaJ* genes, the composition of PHA was altered depending on the type of (R)-specific enoyl CoA hydratase used (Tsuge *et al*., 2003). PhaJ is a powerful tool for PHA production by metabolically engineered bacterium and by selecting different hydratase it would be possible to obtain PHAs with favorable monomer composition. The specific role played by different hydratases of *P. aeruginosa* form the basis of our study presented in chapter 5.

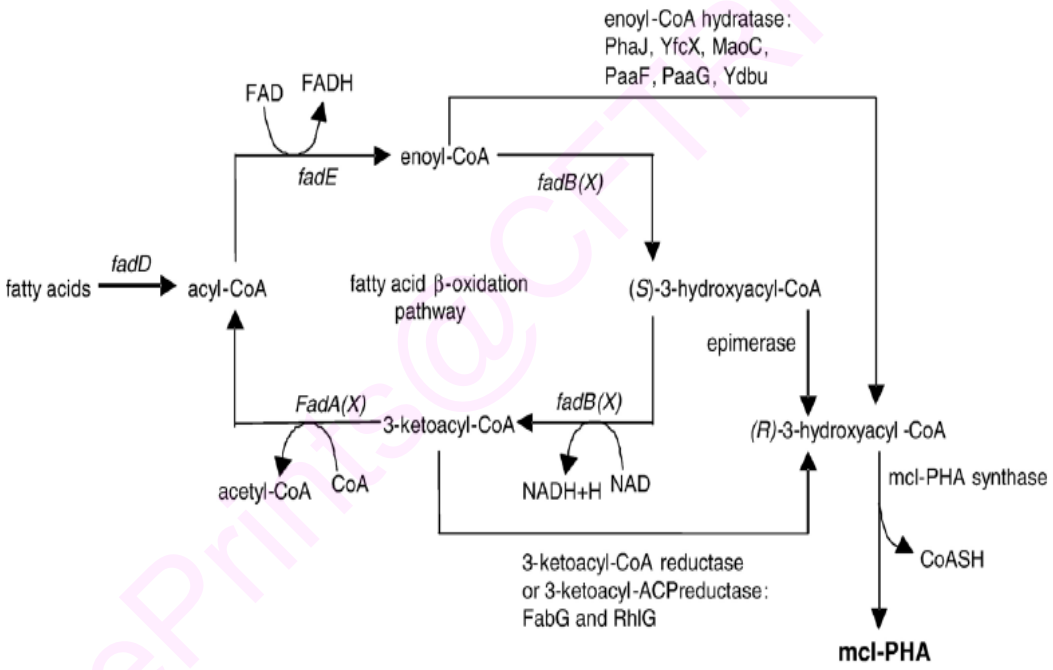


Figure 1.7 Fatty acid β- oxidation pathway and various interconnecting enzymes leading to PHA biosynthesis (Suriyamongkol *et al*., 2007)

1.9.2.4 3-ketoacyl CoA reductase (FabG)

FabG is an enzyme interconnecting fatty acid degradation and PHA biosynthesis by converting 3-ketoacyl-CoA into (R)-3-hydroxyacyl-CoA (Figure 1.7). Screening of 3-ketoacyl-CoA reductase based on the amino acid homology to the *Ralstonia eutropha* acetoacetyl CoA reductase (PhaB) allowed the identification of *fabG* genes encoding 3-ketoacyl-ACP reductase from *E. coli* (Taguchi *et al*., 1999) and *P. aeruginosa* (Ren *et al*., 2000). *fabG* has 30-35% identity to *phaB* of *Raltsonia*

and 32-55% identity to *fabG* sequences from other organisms (Ren *et al*., 2000). There has also been a report on the identification of a NADPH-dependent 3-ketoacyl ACP reductase encoded by the *P. aeruginosa rhlG* gene, which is specifically involved in rhamnolipid synthesis (Campos-Garcia *et al*., 1998). The co-expression of the *fabG* gene from *E. coli*, *fabG* or *rhlG* gene from *P. aeruginosa* along with the mcl-PHA synthase gene successfully established the mcl-PHA biosynthetic pathway in *E. coli* (Taguchi *et al*., 1999; Ren *et al*., 2000; Park *et al*., 2002).

1.9.2.5 Epimerase

Epimerase convert (S)-3-hydroxyacyl CoA into (R)-3-hydroxy acyl CoA (Figure1.7). This enzyme is important in PHA biosynthesis since PhaC can only take up monomers with (R) configuration. (R and S are the two different stereo enantiomers of hydroxy acyl CoA).

1.9.2.6 (R)-3 hydroxyacyl ACP CoA transferase (PhaG)

The *phaG* gene was identified as a metabolic link between *de novo* fatty acid biosynthesis and PHA biosynthesis from *Pseudomonas putida KT2440* (Rehm *et al*., 1998). This enzyme catalyses the conversion reaction of (R)-3 hydroxy ACP intermediates of fatty acid synthesis to the corresponding CoA derivatives, the resultant (R)-3 hydroxyacyl CoA can be utilized as a substrate for PHA synthesis (Figure 1.8). PhaGp_p mediated metabolic pathway was established in a non PHA accumulating bacterium *P. fragi* by co-expressing the *phaC1*p_a gene from *P. aeruginosa* and *phaG*p_p gene from *P. putida* (Fiedler *et al*., 2000). Later three other genes encoding PhaG were cloned from *P. aeruginosa* (Hoffmann *et al*., 2000b), *P. oleovorans* (Hoffman *et al*., 2000a) and *Pseudomonas* sp. 61-3 (Matsumoto *et al*., 2001) and all the four proteins have an average molecular weight of 34kD. PhaG from *P. oleovorans* is functionally active in mcl-PHA synthesis even though it is not transcribed in its natural host under conditions wherein PHA accumulates (Hoffmann *et al*., 2000a). PhaG exhibits a key enzyme activity in mcl-PHA production from non-related carbon sources and thus provides an important tool for establishing this pathway in biotechnologically important organisms.

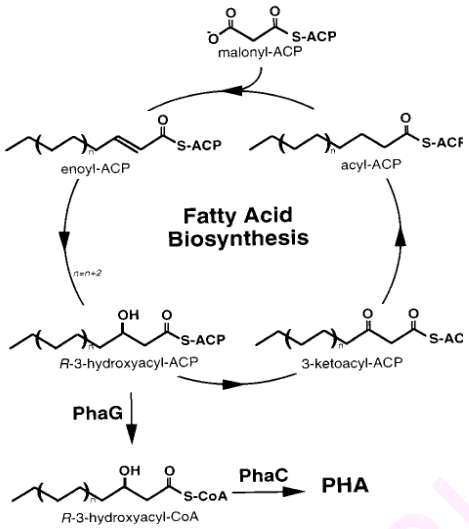


Figure 1.8 Fatty acid biosynthesis leading to PHA production
(Madison and Huisman, 1999)

1.9.3 PHA granule associated proteins

PHA granule consists of a hydrophobic core of amorphous PHA which is surrounded by a membrane consisting of the PHA synthase, PHA depolymerse, amphiphilic phasin proteins and other proteins (Stuart *et al*., 1998) as shown in figure 1.9. Interestingly, it has been proposed that the granules harboring scl-PHA and mcl-PHA have different structures. Granules containing scl-PHA isolated from *B. megaterium* were reported to be enwrapped by a lipid monolayer and proteins on the outside. No evidence was found that proteins or lipids are integrated into the core of granules. Granules containing mcl-PHA were found to be larger and different in their structure from scl-PHA granules. Electron micrographs suggested that the surface consisted of a phospholipid layer separating two crystalline proteins.

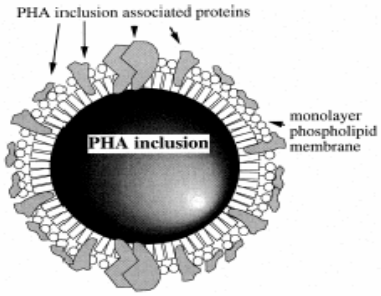


Figure 1.9 Structure of PHA inclusion (Sudesh et al., 2000)

Phasins are a class of proteins, between 14 and 28 kDa in size, that form a layer at the surface of the hydrophobic core of PHA granules; they also influence the number and size of PHA granules (Wieczorek *et al*., 1996; Pieper-Furst *et al*., 1995; Steinbuchel *et al*., 1995). Phasin is coded by *phaP* and the amount of PhaP is depends on amount of PHA synthesized in the cells. PhaR, an ORF adjacent to the *phaCAB* gene cluster has been detected in *R. eutropha* (Slater *et al*., 1998), seems to be involved in the regulation of phasin and PHA biosynthesis (York *et al*., 2002). Two short hydrophobic stretches were identified in the phasin protein of *R. ruber* close to the C-terminus of the protein. These regions have been suggested to be responsible for the binding of the protein to the PHA inclusion. Various PHA-specific regulators such as PhbR from *C. necator* (Potter *et al*., 2002), PhaF from Pseudomonads (Prieto *et al*., 1999; Hoffmann & Rehm, 2004; 2005) and PhaR from *Paracoccus denitrificans* (Maehara *et al*., 1999; 2002) were found to bind non-covalently to the PHA granules. Additional proteins (PhaI, PhaD, PhaS) were found to be granule-associated and co-regulated in Pseudomonads, whose function has not been clarified yet (Prieto *et al*., 1999; Klinke *et al*., 2000; Hoffmann & Rehm, 2004, 2005). All of these non-covalently attached proteins are not essential for PHA granule formation, but serve various biological functions such as PHA granule structure, PHA biosynthesis gene regulation and PHA mobilization.

1.10 MOLECULAR BIOLOGY (GENE ORGANISATION) OF PHA BIOSYNTHESIS IN BACTERIA

The different genes involved in PHA biosynthesis in different pathways are clustered in some bacteria. The organization of the genes involved in PHA biosynthesis is varied among the organisms and these differences make the different PHA operons. The different modes of arrangement of PHA genes are given in Figure 1.10 The PHA operon was first characterized in *R. eutropha* (Peoples and Sinskey, 1989) where the three genes involved in the polyhydroxybutyric acid pathway are organized as *PhbC, PhbA* and *PhbB* down stream to a single promoter form the *phaCAB* operon. The expression of these genes in *E. coli* resulted in the accumulation of PHA to a significant level (Peoples and Sinskey, 1989). In *R. eutropha* and many other scl-PHA producing bacteria three genes are essential for PHA synthesis and

accumulation, where the synthesis is initiated with condensation of two acetyl CoA molecules. Many mcl-PHA producing bacteria possess two *phaC* genes for synthesis.

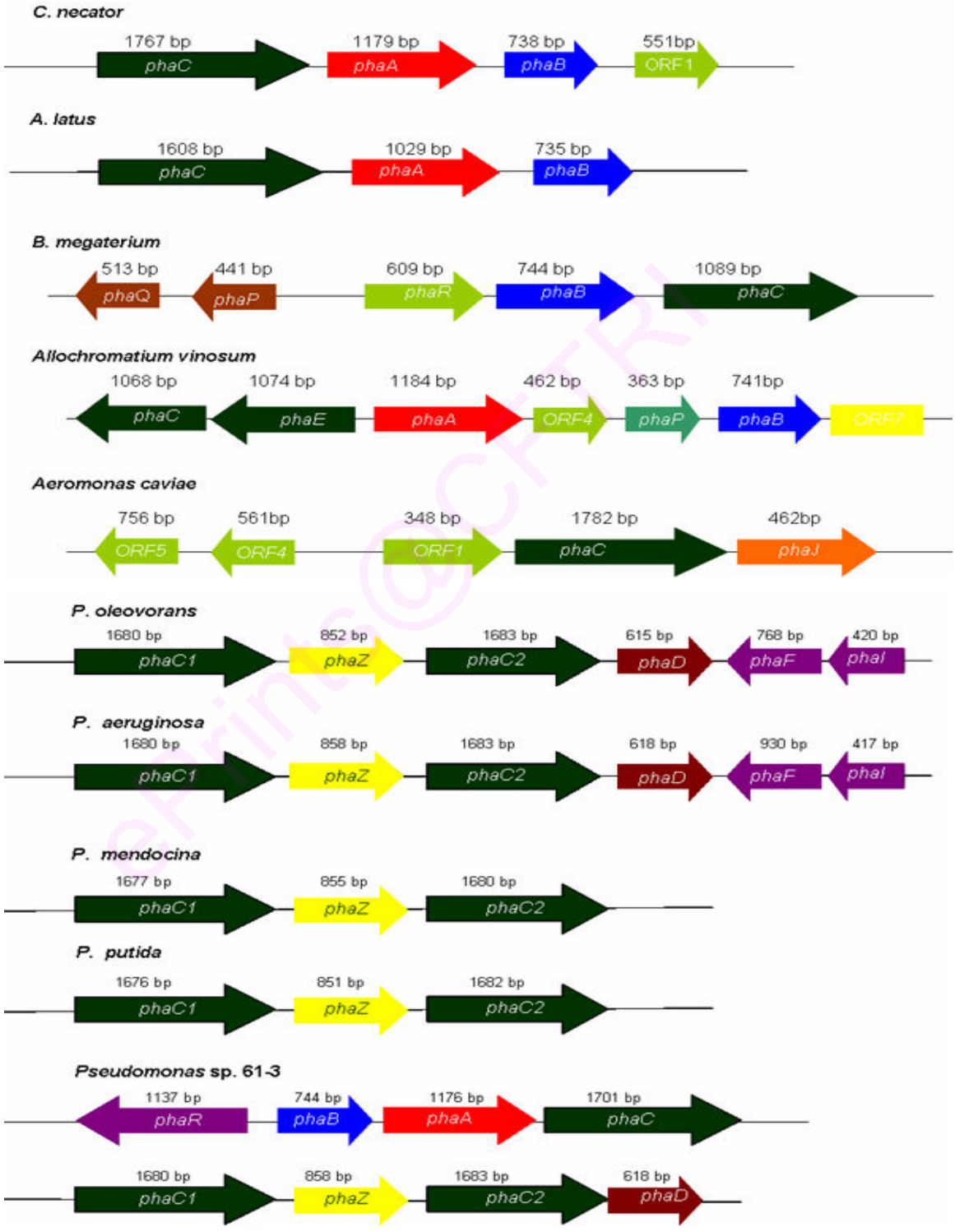


Figure 1.10 PHA biosynthetic operons in various bacteria

Different strategies have been applied towards the identification of PHA synthase genes and other genes involved in PHA biosynthesis. Homologous and heterologous gene probes have been synthesized from a well characterized organism like *R. eutrpoha* to screen the genomic libraries of various PHA producing microorganisms and a number of PHA operons have been isolated. Besides *R. eutropha* the *phaCAB* operon is present in *A. latus, Chromobacterium violaceum, Comamonas acidovorans* and *Burkholderia* sp. In *Acinetobacter* and *Pseudomonas* sp. 61-3 the same genes are arranged in a different fashion even though they are clustered (Figure 1.10). All PHA producing *Pseudomonas* posses two PHA synthase genes (*phaC1 and phaC2*) separated by a *phaZ* gene coding PHA depolymerase. Incorporation of 3-hydroxy butyrate (HB) into PHA by a few *Pseudomonas* sp. led to the identification of another PHA synthase with corresponding substrate specificity in addition to *PhaC1* and *PhaC2* (Lee *et al*., 1995; Matsusaki *et al*., 1998). In *Pseudomonas* sp. 61-3 the additional *phaC* gene is colocalized with *phaA* and *phaB*.

In few bacteria like *Zoogloea ramigera, Rhodococcus ruber, Paracoccus denitrificans, Rhodobacter capsulatus, Sinorhizobium meliloti, Methylobactrium extorquens,* the *phaA, phaB* or other genes involved in PHA biosynthesis are not linked directly to *phaC.* But in *A. caviae, phaJ* codes for enoyl CoA hydratase is located down stream of *phaC* (Fukui and Doi, 1997). It is interesting to note that in many bacteria the *phaP* coding for phasin proteins located close to *phaC.* Bacteria such as *Chromatium vinosum*, *Thiocystis violacea*, *Thiocapsa pfennigii* posses a two component PHA synthase (*phaC* and *phaE*), directly linked in the genome and associated with other genes involved in PHA biosynthesis.

1.11 HETEROLOGOUS EXPRESSION OF GENES INVOLVED IN PHA BIOSYNTHESIS

The major PHA biosynthetic enzymes (PhaA, PhaB and PhaC) and the enzymes interconnecting the major metabolic pathways and PHA biosynthesis can be expressed in different heterologous host for the metabolic engineering of PHA. The most common microbes used as heterologous host are *Escherichia coli, R. eutropha, P. putida* and *P. oleovorans.* There are also reports of the use of *Salmonella enterica, Bacillus subtilis* and *Aeromonas* spp. as host strain for recombinant PHA production.

Other than bacterial system, yeast, insect and plant systems have been tried out for production of PHA.

1.11.1 Recombinant E. coli as a PHA producer

E. coli is the most common heterologous host used for PHA production owing to the following advantages (Lee, 1996; Lee, 1997; Sudesh *et al*., 2000; Aldor and Keasling 2003; Li *et al*., 2007).

(1) Well understood genetics and biochemistry
(2) Genome can be easily manipulated
(3) Easy scale up
(4) Wide range of substrate can be used
(5) Easy lysis
(6) PHA production not tied to natural regulation
(7) Faster growth rate and high productivity
(8) PHA depolymerase is absent.

The first metabolic pathway towards the scl-PHA formation in *E. coli* was established simply by cloning the *R. eutropha* whole *phb* gene operon into *E. coli* (Schubert *et al*., 1988). Later PHB biosynthetic genes from different bacteria have been functionally expressed in *E. coli.* A few such examples are given in the Table 1.5 Productivity was lower in *E. coli* in comparison to that of native producers of PHA. Co-polyesters such as P(3HB-co-3HV), P(3HB-co-4HB) and P(3HB-co-HHx) can also be produced in recombinant *E. coli* by adding corresponding precursors and engineering corresponding metabolic pathways (Rhie and Dennis, 1995; Valentin and Dennis, 1997; Lu *et al*., 2003).

PHA biosynthetic genes of *Pseudomonas* spp. have been widely exploited for the metabolic engineering of *E. coli* for PHA production through the modification of fatty acid β-oxidation or fatty acid *de novo* synthesis and such work is reviewed by Park *et al*., (2005). Report on the use of *Pseudomonas* genes alone or in combination with genes from other sources for PHA production in *E. coli* is summarized in Table 1.6.

Table 1.5 PHA production in recombinant *E. coli*

E. coli strains	Source of gene	Substrate	PHA %CDW	Type of PHA	Reference
DH5α	*R. eutropha phbCAB*	Gluconate	54	PHB	Slater *et al.,* 1988
LS5218	*R. eutropha phbCAB*	Glucose, C3, acetate		PHBV	Slater *et al*., 1992
XL1 blue	*R. eutropha phbCAB*	Glucose	81	PHB	Lee and Chang, 1995
JM109			85		
HB101			75		
MC4100			95		
XL1 blue	*A. latus phbCAB*	Glucose	73	P(3HB)	Choi *et al*.,1998
HMS174	*R. eutropha phbCAB*	Molasses	80	P(3HB)	Liu *et al*.,1998
XL1blue	*R. eutropha, C. kluyveri*	Glucose,4-hydroxybutyrate	36	P(4HB)	Song *et al*.,1999
LS5218	*A. caviae phaCJ*	Dodecanote	38	P(HBHHx)	Fukui *et al*., 1999
XL1 blue	*A. latus phbCAB*	Glucose propionic; oleic acid	78	P(3HBco-3HV)	Choi and Lee, 1999
XL1 blue	*P. denitrificans*	Lactate	43	P(3HB)	Maehara *et al*.,1999
JM109	*T. pfennigi phaEC* *C. acetobutylicum Buk and Ptb*	Glucose+3HB+4HB Glucose+3HB+4HV Glucose+3HB+4HB +HV	68 64 68	P(3HB4HB) P(3HB4HV) P(3HB4HB4 HV)	Liu and Steinbuchel, 2000
JM105	*A. eutrophus phbCAB* *Vitreoscilla vgb* *Lytic gene of* phage λ	Glucose	96	PHB	Yu *et al*., 2000
GCSC4401	*A. latus*	Whey	87	P(3HB)	Ahn *et al*.,2001
LS5218	*A.hydrophila, PhaJ* *A.salmonicida achromogenes PhaC* *R. eutropha. phbB*	Dodecanoate	27	P(HBHHx)	Park *et al.,* 2001a
E. coli LS5218	*A. hydrophila, orf1, phaC, phaJ*	Dodecanoate+odd chain fatty acid	9-33	P(HBHV HHX)	Park *et al*., 2001b
JM109	*Aeromonas caviae phaC and phaJ*	Lauric acid	14	P(HBHHx)	Lu *et al*., 2003
DH5α			15		
XL1 blue	*E. coli yafH*		15		
XL1 blue	*A. hydrophila phaCJ* *E. coli yafH* *Vitreoscilla* (*vgb*)	Lauric acid	60	P(HBHHx)	Lu *et al*., 2004

Table 1.6 Production of PHA in recombinant *E. coli* using PHA synthase of *Pseudomonas* spp.

Source of PHA synthase	Source of other enzyme	Host used	Substrate used	PHA % CDW	Monomer composition	Reference
phaC1 P. aeruginosa	-	Normal and LS1298	Glucose Decanoate	2 21	C6-C14	Langenbach *et al*., 1997
phaC1 and phaC2 P. aeruginosa	-	LS1298	Different fatty acids	15	C6-C14	Qi *et al.,* 1997
P. aeruginosa	-	*E .coli* RS3097	Decanoic acid	38 60	C6-C10	Qi *et al*., 1998
phaC1 Pseudomonas sp. 61-3	*fabG Ec*	*E .coli* HB101	Dodecanoate	8	C6-C10	Taguchi *et al*., 1999
phaC1 P. resinovorans	-	DH5α	Dodecanoate	5	C6-C14	Solaiman, 2000
phaC1 Pseudomonas sp. 61-3	*phaJ1 phaJ2 Pa*	LS5218	Dodecanoate	14-29	C4-C12	Tsuge *et al*., 2000
phaC2 P. oleovorans	*fabG Pa*	JMU193 *fadB* JMU194 *fadA*	Octanoate	20	C6-C10	Ren *et al.,* 2000
phaC2 Pseudomonas sp. 61-3	*rhlG Pa fabG Ec*	Normal and *fadAB*mutant	Decanoate	33	C6-C10	Park *et al*., 2002
phaC1 P.aeruginosa	*PhaJ po*	Normal	Decanoate or Octanoate	9.	C8-C10	Fiedler *et al*., 2002
phaC1 and *phaC2 P.pseudoalcaligenes*	-	Normal	Butyrate to Dodecanoate	1-2	C6-C12	Hang *et al.,* 2002
phaC2 Pseudomonas sp. 61-3	*phaJ1 phaJ2 phaJ3 phaJ4 Pa*	Fad mutant LS5218	Dodecanonate	41	C4 (10-12%) -C12	Tsuge *et al*., 2003
phaC2 Pseudomonas sp. 61-3	*fadD fadE fadL Ec*	Normal and *fadA* mutant	Dodecanoate	44	C4 (2%) - C12	Park *et al*., 2003
phaC2 P. stutzeri	*phaA, phaB Re*	Normal	Glycerol	16	C4	Chen *et al.,* 2004
phaC Pseudomonas sp. 61-3	*phaA, phaB Re*	*fadA/fadB/fadAB Mutant*			C4-C10	Park and Lee , 2004b
phaC1 Pseudomonas sp. 61-3	*fabH Ec*	*fadA fadAB mutant*	Glucose	0.5	C4 –C10	Nomura *et al*., 2004a
phaC1 Pseudomonas sp. 61-3	*fabH Ec Fab G*	*fadA fadAB mutant*	Glucose	0.5	C4 –C10	Nomura *et al*., 2004b
phaC1 and *phaC2 P. putida*		*E. coli fadR fadB mutant*	Hexadecanoate	20	C6-C10	Ren e*t al*., 2005

Ec: *E. coli,* Pa: *P. aeruginosa,* Re: *R.. eutropha,* Po: *P. Oleovorans*

Biosynthesis of PHB in *E. coli* is affected by many factors like choice of vector or host, promoter selection, codon bias and type of culture technique. Several promoters have been used in *E .coli* to control heterologous gene expression. In such systems the transcription initiate only after the addition of inducers like IPTG. In order to avoid the addition of such expensive chemicals, Shi *et al.,* (2001) attempted to construct a recombinant *E. coli* exhibiting thermal induction. They have constructed a vector with λ p_R and p_L promoters, upstream of *phbCAB* genes, and tested the effect of several thermal induction pattern on the production of PHB. Strongest accumulation of PHB was obtained when the temperatures were controlled at 33°C and 37°C, respectively, for cell growth and induction.

The synthesis of PHA in native or recombinant strain is not only depending on gene dosage but also on the availability of acetyl CoA and co factors like NADPH. The effect on NADPH on β-ketothiolase and acetoacetyl CoA reductase activities have been monitored after the supplementation of various intermediates of glycolysis and TCA cycle such as acetyl CoA, citrate, acetate and NADPH (Lee *et al*., 1995; Lee *et al*., 1995; Lee *et al*., 1996; Jung and Lee, 1997).

Many feeding and fermentation strategies have been employed for the optimization of PHA production in *E. coli* (Wang and Lee, 1997; Ahn *et al*., 2001; Lee and Choi, 2001). Addition of valine and threonine to the PHA production media of *E. coli* strain K-12 bearing the genes from *R eutropha* resulted in the incorporation of hydroxy valerate to a level of 4mol% (Eschenlauer *et al*., 1996). Threonine dehydratase converts threonine to α-ketobutyrate which in turn converts into propionyl-CoA by pyruvate dehydrogenase complex of *E. coli* (Eschenlauer *et al.*, 1996). Addition of acrilic acid (inhibits 3-ketoacyl CoA thiolase that catalyzes the formation of acetyl CoA from 3-ketacyl-CoA) results in the accumulation of intermediates from fatty acid β-oxidation that serve as precursors of PHA synthesis (Qi *et al*., 1998; Lu *et al*., 2003). Wang and Lee, (1997) constructed a filamentation-suppressed recombinant *E. coli*, by hyper-expressing an essential cell division protein FtsZ along with *A. eutrophus* PHA biosynthetic enzymes. Using this strategy, high productivity of 3.4g of PHB/l/h could be obtained. With a pH-stat fed-batch culture of *E. coli*, a high-productivity of 4.63 g/l/h was attained (Choi *et al*., 1998). During the fed batch culture of recombinant *E. coli*, a large amount of oxygen was necessary to

maintain the dissolved oxygen concentration. As a solution to this problem *Vitreoscilla* globin gene (*vgb*) was cloned and expressed in *E. coli* (Yu *et al.,* 2000; 2002). The expression of *vgb* induced cell growth and PHB accumulation, especially under low dissolved oxygen concentration conditions during the fermentation process. This methodology was again facilitated by the use of a mutant strain of *E. coli*, *arcA* mutant, which confers high respiratory capacity to the host under microaerobic conditions, resulting in higher growth rate and polymer accumulation (Nikel *et al*., 2006). PHA production from economically cheap substrates like cheese whey (Solaiman *et al*., 2006; Wang and Lee, 1998), molasses (Liu *et al.,* 1998) and xylose (Lee, 1998) using recombinant *E. coli* has gained much attention recently.

1.11.2 Recombinant R. eutropha (presently known as Cupriavidus necator)

R. eutropha is the most widely studied natural producer of PHA. The PHB negative mutant, *R. eutropha* PHB$^-$4, has been used in the genetic engineering approaches for PHA production. Introduction of PHA synthase genes from *Allochromatium vinosum*, *Rhodospirillum rubrum*, *Rhodobactor sphaeroides, Thiocapsa pfennigii* (Liebergesell *et al*., 1993), *Pseudomonas* sp.GP4BH1 (Timm *et al.,* 1994), *Pseudomonas* sp.A33 (Lee *et al*., 1995), *A. caviae* (Fukui *et al.,* 1997; Fukui *et al.,* 1998) and *P. stutzeri* (Chen *et al*., 2005; Chen *et al*., 2006) resulted in the accumulation of PHB or PHA copolymers which depended on substrates used for cultivation of the recombinant bacteria. Introduction of PHA operons of *P. aeruginosa* (Tim and Steinbuchel, 1992), *Thiocystis violacea* (Liebergesell *et al.,* 1993), *Pseudomonas* sp. 61-3 (Matsusaki *et al*., 1998) and *A. caviae* (Kichise *et al*., 1999) resulted in the accumulation of good amount of PHA.

Fukui *et al.,* (2002) designed a sophisticated pathway in *R. eutropha,* using *phaC* and *phaJ* from *A. caviea* and crotonyl-CoA reductase (Ccr) from *Streptomyces cinnamonensis*, to make P (3HB-co-3HHx) from fructose. This pathway enabled the incorporation of 1.5mol% of 3HHx in the polymer, resulted in better material properties. The molar fraction of 3-hydroxy valerate in P (3HB-3HV) has been modulated through the co-amplification of $phbC_{Re}$ gene and *E. coli zwf* gene (encoding Glucose-6-phosphate dehydrogenase generating NADPH from the metabolism of fructose) in *R. eutropha* (Choi *et al*., 2003). PHA content of 60.1% and

molar fraction of P (3HB-3HV) of 71.9mol% was attained through the concurrent feeding of valerate and fructose. A copolymer of 3HB with 5 mol% (R)-3-hydroxyhexanoate(with a high PHA content of 71–74%), could be produced from soybean oil as a sole carbon source, by a recombinant strain of *R. eutropha* PHB^{-}4, carrying the PHA synthase gene of *Aeromonas caviae.* (Kahar *et al*., 2004).

Introduction of *P. stutzeri phaC2* gene into *R. eutropha* PHB^{-}4 mutant conferred the ability to synthesize PHA and the composition varied with respect to the substrate used for growing this recombinant. The recombinants accumulated PHB homopolymers up to 40.9 wt% when fed with gluconate. From fatty acids the recombinant successfully produced copolymers of 3HB and mcl-monomers. After the (R)-3-hydroxydecanol-ACP CoA trans acylase gene *phaG*$_{Pp}$ from *Pseudomonas putida* was introduced into *phaC2*$_{Ps}$-containing *R. eutropha* PHB^{-}4, poly (3HB-co-3HA) co-polyester with a very high 3-hydroxybutyrate (3HB) fraction (97.3 mol %) was produced from gluconate (Chen *et al.,* 2006). Polyhydroxyalkanoate (PHA) copolymers consisting of short-chain-length and medium-chain-length 3-hydroxyalkanoates (3HA) were produced by recombinant *R. eutropha* PHB^{-}4 harboring a low-substrate-specificity PHA synthase PhaC2$_{Ps}$ from *Pseudomonas stutzeri*1317 (Luo *et al*., 2006).

1.11.3 Recombinant Pseudomonas

Several strains of *Pseudomonas* are reported to produce PHA naturally. These strains have two PHA synthase enzymes and different monomer supplying enzymes. Metabolic improvement related to PHA production has been carried out in this bacteria using genetic engineering technique. A PHA-negative mutant *P. putida* GPp104 was used as host strain for the isolation and functional analysis of PHA synthase genes from various organisms. FadA/FadB/FadAB mutation in a PHA producer strain of *P. putida* using transposon Tn5 as a disrupting element resulted in dramatic increase in the polymer content as more than 90% of the cytoplasm was occupied by the polymer (Olivera *et al*., 2001). *Pseudomonas* sp. 61-3, *P. oleovorans* and *P. fragi* have been used as host strain for PHA production through metabolic improvement. A list of recombinant *Pseudomonas* bearing various genes used for PHA production is given in Table 1.7.

Table 1.7 PHA production in recombinant *Pseudomonas*

Host	Introduced gene	Carbon source	PHA %CDW	PHA composition	Reference
P. putida GPp104	*phaC1*$_{Po}$	Decanoate	29	C6-C10	Huisman *et al.,*1991
	phaC2$_{Po}$	Decanoate	36	C6-C10	
	phaC1$_{Pa}$ *phaC2*$_{Po}$	Gluconate	32	C6-C10	Timm and Steinbuchel, 1992
		Octanoate	41	C6-C8	
	phaC2$_{Pa}$	Gluconate	26	C6-C12	
		Octanoate	16	C6-C8	
	phaA$_{Tv}$ *phaC*$_{Tv}$ *phEC*$_{Tv}$	Octanoate	14	C4-C6	Liebergesell *et al*., 1993
	phaC1$_{Pc}$	Gluconate	24	C6-C12	Timm *et al*., 1994
		Octanoate	47	C6-C10	
	phaC1$_{Pm}$	Gluconate	0		
		Octanoate	40	C6-C8	
	phaC1$_{PsG}$	Gluconate	21	C6-C12	
		Octanoate	62	C6-C10	
	phaC1$_{PsA}$	Gluconate	29	C4-C12	Lee *et al*., 1995
		Octanoate	89	C6-C12	
	phaC$_{Ac}$ *phaJ*$_{Ac}$	Hexanoate	38	C4-C6	Fukui and Doi,
	phaP$_{Ac}$	Octanoate	48	C4-C6	1997
	phbA$_{Ps6}$ *phbB*$_{Ps6}$ *phbC*$_{Ps6}$ *phbR*$_{Ps6}$	Gluconate	20	C4	Matsusaki *et al*., 1998
	phaC1$_{Ps6}$	Gluconate	9	C6-C12	
		Octanoate	43	C4-C10	
	PhaC1$_{Ps6}$*phbA*$_{Re}$	Gluconate	10	C4-C12	Matsusaki *et al*., 2000b
	phbB$_{Re}$	Octanoate	18	C4-C12	
	PhaCAB$_{Re}$	Gluconate	41	C4-C12	Shin *et al*., 2002
		Octanoate	33	C4, C8-C12	
		Decanoate	45	C4, C8-C12	
		Oleate	27	C4-C12	
	phaC A. hydrophila, phaB R. eutropha phaG P. putida,	Glucose	19	C4-C6	Qiu *et al*., 2005
P. oleovorans	*phbC*$_{Re}$	Gluconate	11	C4	Timm and Steinbuchel,
		Octanoate	77	C4-C10	1990
Pseudomonas sp.61-3	*PhaC1*$_{Ps6}$*phbA*$_{Re}$ *phbB*$_{Re}$	Gluconate	45	C4, C8-C12	Matsusaki *et al*., 2000a
P. fragi	*phaC1*$_{Pa}$ *phaG*$_{Pp}$	Gluconate	9	C6-C12	Fiedler *et al*., 2000
		Oleate	6	C6-C12	

Ac: *Aeromonas caviae*, Pa: *P. aeruginosa*, Pc: *P. citronellosis*, Pm: *P. mendocina*, Po: *P. oleovorans,* Pp: *P. putida,* Ps6: *Pseudomonas* sp. 61-3, PsA: *Pseudomonas* sp. , PsG: *Pseudomonas* sp., Re: *R. eutropha,* Tv: *Thiocystis violacea*

1.11.4 Other Bacteria

Many bacteria such as*, Bacillus, Aeromonas, Salmonella* have been used as host strain for recombinant synthesis of PHA because of the unique properties of these bacteria.

Bacillus subtilis has many advantages such as well known genetics and biochemistry, short generation time, can grow to very high cell density, capable of

using inexpensive carbon and nitrogen sources, capable of secreting large amount of enzymes, lack of pathogenicity, no major codon bias. The first attempt of PHA production in *B. subtilis* recombinant has been made by cloning *B. megaterium phaPQRBC* genes (Law *et al*., 2003). Even though PHB homopolymers was detected in the recombinant the amount was found to be very low. Recently Wang *et al*., (2006) constructed a *B. subtilis* recombinant bearing *Pseudomonas* PHA synthase and *Ralstonia PhaAB* genes capable of producing mcl-PHA from malt waste. In order to establish a novel recovery system for polyhydroxyalkanoates, a self-disruptive strain of *B. megaterium* that responds to substrate exhaustion has been constructed. A gene cassette carrying the lysis system of *Bacillus amyloliquefaciens* phage - holin and endolysin was inserted into the *E. coli-B. subtilis* shuttle vector, under the control of a xylose-inducible expression system, xylR-xylA' (Hori *et al*., 2004).

Properties such as robust growth, simple growth requirements and convenience for genetic engineering make *Aeromonas hydrophila* 4AK4 very useful for the production of P (HB-HHX), a polymer with different properties and which support various applications. Recently, many recombinant *A. hydrophila* 4AK4 strains were constructed that produced P (HB-HHx) containing various molar fractions of HHx. Fukui *et al*., (2001) reported that a trans conjugant of *A. punctata* harboring additional copies of *phaPCJ* genes accumulated P (HB-HHx) with much higher 3HHx fraction (46–63 mol%) from fatty acids or olive oil. In order to study the factors affecting P (HB-HHX) content and composition, genes encoding phasin (phaP), PHA synthase and (R)-specific enoyl-CoA hydratase from *A. punctata* were introduced individually or jointly into *A. hydrophila* 4AK4 (Han *et al*., 2004). The results suggest that a higher PHA synthase activity could lead to an increased 3HHx fraction and P (HB-HHx) content (50-63%). *A. hydrophila* 4AK4 synthesized 15% PHA (19% (mol/mol) 3-hydroxyhexanoate) when additional P (HB-HHx) synthesis genes, *phaPCJ*, were over-expressed along with the truncated *tesA* gene, encoding cytosolic thioesterase I of *E. coli* which catalyzes the conversion of acyl-ACP into free fatty acids(Qiu *et al*., 2005).

Salmonella enterica serovar typhimurium is a close relative of *E. coli* and offers the same advantages as host strain as does *E. coli*. When *Acinetobacter phaCAB* genes and *Salmonella typhimurium* propionyl CoA synthetase gene (*prpE*)

have been introduced into a mutant strain of *S. enterica* (mutant in propionate activation activity A), under the control of arabinose and *tac* promoters, respectively, led to the synthesis of P(3HB-co-3HV) with a high percentage of valerate (Aldor and Keasling, 2001). This pathway has been further improved by introducing the newly discovered *E. coli* enzymes, sleeping beauty mutase (Sbm) and (R)-methyl malonyl-CoA decarboxylase (YgfG) into the same *Salmonella enterica* recombinant (Aldor *et al*., 2002). These two enzymes help in the conversion of succinyl-CoA derived from tricarboxilic acid cycle, to propionyl CoA. This recombinant was capable of synthesizing copolymer, up to 40% cellular dry weight containing 30 mol% valerate, from glycerol. The novel pathway constructed by Aldor *et al.,* (2002) is shown below (Figure 1.11).

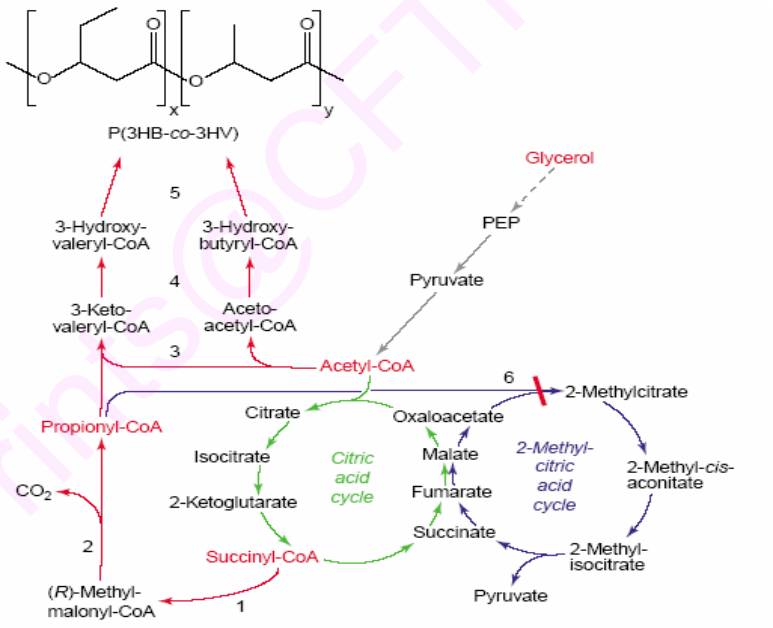


Figure 1.11 Novel metabolic pathway for P (HB-co-HV) synthesis (Aldor *et al*., 2002)

1.11.4 Eukaryotic systems

The high production cost of PHA in bacteria has driven the attention of people working in this area into eukaryotic system for PHA production. PHA production has been already achieved in insect cells of *Spodoptera frugiperda* (Williams *et al.,* 1996) and yeast cells of *Sacharomyces cerevisiae* (Leaf *et al*., 1996). PHA accumulation was very low in these recombinants due to insufficient intermediary enzymes.

Modified PHA synthase *phaC1* of *P. aeruginosa,* with the ability of peroxysomal targeting, was introduced into *S. cerevisiae* (Poirier *et al*., 2001) and *Pichia pastoris* (Poirier *et al*., 2002) which led to the accumulation of mcl-PHA by these recombinants grown on fatty acids.

Plants may serve as an alternative production system for PHA to a higher level, which are difficult to reach by bacterial fermentation. Poirier *et al.,* (1992) demonstrated the ability of *Arabidopsis* plant to synthesize PHA when *phbB* and *phbC* genes of *Ralstonia* were expressed. But the production was less and it had adverse effect on the plant growth. So later the work was targeted on the expression of these proteins in plastids of *Arabidopsis thaliana* (Nawrath *et al.,*1994), cotton fibers (John and Keller,1996), Brassica (Slater *et al*., 1999), hairy roots of sugar beet (Menzel *et al.,* 2003) and tobacco (Lossl *et al*., 2003).

1.12 COMERCIAL PRODUCTION OF PHA

A number of different companies are developing PHAs worldwide including Kaneka in Japan, and P&G Chemical, BioPol and Metabolix in the US and Imperial Chemical Industries in the UK. Kaneka and P&G Chemical have teamed up to commercialize a product called Nodax (also known as Nodak™), which is a specialized PHA. Nodak ™ has already been made into a variety of different prototype objects such as plastic fiber or twine and molded plastic ware such as plates and cup. The cost of PHA using the natural producer *R. eutrophus* is US$16 per Kg which is 18 times more expensive than polypropylene. With recombinant *E. coli* as producer of PHA, price can be reduced to US$4 per Kg, which is close to that of other biodegradable plastic materials such as PLA and aliphatic polyesters. Metabolix, the pioneer company manufacturing biodegradable plastic, has brought down the price of PHA ($0.50/lb) through their technologies (Metabolix, Inc. and Tepha, inc. 2003). Metabolix aims to produce PHAs in plants at a cost below $0.30/lb. These products are expected to be economically competitive with polyethylene and polypropylene.

1.13 REGULATION OF PHA BIOSYNTHESIS

PHA synthesis is regulated at the enzymatic level (Senior and Dawes, 1971) and transcriptional level. Many proteins involved in the regulation of PHA biosynthesis and promoters upstream of PHA biosynthetic enzymes have been cloned and characterized from different bacteria. Intra cellular concentration of carbon to nitrogen ratio, various biosynthetic enzymes, degrading enzymes, acetyl CoA and NADH play a major role in the regulation of PHA synthesis (Haywood *et al*., 1988). The TCA cycle is an important control point since it provides acetyl CoA and NADPH for the biosynthesis of PHA. A recent work with recombinant strains demonstrated that the biosynthesis rate of PHB is controlled by 3-ketothiolase and acetoacetyl- CoA reductase, whereas the content of PHB is controlled by PHB synthase (Jung *et al*., 2000). Increased activity of PhaA and PhaB tend to increase the size of the granules and the enforced activity of PhaC tends to increase the number of granules. Han *et al*., (2004) suggest that a higher PHA synthase activity could lead to a higher 3HHx fraction and PHB-HHx content in *A. hydrophila.* PhaP is a natural PHA-binding protein that determines the size of PHA granules (Potter *et al*., 2005; York *et al*., 2001). The hyper expression of PhaP and PhaC in *A. hydrophila* resulted in the increase in granule number and HHX fraction (HHX increased to 21mol% compared to 14mol%HHX produced by the wild type) (Tian *et al*., 2005). RT-PCR analysis by these workers suggested that phasin PhaP, regulates *phaC* gene at the transcription level in *A. hydrophila*.

The regulatory proteins identified so far from different bacteria are PhaF1, a histone like protein from *P. aeruginosa, P. putida* and *P. oleovorans,* PhaR, from *Pseudomonas* sp. 61-3, *Paracoccus denitrificans* and *R. eutropha,* PhaS from *P. putida*, GacS from *Acetobacter vinelandi,* NtrB/NtrC from *Azospirillum brasilense* Sp7, Lux R , a quorum sensing regulator in *Vibrio harvey.* A few reports postulated that the involvement of global regulators in the degradation of PHA such as *ptsH* and *ptsI* in *R. eutropha* (Pries *et al*., 1991) and rpoS in *P. oleovorans* (Ruiz *et al*., 2004).

PhaR plays a role in the regulation of *phaP* expression as well as auto regulation. The mechanism of action of PhaR has been studied in *R. eutropha* (Potter *et al*., 2002) and *P. denitrificans* (Maehara *et al*., 2002) and as follows. PhaR is able to sense both the onset of PHA synthesis and the enlargement of the granules through

direct binding to PHA. When conditions are not favorable for PHA biosynthesis PhaR binds to the *phaP* promoter region and represses transcription of this gene. After the onset of PHA biosynthesis, under conditions that are permissive for the formation of nascent granules, PhaR binds to PHA granules and *phaP* will be transcribed. At the later stages of PHA accumulation, PhaR no longer binds to the granules and the transcription of *phaP* will be again repressed. Excess PhaR binds to the *phaR* upstream region, there by repress its own transcription.

Regulation of PHA synthesis in *Pseudomonas* has been studied to a limited extent. Many Pseudomonads are able to synthesize PHAs by two different pathways: through fatty acid biosynthesis when grown on gluconate or through fatty acid degradation when grown on fatty acids. *Pseudomonas* spp. PHA operon contains two PHA polymerases with slightly different substrate specificity, which is encoded on the chromosome together with a PHA depolymerase and *phaD*. In most of the *Pseudomonas* spp. the two PHA synthases function independently of pathways. But the two PHA polymerases that have been identified in *P. putida* are functional in either of the two biosynthetic pathways. Qi *et al.,* (1997) had indicated negligible differences between *phaC1* and *phaC2* in terms of substrate specificity. In contrast to this Chen *et al*., (2004) had investigated the function of *phaC1* and *phaC2* gene by expressing the genes in *E. coli* individually and concluded that both have different specificity for substrates.

In various microorganisms promoters have been experimentally identified or postulated based on the sequence upstream of the PHA and PHB biosynthetic genes (Huisman *et al*., 1991; Schubert *et al*., 1991; Timm and Steinbuchel, 1992; Liebergesell and Steinbuchel, 1992; 1993; Schembri *et al.,* 1995; Matsusaki *et al.,* 1998; McCool and Cannon, 1999). A promoter region containing one or more promoter sequences has been found upstream of the PHA gene cluster in three different *Pseudomonas* strains. In *P. aeruginosa,* the structural genes for PHA biosynthesis are organized as an operon, i.e *phaC1* and the *phaZ* driven by one promoter and the *phaC2* and *phaD* driven by a separate promoter (Timm and Steinbuchel, 1992). Two transcriptional start sites, which were preceded by sequences resembling the *Escherichia coli* consensus sequences for σ^{54} and σ^{70} promoters, were

identified experimentally upstream of *phaCl*. The *phaC2* promoter bears resemblance to that of *E. coli* σ^{70} dependent promoters.

Hoffmann and Rehm, (2004), through conducting PHA accumulation experiments and transcriptional analysis of PHA biosynthesis genes, had suggested that the PHA accumulation depends on RpoN (σ^{54}, an alternative sigma factor of RNA polymerase), regardless of type of carbon source (either gluconate or octanoate) in *P. aeruginosa.* The lower yield of PHA in alginate over producing strains of *P. aeruginosa* from gluconate, indicated that biosynthesis pathways of PHA and alginate compete for the central metabolite acetyl-CoA. In *P. putida* the pathway from gluconate and fatty acids is completely σ^{54} independent (Hoffmann and Rehm, 2004). In contrast to *P. aeruginosa, P. putida* shows a stronger nitrogen-dependency when cultivated on gluconate. Also the *phaG* gene in *P. putida* was strongly induced during nitrogen starvation (Hoffmann and Rehm, 2004). This would indicate that the genes which are involved in PHA biosynthesis from acetyl CoA might be induced under nitrogen starvation. This report throws light on the mechanism of regulation of the expression of the PHA synthase gene.

The *P. aeruginosa* PHA gene locus also includes the *phaD* (encoding putative transcriptional regulator), *phaI* gene (unknown function) whose homologue in *P. oleovorans* are proposed to be PHA granule-associated structural proteins whereas *phaF* has also been considered as a negative regulator of PHA biosynthesis genes (Prieto, *et al*., 1999). Comparative transcriptional analysis of PHA biosynthetic genes with a wild type and RpoN negative mutant have suggested that phaI and phaF are co-transcribed in *P. putida* and *P. aeruginosa* (Hoffmann and Rehm, 2005). In agreement with Prieto *et al*., (1999), Hoffmann and Rehm, (2005) suggested that PhaF is a negative regulator of PhaC1 and RpoN is a negative regulator of PhaF transcription.

Based on all the information collated here the objectives of the present work were formulated and are presented subsequently:

SCOPE AND PLAN OF THE PRESENT INVESTIGATION

Polyhydroxyalkanoates (PHAs), produced by different bacteria have acquired focus in recent times because of their use as biodegradable thermoplastics. A variety of *Pseudomonas* spp. are known to produce PHAs composed of medium chain length (mcl) 3-hydroxy fatty acids, from a wide range of substrates, using different metabolic pathways. Mcl-PHA and its copolymers have better material properties and have wide range of application. Many powerful monomer supplying enzymes like (R)-specific enoyl CoA hydratases, 3-ketoacyl CoA reductase, epimerase and PHA synthases of broad substrate specificity, are present in *Pseudomonas.* These features of *Pseudomonas,* making it a significant organism in the area of PHA research. So the primary objective of this investigation was to isolate *Pseudomonas* spp. from different sources and characterize the polymer produced.

The physical and thermal properties of PHA copolymers can be regulated by varying their molecular structure and copolymer compositions. Copolymers of PHB with PHV and mcl-PHA have better film forming and mechanical properties than homopolymers. A survey of the literature especially on the production of polyhydroxyalkanoates by recombinant bacteria had indicated that there are only a few reports on the production of ter polymers in *E. coli* (Table 1.5 and 1.6). The yield of PHA obtained by these workers is not satisfactory. It has been our main objective to produce PHA co-polymers in *E. coli* using genes from different organisms utilizing different biosynthetic pathways. It was envisaged that cloning of PHA synthase and (R)-specific enoyl CoA hydratase genes from *Pseudomonas aeruginosa* into *E. coli* would enable production of mcl-PHA. It was expected that *fadB* mutant of *E. coli* would allow enhanced mcl-PHA synthesis since the availability of the enoyl CoA would be enhanced. It was also presumed that addition of β-ketothiolase and acetoacetyl CoA reductase from *Bacillus* sp. would improve the yield and quality of PHA produced in *E. coli*. There are many constraints to heterologous production of proteins in *E. coli* such as nature of the promoter, processing of the signal peptide and solubility of the protein product. These factors have been taken into account while designing the cloning strategy. It was also of our interest to record the changes in the quantity and monomer composition of PHA on addition of different fatty acids and inhibitor (acrylic acid) to the medium of recombinant *E. coli*.

A survey of the literature suggested the presence of isoforms of PHA biosynthetic enzymes such as PHA synthase and (R)-specific enoyl CoA hydratase in *Pseudomonas aeruginosa.* There are four (R)-specific enoyl CoA hydratases present and the exact role of these hydratase in *Pseudomonas* is unknown. Earlier workers have studied the hydratases hyper expressed in *E. coli* (Tsuge *et al*. 2000; Tsuge *et al*. 2003). There has been no earlier attempt to down regulate the hydratases either *in situ* or elsewhere. Therefore, we have used antisense technique as well as hyper-expression to study the substrate specificity of two of these enzymes in *P. aeruginosa.*

A survey of the literature especially that on the gene organization and regulation of PHA synthesis in *Pseudomonas* have indicated that the difference in function of *phaC1* and *phaC2* genes was unclear. Some workers have reported about the differential expression of PHA synthase genes in the presence of different substrates. PhaC2 of *Pseudomonas* sp. 61-3 and *P. putida* have shown broader substrate specificity when co- expressed with enzymes capable of providing monomers with different carbon number. (Tsuge *et al*., 2000; Chen *et al* 2004; Chen *et al* 2006). Most studies regarding the specificity of these enzymes have been carried out with the heterologously expressed enzymes. The naturally occurring global antisense RNA regulators of translation in *E. coli* often target the Shine Dalgarno (SD) region of the promoter, e.g. OxyS RNA-*fhl*A gene system (Argaman and Altuvia, 2000)**.** It is envisaged that expression of RNA antisense to the SD region could control protein synthesis as much as antisensing the coding region. Based on this hypothesis, the specificity of two PHA synthases in *P. aeruginosa* was investigated. Literature survey indicated that *phaC1* and *phaC2* genes are under the control of two different promoters and was identified from three strains of *Pseudomonas.* A reporter gene mediated system for the study of these promoters has not been hitherto reported and hence became an objective of this investigation. This study may help in the understanding of PHA synthase gene regulation and further study on transcriptional factors which recognize these promoters.

The following constitutes the main objectives of the present study:

1.Isolation and identification of PHA producing *Pseudomonas* strains and characterization of the PHAs produced.

2. Cloning of genes essential for heterologous synthesis of PHAs.
3. Investigations into the regulation of PHA synthesis in *Pseudomonas* species.

The work plan to achieve the above mentioned objectives was divided into the following 4 phases.

1) Isolation of *Pseudomonas* spp. and identification of PHA producers by PCR and colony hybridization. PHA extraction, quantification and characterization using GC, FTIR, and GCMS. Cloning of PHA synthase1 gene (*phaC1*) from *P. aeruginosa* and its expression in heterologous and homologous host.
2) Cloning of (R)-specific enoyl CoA hydratase 1 and 4 *(phaJ1* and *phaJ4*) from *Pseudomonas* sp. Co-expression of PhaC1 and hydratases in *E. coli* of different genetic backgrounds. Quantification and characterization of PHA produced by the recombinant *E. coli* strains. Construction of a recombinant *E .coli* using two genes (*phaJ1* & *phaC1*) from *Pseudomonas* sp. and two genes (*phaA* & *phaB*) from a *Bacillus* sp. for the synthesis of PHA copolymer. Optimization of PHA production and characterization of PHA produced by the recombinant.
3) Antisensing and hyper-expression of *phaJ1* and *phaJ4* genes in *P. aeruginosa* to study the role of these enzymes in PHA biosynthesis.
4) PCR cloning of *phaC1* and *phaC2* promoters. Antisense construct of promoter with SD sequences to study the differential expression of *PhaC1* and *phaC2*, reporter gene mediated study of deletion constructs of *phaC1* promoter under various growth conditions.

2.1 MATERIALS USED

2.1.1 Chemicals (General):

Acetic acid, Acrylamide, Agar, Agarose, Ammonium persulphate, Beef extract, Benzoic acid, β-mercapto ethanol, Bis acrylamide, Boric acid, $CaCl_2$ $2H_2O$, Citric acid, Crotonyl CoA, different fatty acids such as butyric acid, valeric acid, hexanoic acid, octanoic acid, heptanoic acid, nonanoic acid, decanoic acid, dodecanoic acid, Dinitrosalicylic acid, EDTA, Ethidium bromide, Glucose, glycerol, H_2SO_4, HCl, IPTG, KH_2PO_4, lactose, $MgSO_4$ $7H_2O$, Na_2HPO_4 $2H_2O$, NaCl, NaOH, $(NH_4)_2SO_4$, Peptone, PMSF, Polyhydroxybutyrate, Polyhydroxybutyrate-co-hydroxyvalerate (5 mol%), SDS, Sodium hypochlorite, Sucrose, TEMED, Tris base, Tris chloride, X-gal and Yeast extract.

The given list of chemicals used in the study was obtained from known companies such as SRL, Qualigenes, Himedia, SISCO, Merck, ICN and Sigma aldrich.

2.1.2 Solvents

The solvents used in the study listed below were obtained from reputed companies such as SRL, SISCO, Merck, and Qualigen.

Chloroform, Acetone, Ethanol, Methanol, Hexane, Phenol, Butanol and Toluene.

2.1.3 Filters, Filter papers, membranes

Filter papers of different pore sizes (Whatman) and Nylon membranes have been procured from Labmate Asia and Sigma Chemical Company, respectively. Autoclavable and disposable filters (syringe filter) of different pore sizes were purchased from Gelman Laboratory and HiMedia.

2.1.4 Equipments

Equipments used in the present study are listed below

Autoclave (Omega scientific instrument), Deep freezer (Blue star), Electronic balance (Shimadzu), Electrophoresis system for DNA (Bangalore Genei), Electrophoresis system for protein (Banglore Genei and Hoefer), Fermentor: Bioflo 110 3 L. jar fermentor, (New Brunswick Scientific Co. USA), Fourier Transform Infrared Spectrophotometer (Nicolet Magna 5700 spectrophotometer), Gas chromatography (Fison's), Gas chromatography mass spectrometer (Turbo Mass Gold Mass Spectrometer, Perkin Elmer Instruments), Gel documentation system (Biorad), Hybridization oven

(GATC-HYBE), Incubator shaker (Remi), Incubators (Remi Instruments), Laminar hood (Klenzoids), Mettler balance (Shimadzu), NMR: AMX 400 spectrophotometer (Brucker), PCR machine (Gene Amp PCR System 9700, PE Applied Biosystems), pH meter (Control dynamics), Phase contrast microscope (Kryptone), Pipetteman (Accupipet, Tarson), Refrigerators (Voltas), Scanning Electron Microscope (LEO-435), Sonnicator (dr. shielscher), Spectrophotometer (Shimadzu, UV 160, Japan), Vortexer (Spinix) and Water bath (Neutronics).

2.1.5 Media Composition

2.1.5.1 Nutrient Agar:

Components in g/l of distilled water

Peptone: 5.0, NaCl: 5.0, Beef extract: 1.5, Yeast extract: 1.5, Agar: 1.5, (pH 7.0)

2.1.5.2 PHA production medium (minimal media)

Components in g/l of distilled water

$Na_2HPO_4 2H_2O$: 4.4, KH_2PO_4:1.5, $(NH_4)_2SO_4$:1.5, $MgSO_4\ 7H_2O$:0.2, Sucrose: 20, (pH 7.0)

2.1.5.3 Luria Bertani medium

Components in g/l of distilled water

Tryptone: 10, Yeast extract: 5.0, NaCl: 10, (pH 7.0)

2.1.5.4 PHA production medium for recombinant E. coli (Modified medium of Wang and Lee 1998):

Components in g/l of distilled water

KH_2PO_4: 13, $MgSO_4\ 7H_2O$: 1.2, Citric acid: 1.7, $(NH_4)_2HPO_4$: 4.0, Glucose: 20, Yeast extract (Optional): 2.0, Tryptone: 2.0, Trace metal solution: 1.0 ml / l

[*Trace metal solution* **(**In a Liter of 5 M HCl**):** 10 g $FeSO_4$. $7H_2O$; 2 g $CaCl_2\ 2H_2O$; 2.2 g $ZnSO_4\ 7H_2O$; 0.5 g $MnSO_4\ 4H_2O$; 1 g of $CuSO_4\ 5H_2O$; 0.1 g $(NH_4)_6Mo_7O_{24}\ 4H_2O$ and 0.02 g $Na_2B_4O_7\ 10H_2O$.]

2.1.5.5 Pseudomonas Isolation Agar

Components in g/l of distilled water

Peptic digest of animal tissue, 20; Magnesium chloride, 1.4; Potassium sulphate, 10; Triclosan: 0.025, Agar: 1.3, (pH 7.0)

2.2 MICROBIOLOGICAL METHODS

2.2.1 Sterilization of the media, glass ware and plastic wares

Liquid and solid media used in various experiments were autoclaved in flasks or tubes that were plugged with absorbent cotton, at 15-lbs of steam pressure and 121^{o}C for 20 min. Heat sensitive ingredients were sterilized by filtration. Glass wares were wrapped with paper and sealed in polypropylene bags before sterilization.

2.2.2 Maintenance of microorganisms

The purified culture of *Pseudomonas* spp*., Bacillus subtilis* and *Escherichia coli* strains were maintained at 4°C on nutrient agar slants or semi solid Luria Bertani slants. The cultures were sub cultured once in a month for maintenance.

Recombinant strains of *E. coli, B. subtilis* and *P. aeruginosa* were maintained on Luria Bertani semi-solid agar slants as stab cultures and on agar plate. The medium contained Ampicillin sodium salt (100 mg/l) or Tetracycline (12mg/ml) or Kanamycin (50mg/ml) for sustenance of the plasmids in *E. coli*. Carbenicillin sodium salt (200mg/ml) or Tetracycline (150mg/ml) was used for maintenance of plasmids in *P. aeruginosa*. The culture was sub cultured on monthly basis and stored at 4°C.

2.2.3 Inoculum

Inoculum of *Pseudomonas* spp., recombinant *P. aeruginosa*, recombinant *B. subtilis,* different strains of *E. coli* and recombinant *E. coli* were prepared by transferring single colony from a 24 h old culture plate into 5 ml of nutrient broth or LB broth. The culture was allowed to grow at 37°C, 250 rpm for overnight. Recombinant bacteria were cutivated with appropriate antibiotic. The inoculum of recombinant *E. coli fadB* mutant, *E. coli* BL21, *E. coli* JC7623 and *P. aeruginosa* contained 70x10^{6} cfu/ml, 76x10^{6} cfu/ml, 68x10^{6} cfu/ml, 116x10^{6}cfu/ml, respectively.

2.2.4 PHA production

2.2.4.1 Flask culture

PHA production by *Pseudomonas* spp. was carried out in triplicate in 250 ml Erlenmeyer flasks in liquid medium (50 ml Mineral salt medium). The flasks were inoculated with 10% (v/v) of inoculum and incubation was performed at 250 rpm and

37°C for 48-72 h. Hexanoic, octanoic and decanoic acids were added at 1 g l^{-1} of medium, as neutralized and sterilized salt solutions in 5 ml portions of water to 12 h old growing cultures. Shake flask experiments were carried out in triplicate. Recombinant *P aeruginosa* was also cultivated in a similar way with the addition of Carbenicillin as a selective agent and IPTG as an inducer.

Recombinant *E. coli* strains were cultivated in shake flasks in a) LB medium with 2% (w/v) glucose and 10 mg% Ampicillin and b) modified synthetic medium of Wang and Lee (1998). Medium (50 ml) contained in 250 ml Erlenmeyer flasks were inoculated (10%, v/v) with 8 h old inoculum and incubated at 30°C at 250 rpm for 48 h. Isopropyl-β-D-thiogalacto pyranoside (IPTG) was added (0.4 mmol) after6 h of growth. Sodium butyrate, valeric acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid and decanoic acid were added (0.2 g%, w/v) in 5 ml portions of neutralized and filter sterilized solutions after 12 h of growth.

2.2.4.2 Fermentative production of PHA

Recombinant strain was cultivated in a jar fermentor (Bioflo 110, New Brunswick Scientific Co. USA) of 3 l capacity, containing 2 l of synthetic medium containing 20 g/l of glucose. Medium was inoculated with 200 ml inoculum, which was prepared according to the method mentioned above. Cultivation was carried out for 48 h and culture pH was controlled at 7 automatically, by the addition of 1 M NaOH. The dissolved oxygen was maintained above 40% of the air saturation level by cascading effect.

2.2.5 Viable plate counts

Total plate counts or viable cell count or cell forming units of inoculum was estimated by plating diluted fresh growing culture on nutrient medium. One ml of the broth was serially diluted six times in test tubes containing 9 ml of sterile water and homogenized 0.1 ml sample from individual dilutions were plated in duplicates on nutrient agar medium. Plates were incubated at 37^{o}C for 24 h and the number of colonies developed was counted. Number of viable cells was presented as colony forming units or viable cell counts/ml.

2.2.6 Staining Methods

2.2.6.1 Gram staining

Reagents:

Crystal violet stain: 2 g of crystal violet was dissolved in 20 ml of 95%ethanol and the volume was made up to 100ml with distilled water.

Gram's iodine: 2 g of potassium iodide, was dissolved in 300 ml of distilled water, 1 g of iodine dissolved in this KI solution.

Decolorizer : 50% ethyl alcohol, 50% acetone

Safranin stain: 2 g of safranin was dissolved in 20 ml of 95%ethanol and the volume was made up to 100ml with distilled water.

*Method***:** A thin smear of the culture was prepared on a glass slide and heat fixed. The primary stain crystal violet was added and incubated for 1 minute. Excess stain was washed off with distilled water. Gram's iodine was added and incubated for 30 seconds. The smear was rinsed with water and ethanol. The secondary stain, safranin, was added and incubated for 30 seconds. The slide was then washed with water and observed under the light microscope.

2.2.6.2 Sudan Black staining **(**Byrom and Byrom, 1991**)**

Reagent**:** 0.3 % of Sudan black was prepared in ethylene glycol and was filtered before use.

Method: A thin smear of 24 h grown culture (PHA production medium) of bacteria was prepared, air dried and heat fixed. Sudan black stain was added to the cells and incubated for 20 min. The slides were then washed with distilled water and counter stained with safranin (0.5% in water) for 30 seconds. The slides were washed with distilled water, air dried and observed under light microscope. Presence of polyhydroxyalkanoates was visualized as blue stained granules inside the cells

2.2.7 Catalase test

One drop of hydrogen peroxide solution was placed on a slide. A small portion of the suspect colony from freshly grown NA plate was spotted onto the centre of a cover-slip. The cover-slip was inverted and placed it on the drop of hydrogen peroxide solution. Vigorous bubbling occurring within 10 seconds indicated positive test for catalase.

2.2.8 Oxidase test

Reagent: 1% *N, N, N', N'*-tetramethyl-*p*-phenylenediamine dihydrochloride in distilled water.

Method: A piece of filter paper was soaked in the reagent solution. Some freshly grown colonies from the NA plate was scraped with a disposable loop or stick and rubbed onto the filter paper. Development of Blue color within 10 seconds indicated positive test for oxidase.

2.3 ANALYTICAL TECHNIQUES

2.3.1 Estimation of biomass

Dry weight of the cells was estimated by centrifugation of fermented broth at 8000 rpm for 15 min followed by washing and drying of the cell pellet at 60°C to a constant weight.

2.3.2 Quantification of PHA

2.3.2.1 Gravimetric method:

PHA content of bacterial cells was determined gravimetrically by sodium hypochlorite method [Law and Slepecky, 1969; Williamson and Willkinson, 1958). Sodium hypochlorite (1ml/20 mg of biomass) was added to the biomass and allowed for digestion for 1h at 37^{o}C. After 1h the reaction was stopped by adding distilled water and the suspension was centrifuged at 6000 rpm for 15 min. The pellet obtained was washed successfully with 5ml each of water, acetone, diethyl ether and absolute alcohol. The final white powder obtained was dissolved in chloroform, air-dried and weighed. PHA concentration was expressed as % PHA of Cellular dry weight (CDW).

$$\% \text{ PHA} = \frac{\text{Weight of PHA X 100}}{\text{Weight of biomass}}$$

2.3.2.2 Isolation of PHA by solvent extraction

PHA was isolated from the lyophilized cells by refluxing with chloroform for 5-6 h. The polymer was recovered using hexane in 1:2 proportions. The product was air dried to a constant weight.

2.3.3 Preparation of cell lysate

Cell lysate of recombinant *E. coli* and *Pseudomonas aeruginosa* were prepared by sonication of the pellet in lysis buffer (100 mM EDTA, 100 mM β- mercapto ethanol and 10 mM Phenyl Methyl Sulfonyl Fluoride (PMSF). Sonication was repeated twice for 1min while incubated on ice with an interval of 1min

2.3.4 Estimation of protein (Bradford, 1976)

The Method of Bradford's was used for the quantification of total protein in the cell lysate of recombinant *P. aeruginosa* and *E. coli.*

Bradford Reagent

50 mg Coomassie Blue G250, 25ml 95% Ethanol, 50ml 85% Phosphoric acid; Reagent was prepared by dissolving Coomassie blue G250 in 95% ethanol, followed by addition of phosphoric acid and the volume was made up to 500ml.

Standard Curve

Bovine serum albumin (BSA) was chosen as the standard. A stock solution of 100µg/ ml BSA was prepared. Known concentrations of standard ranging from 1-10µ g in a total volume of 100µl were pipetted out on to the test tubes. 100 µl distilled water was taken as blank. 1ml of Bradford reagent was added to each tube and mixed well by vortexing. The samples were transferred to cuvette and using reagent blank to zero the spectrophotometer, OD measurements were taken at 595nm and a standard graph was plotted.

Analysis of sample

Appropriate dilutions of the samples (cell lysate) was prepared and 10 µl of the prepared sample was pipetted and made up to 100 µl with distilled water in a clean test tube and 1ml of Bradford reagent was added and mixed thoroughly before measuring the OD at 595nm. Experiments were conducted in duplicates and using the standard curve equivalent protein concentration for each sample was evaluated.

2.3.5 Fourier Transform Infrared Spectroscopy

a) Standard PHA, PHA from bacterial isolate or recombinant bacteria(5 mg each) were mixed with 100 mg of FTIR grade KBr and pelletized. The FTIR spectrum was recorded at 400-4000 cm^{-1} in FTIR Nicolet Magna 5700 spectrophotometer.

b) PHA samples (10 mg) were dissolved in 200 µl of chloroform and placed between the KBr windows without any air bubble formation and the clamp was closed and fixed in the detector unit and the spectrum obtained for each sample were recorded.

2.3.6 Gas chromatography

Composition of PHA was analyzed by gas chromatography using either lyophilized cells or purified polymer (Brandl, 1988). 10-12 mg lyophilized cells or 5 mg of PHA was taken in test tubes and in to that 1ml chloroform, 850µl methanol and 150µl concentrated sulphuric acid were added, sealed and subjected to methanolysis at 100^{o}C for 150 min. After cooling, the test tubes were broken and 1ml water was added and mixed well for 2 min. 1µl of the bottom phase (chloroform) was used for GC analysis (FISONS, GC 8000 series with a flame ionization detector).The conditions for GC were as follows: BP1 capillary column 30 m by 0.32 mm; inner diameter 0.25 mm, injector temperature 220°C, detector temperature 230°C, initial column temperature was maintained at 54°C for seven min and then raised at a rate of 4°C per min up to 100°C and then 10°C per min up to 200 °C. PHB and P(HB-Co-HV) of Sigma Aldrich, USA were used as standards. Benzoic acid was used as an internal standard. Identification of the PHA monomeric units was carried out by gas chromatography/mass spectroscopy (GC/MS) using a Turbo Mass Gold Mass Spectrometer (Perkin Elmer Instruments).

2.3.7 Nuclear Magnetic Resonance analysis

Lyophilized cells (100 mg) were suspended in 10 ml of chloroform and extracted overnight at 40°C. Cell sediment was separated by centrifugation at 1900 x g for 20 min and PHA was isolated from clear chloroform layer by the addition of 2 volumes of hexane. Precipitated polymer was air-dried at 40 °C. ^{1}HNMR of the polymer was carried out in deuterated chloroform at 400 MHz on an AMX 400 spectrophotometer. P(HB) and P(HB-co-HV) from Sigma Aldrich, USA were used as standards.

2.4 MOLECULAR BIOLOGY METHODS

All laboratory reagents used were of molecular biology or A.R. grade or were of higher grade. Stock solutions and media were sterilized by autoclaving at 121°C for 20 min wherever necessary. Reagents and buffers not suitable for autoclaving were sterilized

by filter sterilization using Millipore disposable filters (0.4 μm). Standard procedures described by Sambrook and Russell, (2001) or manufacturer's instructions in the case of commercial kits were followed for all the experiments, or unless specified otherwise. The stock solutions used in molecular biology protocols are given below

a) 0.1M $CaCl_2$ stock solution

1.47g of $CaCl_2$ was dissolved in 100 ml of deionized water, filter sterilized and stored as 20 ml aliquots at -20°C.

b) Ampicillin stock solution

100 mg Ampicillin sodium salt was dissolved in 1.0 ml of deionized water, sterilized by filtration and stored at 4°C. The prepared solution was used at a working concentration of 100 μg ml^{-1}.

c) Carbenicillin stock solution

1g Carbenicillin was dissolved in 5.0 ml of deionized water, filter sterilized, stored at 4°C and used at a working concentration of 200 -400μg ml^{-1}.

d) Tetracycline stock solutions

500mg of tetracycline was suspended in 5 ml of distilled ethanol, stored at -20°C and used at a working concentration of 15 μg ml^{-1} for *E. coli* and 150 μg ml^{-1} for *Pseudomonas* strains.

e) Kanamycin stock solutions

500mg of Kanamycin was taken in 5ml of distilled water, solution was filter sterilized, stored at 4°C and used at a working concentration of 50 -100μg ml^{-1} for *E. coli*

f) 0.1 M IPTG stock solution

0.12 g of IPTG was dissolved in 5.0 ml of deionized water, solution filter-sterilized and stored as aliquots at -20°C.

g) X-Gal stock solution

Stock solution was prepared by dissolving 100 mg of X-Gal in 2.0 ml of N, N'-dimethylformamide (DMF). The solution was stored at -20°C in a micro centrifuge tube wrapped with aluminium foil.

h) 50X TAE

Components per litre of solution : Tris base 242.0g, Glacial acetic acid 57.1 ml & 0.5 M EDTA (pH 8.0). Solution was sterilized by autoclaving.

i) DNA loading dye (6X)

Xylene cyanol blue 0.25 %, Bromophenol blue 0.25%, Glycerol 30.0% were prepared in deionised water and stored at 4°C.

j) Ethidium bromide

Ethidium bromide (10 mg/ml in distilled water) was stored at 4°C and used at a working concentration of 0.5µg/ml.

2.4.1 Methodology for PCR amplification

Various combinations of primers were used to get the required amplicons of the gene. The PCR reaction was carried out by combining the following reaction components in a 25µl reaction volume:

Components concentration	Volume (µl)	Final concentration
Nuclease-free water	18.7.5	
10 X Reaction Buffer	2.5	1 X
dNTP mix (10 mM)	0.5	0.2 mM
XT-Taq system (3U/ µl)	0.3	0.03U/µl
Primer (Forward)	1.0	0.2 µM
Primer (Reverse)	1.0	0.2 µM
Template (~100 ng)	1.0	

10X reaction buffer of XT-Taq system (Bangalore genie, India) contained 15 mM $MgCl_2$ and 0.1% gelatin. The contents of the tube were mixed by a brief spin in a micro centrifuge. The reaction was carried out in a thermocycler GeneAmp PCR System 9700 (Perkin-Elmer, USA) and the reaction parameters were as follows:

a. Initial Denaturation : 94°C for 4 min
b. Denaturation : 94°C for 60 sec
c. Annealing : X °C for X min
d. Extension : 72° C for X min
(b–d) 30 cycles
e. Final extension : 72°C for 8 min

2.4.2 Analysis of PCR product by agarose gel electrophoresis

A 10 µl aliquot of the PCR product was analyzed by agarose gel (1.2%) electrophoresis as described below. The size of the amplicons was checked by comparing with a 3kb or 100bp DNA Marker (MBI Fermentas, Lithuania and Bangalore genei, India).

Materials:

- Agarose
- 50X TAE buffer
- DNA loading dye
- Standard DNA size marker
- Gel running boat
- Electrophoresis unit
- Ethidium bromide stock solution

Procedure:

The boat was sealed with adhesive tape and comb was placed for wells.

1. Agarose was used at 0.8 % to 1.2% level and melted in 1X TAE.
2. Agarose was allowed to cool to about 50°C and then poured into the sealed boat.
3. Then gel was allowed to set (30 min), the comb and adhesive tape were removed and the gel was placed in the electrophoresis tank.
4. 1X TAE buffer was added to the electrode chamber to cover the gel to a depth of about 1 mm.
5. The samples were loaded by mixing a 5-10 µl aliquot of the total reaction with 2 µl of loading dye.
6. Samples were run at 120 volts until the dye reached 3/4th length of the gel.
7. The gel was removed from the tank and placed in ethidium bromide solution for 10 min.
8. The gel was destained in distilled water and was examined on a UV trans-illuminator

2.4.3 Purification of PCR product using SIGMA gene elute PCR clean up kit:

Procedure:

- A Gene Elute Mini prep Binding column was inserted into collection tube provided with the kit.

- 5 volumes of binding solution was added to 1 volume of the PCR reaction and gently mixed.
- The solution was then transferred into the binding column.
- Column was centrifuged at maximum speed for 1 min.
- Flow- through liquid was discarded and the collection tube was retained.
- The binding column was placed into the collection tube. 0.5 ml of diluted wash solution was applied to the column and centrifuged at maximum speed at 11,000 rpm for 1 min.
- Flow- through liquid was discarded again and collection tube was retained.
- Column was placed in the collection tube and centrifuged at maximum speed at 11,000 rpm for 2 min, without any additional wash Solution, to remove excess ethanol. Any residual flow- through as well as the collection tube were discarded.
- The column was transferred to a fresh 2 ml collection tube. 50 µl of Elution Solution or deionised water was applied to the center of each column, incubated for 1 minute at room temperature and centrifuged to collect purified PCR product.

2.4.4 Colony Hybridization

2.4.4.1Transfer of colonies onto nylon membrane

1. The nylon membrane and the petri plates containing colonies developed on agar media were marked at three asymmetric positions.
2. The membrane was placed over the colonies and was removed as soon as it moisted evenly.
3. The plates were incubated overnight again at 37°C for the colonies to grow again.
4. The plates were sealed and stored at 4°C.

2.4.4.2 Lysis of colonies and binding of DNA to nylon membrane

1. The nylon membrane was wetted with 300 µl lysozyme solution and incubated at 37°C for 1 h with colonies on the upper surface.
2. Membrane wetted sequentially with 300µl of 10% SDS, denaturation solution, and neutralization solution and incubated for 10 min each respectively.
3. The membrane was air dried and placed in a polythene bag and the membrane surface was exposed to UV radiation bearing the bacterial colonies for 2 min to fix single stranded DNA on the membrane.

4. The membrane was then stored at 4°C prior to hybridization.

Solutions used:

1. Lysozyme 10 mg/ml in 25mM Tris-Cl (pH 8.0).
2. 10%SDS
3. Denaturation solution 0.5M NaOH
4. Neutralization solution 1.5M NaCl/0.5M Tris-HCl, pH 7.0

2.4.4.3 Preparation of probe using Psoralen-Biotin Nonisotopic Labeling Kit (Ambion, USA).

Protocol for labeling:

- A clean untreated 96-well plate was placed on ice bath.
- The sample was denatured by heating at 100°C for 10 min, and then the mixture was rapidly cooled in a dry ice/alcohol bath thereby freezing the sample.
- The vial containing the lyophilized Psoralen-Biotin was centrifuged for 15 seconds at 7000 *x* g. The Psoralen-Biotin was reconstituted in 33µl DMF. Pipetted up and down a few times to get the Psoralen-Biotin into solution. The solution was then stored at 4°C well protected from light.
- The frozen sample was thawed by rolling it between gloved hands and immediately 1µl of the Psoralen-Biotin was added to 10 µl of the nucleic acid solution in a microfuge tube. The sample was mixed and transferred to a well in a 96 well plate. Alternatively, Psoralen-Biotin was added to nucleic acid contained already in the 96 well plate and mixed well with the help of a pipette.

  (Note: The nucleic acid solution should have a final concentration of 0.5 - 50 ng/µl, pH of the nucleic acid solution should be between 2.5 and 10, and the salt concentration should be less than 20mM).
- A 365 nm UV light source was placed directly over the plate with the sample and irradiated for 45 min.
- The sample was diluted to 100 µl by adding 89 µl of TE Buffer and the mixture was transferred to a clean microfuge tube.

- 200 µl of water-saturated n-Butanol (shaken well before use) was added to the tube, vortexed well and centrifuged for 1 minute at 7000 *x* g. The upper n-Butanol layer was aspirated and the procedure repeated twice.
- The biotin-labeled nucleic acid was stored at -20°C for short-term storage (up to a few weeks) or at -80°C for long term storage (months to a year).

*Reagents (provided with the Kit)***:**

1. Psoralen-Biotoin
2. Dimethylformamide (DMF)
3. Water- saturated n-Butanol
4. TE buffer

2.4.4.4 Hybridization and processing of nylon membrane

Prehybridization

1. Membrane was placed in a polythene bag and 15 ml prewarmed (68°C)* hybridization buffer was added.
2. The bag was sealed and incubated overnight at 68°C with mild agitation.

Hybridization:

1. 5µl of probe was denatured in boiling water for 5 min followed by snap cooling on ice.
2. Hybridization buffer was discarded from the bag.
3. 2 ml of prewarmed hybridization buffer (68°C) having 2µl of denatured probe was added to membrane and the membrane was placed in a polythene bag and sealed.
4. The membrane was incubated at 68°C with mild agitation for 6 h.

Detection procedure:

1. Membrane was washed for 2 X 5 min at room temperature in approximately 1 ml Ambion wash buffer/cm^2.
2. Membrane was then incubated twice for 5 min in approximately 0.5ml blocking solution / cm^2 membrane.
3. Membrane was then incubated for 30 min in blocking buffer (approximately 1ml blocking buffer/ cm^2 membrane).
4. Diluted Strep-alkaline phosphates (Strep-AP) was prepared by gently and thoroughly mixing together 10ml blocking buffer and 1ul Strep-AP for 100cm^2-size membrane.

5. Membrane was incubated for 10 –15 min in blocking buffer. (0.5ml Blocking buffer / cm^2 membrane)
6. Membrane was washed trice for 5 min (or up to 15 min) in 1ml wash buffer/ cm^2 membrane /incubation.
7. 2 X 2 min incubations was given with 0.5 ml 1X assay buffer/ cm^2 membrane /incubation.
8. 10 ml freshly prepared colour solution (45 µl NBT and 35 µl X-phosphate in 10ml detection buffer) was added to membrane.
9. for the development of colour, the membrane was kept in the dark without shaking overnight.
10. Reaction was stopped by washing the membrane for 5 min with 50 ml of deionized water.

Solutions and Reagents:

20XSSC: 3M NaCl , 0.3M sodium citrate pH 7.2.

Hybridization buffer: 5X SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS

* Blocking reagent

Post-hybridization washing buffer I : 2XSSC, 0.1% SDS

Post-hybridization washing buffer II : 0.1% SSC, 0.1% SDS

* Alternatively Ambion wash buffer can be used

* Streptavidin Alkaline Phosphatase

Detection buffer : 0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5

Color-substrate solution: Prepared freshly by adding 45 µl NBT-solution and 35 µl X-phosphate solution in 10 ml of detection buffer.

* Supplied with the kit

2.4.5 Cloning of PCR product

The purified PCR product was cloned into a T tailed vector viz. pTZ57R/T after A tailing using MBI Fermentas Inst T/A cloning kit.

2.4.5.1 'A' tailing of PCR product

A-tailing of the purified amplicon was carried out by the method described by Kobs, (1997). To 5 µl of purified PCR fragment, 1 µl of *Taq* DNA Polymerase reaction buffer (1X) and 1 µl of 25 mM $MgCl_2$ were added. dATP to final concentration of 0.2

mM and 5 Units (2 µl) of *Taq* DNA polymerase (Bangalore Genie, India) were added to the reaction. The reaction mixture was incubated at 70°C for 20 to 30 min. To remove the residual dATP present in the reaction mixture, the PCR product was purified using Gene Elute PCR Clean-Up Kit (Sigma, USA).

2.4.5.2 Ligation of PCR product into T- tailed vector

Solutions

10 x Ligation Buffer[*]

400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5.0 mM ATP (pH 7.8)

PEG 4000[*]

10 x (50% w/v) PEG 4000 solution

T4 DNA Ligase, 5U/µl[*]

Prepared in 20 mM Tris-HCl (pH 7.5), 1 mm DTT, 50 mM KCl, 0.1 mM EDTA and 50% glycerol.

*Supplied with the kit

Methodology

The following components were added in a thin-walled 0.2 ml PCR reaction tube

'T' tailed vector pTZ57R/T	1µl
Purified 'A' tailed PCR product	15 µl
T_4 DNA ligase	0.5 µl
Ligase buffer	3 µl
PEG	1 µl
Sterile water	9.5 µl

The reaction mixtures were mixed properly in the PCR tubes and were incubated at 22°C for over night. The ligated products were used for transformation. Ligation was confirmed by agarose gel electrophoresis of the isolated plasmid from the transformed colonies.

2.4.6 Transformation of *E. coli* using ligation reaction mix (Sambrook and Russell, 2001)

2.4.6.1 Competent cell preparation using $CaCl_2$

1. A single colony of *E. coli* DH5α was picked from an agar plate and was grown overnight at 37°C.

2. The primary inoculum was then transferred into 50 ml of LB broth contained in a 500 ml conical flask and the culture was incubated at 150 rpm and 37°C untill OD_{600} reached 0.4-0.5.
3. Then cells were transferred aseptically to sterile polypropylene tubes. Cultures were cooled by storing the tube on ice for 10 min.
4. Cells were recovered by centrifugation at 4000 rpm for 10 min at 4°C.
5. Medium was decanted from the cell pellet and kept in an inverted position for 1 minute to allow the last traces of medium to drain away.
6. Each pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice.
7. Again, the cells were recovered by centrifugation at 4000 rpm for 10 min at 4°C, decanted and kept in an inverted position for 1 minute for draining of liquid.
8. Then cell pellets were resuspended in 2.0 ml of ice-cold 0.1 M $CaCl_2$.
9. The cells were stored at 4°C overnight.

2.4.6.2 Transformation of competent cells

1. 200 µl suspension of competent cells was transferred to a sterile micro-centrifuge tube.
2. DNA (~50 ηg) was added to each tube. The contents of the tube were mixed by swirling gently and stored on ice for 30 min.
3. The tubes were transferred to water bath set at 42°C and incubated for 90 seconds.
4. The tubes were rapidly transferred to ice and the cells were allowed to chill for 1-2 min.
5. 800 µl of LB medium was added to each tube and the cultures were incubated for 45 min at 37°C and 150 rpm.

Selection of transformants/recombinants

100µl of transformation mix was plated onto LB agar plates containing 100 µg ml^{-1} ampicillin. The plates were incubated at 37°C overnight for the development of colonies.

Analysis of transformants/recombinants

The transformants were checked

1) For the presence of recombinant plasmid by using agarose gel electrophoresis
2) For insert release from recombinants on restriction digestion
3) Through PCR

2.4.7 Rapid plasmid isolation from *E. coli* (Birnboim and Doly, 1979)

1. Single colony of *E. coli* DH5α was inoculated in 2 ml of LB broth containing 2 µl Ampicillin (stock solution 100 µg/µl) and grown overnight in a shaker incubator at 37°C and 180 rpm.
2. Overnight grown culture was transferred to a 1.5 ml micro centrifuge tube, cells were harvested by centrifugation at 10,000 rpm for 30sec, and supernatant was discarded.
3. 100 µl of solution I was added and vigorously vortexed until no visible clumps of cells are observed, then samples were kept on ice for 5 min.
4. 200 µl of freshly prepared alkaline solution (solution II) was then added and mixed gently by inverting the tubes several times (no vortexing). Then samples were kept on ice for 5 min to denature the plasmid and chromosomal DNA, and proteins.
5. 300 µl of ice-cold solution III was then added and tubes were inverted gently.
6. Tubes were centrifuged at 10,000 rpm for 12 min and supernatant was transferred to a fresh tube.
7. 800 µl of Isopropyl alcohol was added and kept at room temperature for 10 min. This precipitated the plasmids.
8. Suspension was centrifuged at 10,000 rpm for 12 min and supernatant was discarded carefully without dislodging the pellet.
9. 150µl of 70% ethanol was then added and centrifuged to remove salts.
10. The pellets were air dried and dissolved in 20 µl of TE buffer.
11. Each sample was tested by agarose gel (0.8%) electrophoresis.

Solution I: 50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)

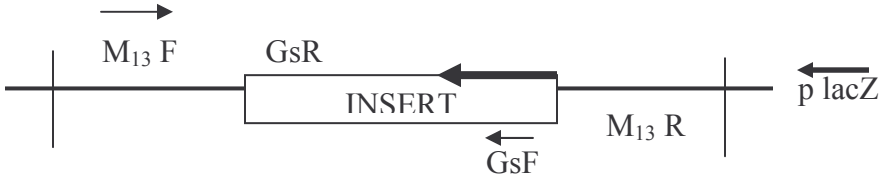
Solution II: 0.2 N NaOH (freshly prepared from 10 N NaOH), 1.0% SDS

Solution III : 5.0 M Potassium acetate, 60.0 ml; glacial acetic acid, 11.5 ml; Distilled water 28.5 ml.

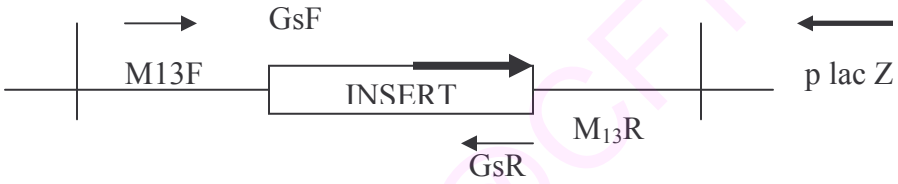
2.4.8 Checking the direction of insert

The direction of the insert with respect to *lacZ* promoter of the T tailed vector was checked by a PCR, using M13 sequence primers. M_{13} is a sequence present in the T tailed

vector. Amplification in PCR with $M_{13}F$ and GsF (gene specific forward) primers will occur only if the insert is in "sense" direction and would not occur if the inserts were in the opposite direction. Similarly, PCR with $M_{13}F$ and GsR (gene specific reverse) primers will occur only if the insert is in "antisense" direction. This is figuratively described below (Figure 2.1):



A) Insert in sense direction to lac Z promoter



B) Insert in antisense direction to *lacZ* promoter

Figure 2.1 Schematic representation of determination of direction of the insert with respect to *lacZ* promoter in pTZ57R vector

2.4.8 Restriction digestion of plasmid DNA

2.4.8.1 Single digest

1. A 20 µl reaction mixture was prepared by adding the reagents in the following order:

Plasmid DNA	5 µl (~1 µg)
10X Restriction enzyme buffer	2 µl
Sterile distilled water	12 µl
Restriction enzyme	1 µl (10 U)

2. The contents were mixed gently with a pipette tip and spun briefly.

3. The content was incubated at 37°C for desired length of time (~6h).

4. The reaction was stopped by heating at 65°C for 20 min and analyzed by agarose gel (0.8%) electrophoresis.

2.4.8.2 Double digest

Double digestion was performed after consulting the restriction enzyme buffer compatibility chart and chosen the best one, by comparing the enzyme activities. The units of enzyme to be added were depended on the activity of enzyme in selected buffer. Most double digestions were performed with the use of universal buffers (specially designed for double digestions).

2.4.9 Transformation of *Pseudomonas aeruginosa* (Chakrabarty *et al.,* 1975)

2.4.9.1 Preparation of Pseudomonas aeruginosa competent cell

- Single colony of *P. aeruginosa* was inoculated into 2ml LB broth and were grown overnight
- Culture was transferred into 50 ml of LB broth contained in a 250 ml conical flask and was incubated at 150 rpm and 37°C till OD_{600} reached 0.40-0.50
- Culture was cooled by storing the tube on ice for 10 min.
- Cells were recovered by centrifugation at 4000 rpm for 10 min at 4°C.
- Sedimented cells were resuspended in 10 ml of ice-cold 10 mM NaCl and stored on ice.
- Cells were recovered by centrifugation at 4000 rpm for 10 min at 4°C.
- The pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice for 20 min and again cells were recovered
- Pellets were resuspended in 2.0 ml of ice-cold 0.1 M $CaCl_2$ and stored at 4°C overnight.

2.4.9.2 Transformation of Pseudomonas aeruginosa

- 200 µl suspension of competent cells was transferred to a sterile micro-centrifuge tube.
- Then DNA (~50 ηg) was added to each tube. The contents of the tube were mixed by swirling gently and stored on ice for 60 min.
- The cells-DNA mixture was subjected to a heat pulse at 40°C for 2 min in water bath.
- The tubes were rapidly transferred to ice and the cells were allowed to chill for 1-2 min.
- 800 µl of LB medium was added to each tube and the cultures were incubated for 45 min at 37°C in a shaker incubator set at 150 rpm.

2.4.10 Isolation of genomic DNA from *Pseudomonas* (Syn and Swarup, 1999)

Materials

1. LB Medium, 2. Lysis Buffer (50mM Tris, 50mM EDTA, 3% SDS,1% mercaptoethanol, pH 8), 3. TE Buffer, 4. Tris Phenol, 5. Chloroform, 6. 3M Sodium Acetate, 7. Cold ethanol, 8. 70% ethanol

Procedure

- Single colony of *Pseudomonas* was inoculated in to 2 ml of LB broth and incubated at room temperature for overnight in a shaker.
- The culture was transferred to vials and centrifuged at 10000 rpm for 5 min at room temperature.
- Pellet was collected and washed with sterile distilled water.
- The pellet was resuspended in 500µl of prewarmed lysis buffer and vortexed.
- Suspension was incubated at 65°C for an hour.
- Cell lysate was extracted with equal volume of phenol and chloroform with intermittent vortexing and centrifuged at 10000 rpm for 5 min each.
- The upper phase was collected followed by addition of 50µl of 3M sodium acetate.
- DNA was precipitated using 1 ml cold ethanol.
- DNA was washed with 70% ethanol and pellet was air-dried.
- The pellet was suspended in 20µl TE buffer.
- Quality of the DNA was checked by agarose gel electrophoresis.

2.4.11 Transformation of *Bacillus* by electroporation

Materials:

Brain Heart Infusion (BHI):

BHI (37gm, HiMedia) was suspended in 1000 ml of water and autoclaved.

EP Buffer:

Dipotassium hydrogen phosphate (0.1M)	: 810µl
Potassium dihydrogen phosphate (0.1 M)	: 190µl
Magnesium chloride (1 M)	: 100µl
Sucrose (1 M)	: 54.4 ml

Final volume was brought to 200 ml with Milli Q water. pH was adjusted to 7.4. The buffer was filter sterilized and stored at 4ºC.

2.4.11.1 Preparation of electro competent cells

- 5 ml of overnight grown culture was inoculated in to 50 ml BHI broth and incubated till OD_{600} reached 0.3 at 30ºC under agitation.
- After chilling on ice for 10 min, the culture was transferred the into 50 ml sterile tubes, centrifuged at 10,000 rpm at 4ºC for 10 min and the supernatant was discarded.
- Pellet was resuspended in 25 ml of the EP buffer and centrfuged at 10,000 rpm.
- The supernatant was discarded and the pellet was again resuspended in 15ml of ice cold EP buffer and centrifuged at 4ºC for 10 minute at 10,000 rpm .
- The supernatant was discarded and resuspended in 0.5 ml of ice cold EP buffer.
- The cells was placed on ice and used for electroporation.

2.4.11.2 Electroporation

- To 3µl each of plasmid DNAs taken in a cool and sterile 1.5 ml microcentrifuge tubes, 100µl of electro competent cells were added, mixed and incubated on ice for 5-10 min.
- From this cell suspension, 100µl was transferred into chilled 0.2 cm electroporation cuvette.
- Cuvette was kept in the shock pod and pulse was applied. [Parameters used were:- Volt: 2.5 KV; Resistance: 200 Ohms; Capacitance: 20µf].
- Immediately a portion of L broth was added to the cuvettes and cells transferred into 1ml LB broth taken in a sterile 1.5ml micro centrifuge.
- These tubes were then incubated for 2h at 37ºC under agitation.
- Cells were then plated on LB agar plates with suitable antibiotic (Kanamycin 5mg/ml) and incubated overnight at 37ºC.

2.4.12 Plasmid isolation from *Bacillus* (Voskuil and Chambliss, 1993)

Materials:

1) SET Buffer: 25% Sucrose, 50 mM EDTA, 50mM Tris HCl.

2) Lysozyme: 10mg/ml (prepared in SET buffer).

3) Solution II: 0.2 N NaOH (freshly prepared from 10 N NaOH), 1.0 % SDS.

4) Solution III: 5.0M Potassium acetate, 60.0 ml, Glacial acetic acid 11.5ml, Distilled water 28.5ml.

5) Phenol: Chloroform: Iso amyl alcohol; In the ratio of 25:24:1.

6) Chloroform: Iso amyl alcohol; In the ratio of 24 : 1.

7) Cold isopropanol.

8) 70% ethanol.

9) TE buffer (pH 8.0).

Procedure:

- Colonies were picked up from the transformed plates and inoculated in to 2ml of LB broth containing Kanamycin.
- The tubes were incubated overnight at 37ºC under agitation.
- Cells were pelleted by centrifugation at 6000 rpm for 6 min.
- To the pellets, 200 µl of SET buffer was added and vortexed thoroughly.
- 3 µl of lysozyme was added (prepared in SET) and incubated for 30 min at 37ºC.
- 350 µl of solution II was added and inverted repeatedly to get a clear solution.
- 350µl of solution III was added and vortexed.
- Sample was centrifuged at high speed (12,000 rpm).
- 750µl of the supernatant was transferred to fresh Eppendorf tubes.
- 650µl of cold phenyl chloroform isoamyl alcohol was added and vortexed for 1 minute.
- Sample was centrifuged for 5 min at high speed.
- 620µl of aqueous phase was transferred to fresh Eppendorf tube.
- 620µl of cold chloroform isoamyl alcohol was added and vortexed for 30 seconds.
- The samples were centrifuged again at high speed (12,000 rpm) for 3 min.
- 550 µl of aqueous phase was transferred in to fresh Eppendorf tube.
- Equal volume of ice cold isopropanol was added for precipitation and the Eppendorf tube was inverted several times.
- Samples were centrifuged at high speed (12,000 rpm) for 5 min.
- The supernatant was discarded and the pellets were washed with 70% ethanol.
- The pellet was dried and TE buffer was added prior to use.

2.4.13 Sodiym Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970)

Chemicals and solutions

A. Separating gel buffer

Sodium dodecyl sulphate: 1 g and Tris: 45.4 g were dissolved in 500 ml double distilled water, pH was adjusted to 8.9.

B. Stacking gel buffer

Sodium dodecyl sulphate: 0.40 g and Tri: 6.06 g were dissolved in 190ml of double distilled water. pH was adjusted to 6.8 with 1N HCl, the volume was made up to 200 ml.

C. Tank Buffer

Glycine: 8.64 g, Tris: 1.8g and Sodium dodecyl sulphate: 0.6 g were dissolved in 600 ml double distilled water. pH was adjusted to 8.3 with 1N HCl

D. Sample Buffer (5X):

60mm tris HCl pH 6.8, 25% Glycerol, 2% sodium dodecyl sulphate, 14.4mm 2-Mercaptoethanol, 0.1% Bromophenol blue, was made up to 10 ml

E. Stock acrylamide for separating gel:

Acrylamide : 30.0 g and bis -acrylamide: 0.4 g were dissolved in 50 ml of double distilled water and the volume was made up to 100 ml. The solution was filtered through whatman No.1 filter paper.

F. Stock acrylamide for stacking gel:

Acrylamide :15 g and bis-acrylamide: 0.4 g were dissolved in 30 ml of distilled water and the volume was made up to 50 ml. The solution was filtered through Whatman No.1 filter paper and was stored at 4°C in a dark brown bottle.

G. Ammonium persulfate (APS):

100 mg of Ammonium persulfate was dissolved in 1ml of distilled water.

Preparation of separating gel (30 ml)

A cocktail was prepared with the following solutions:

Solution A: 15ml, E; 12 ml, G; 50 µl, TEMED; 50 µl. The solution was poured between the two clean glass plates and layered with 5ml of n-butanol, and was allowed to polymerise for 30 min. After polymerization n-butanol was removed and the gel surface was rinsed with water. The comb was inserted carefully into gel sandwich until bottom of teeth reached top of front plate.

Preparation of stacking gel (10 ml)

Following solutions were mixed together: solution B: 1.25ml, F: 0.75ml, G: 50µl, TEMED: 50µl, distilled water 6ml. The solution was poured over the separating gel and allowed to polymerize for 30 min.

Sample preparation

To the sample (200µl of the *E. coli* total cell extract or any other sample), sample buffer was added (to get 1X sample buffer in a mixture), vortexed thoroughly and boiled for 3 min, cooled and centrifuged. 30 µl of sample was loaded (depending on the protein concentration) into each well.

Electrophoresis conditions

The gel was run at 30mA constant current until the tracking Bromophenol blue dye reached the end of the gel (about 3 h).

Staining and destaining of the gel

The gels were stained in 0.05% (w/v) Coomassie Brilliant Blue R-250 in acetic acid/methanol/water (10:25:65%v/v), for ½ h to 16 h and destained repeatedly in the same solution without dye.

3.0 ABSTRACT

Different *Pseudomonas* specieses were isolated from various soil samples on *Pseudomonas* isolation agar. Presumptive *Pseudomonas* colonies were screened for the presence of *phaC1* gene, by PCR, using primers designed based on the multiple sequence alignment of sequences available in the data bank. A 700bp fragment of the *phaC1* amplified through PCR of DNA from *Pseudomonas aeruginosa* was used as a probe in colony hybridization for the rapid screening of PHA producing *Pseudomonas* spp. Colonies that reacted positively to PCR and to the probe were randomly selected for a study of their ability to produce PHA from various carbon substrates. *Pseudomonas* spp. grown on various fatty acids, glucose or in both substrates, were found to produce 18-33% PHA. The PHA was characterized using FTIR, GC and GCMS. The composition of the PHA depended on the substrate used for cultivation of the host bacteria. Hydroxy octanoate and hydroxy decanoate were the major monomers found in the PHA produced by different isolates on most of the substrates used. Minor variation in PHA composition was observed among different isolates.

Six isolates producing the highest amount of PHA were selected for further studies. The amount of PHA and its composition was estimated at different intervals of time. There was a noticeable increase in concentration of PHA at the 48th h rising up to the 72nd h and a decrease in some strains at the 96th h. Addition of acrylic acid, an inhibitor of β-oxidation resulted in increased production of PHA when cells were grown in the presence of fatty acids. Restriction length polymorphism for the 700bp amplicon of *phaC1* was not found among the different isolates. Efforts at cloning the *PhaC1* or *PhaC2* from soil isolates were however unsuccessful.

A 1680bp (Figure. 3.1) PCR amplicon of the *PhaC1* was obtained from genomic DNA of *P. aeruginosa*, cloned initially into the T-tailed vector pTZ57R/T and authenticated by sequencing. *phaC1* was sub-cloned into the broad host range vector pBSPIIKS- for functional expression in *E. coli* strains BL21, LS1298Kan:*fadB1* and *P. aeruginosa* under control of the *lac* promoter. *PhaC1* was also sub cloned into the *Bacillus* expression vector pCE20 for expression in *Bacillus subtilis*. The content and composition of the PHA in *E. coli* varied depending on the substrate used. 2-6% increase in the content of PHA and significant change in the molar fraction of the different

constituent monomers was observed, in comparison to that of the control, when *phaC1* was over expressed in *P. aeruginosa*. An increase in the hydroxy decanoate content of the PHA in these recombinants indicated that the PhaC1 enzyme is more specific towards the polymerization of hydroxy decanoyl CoA. Expression of *phaC1* in *B. subtilis* resulted in the accumulation of PHA (2-8%CDW) containing PHB and mcl-monomers which revealed that PhaC1 of *P. aeruginosa* can polymerize a greater variety of monomers on expression in heterologous host thanin *in vivo* expression.

3.1 INTRODUCTION

From the discovery of PHA to 1974, the work on PHA was concentrated on hydroxy butyrate. Wallen and Rohwedder in 1974 reported the presence of 3-hydroxyvalerate and 3-hydroxy hexanoate in activated sludge. Capillary chromatography of marine sediments by Findlay *et al.*, (1983) revealed the presence of 3-hydroxyvalerate and 3-hydroxyhexanoate as the predominant components among 11 other hydroxyalkanoates units. Batch-grown *B. megaterium* cells accumulated a polymer consisting of 95% 3-hydroxybutyrate, 3% 3-hydroxyheptanoate, 2% of an 8-carbon HA and trace amounts of three other HA compounds (Findlay *et al*., 1983). *P. oleovorans* cultivated on n-octane accumulated PHA that consisted principally of 3-hydroxyoctanoate units (De Smet *et al*., 1983) and small amounts of 3-hydroxyhexanoate units (Lageveen *et al*., 1988). PHB homopolymers have thermoplastic properties but generally they are brittle and addition of mcl units could significantly enhance its useful properties (Doi *et al*., 1995). This finding laid the foundation for the identification and characterization of this bacterial polyester and for the isolation of potent bacterial species capable of producing them. Because of the many possible applications of scl-co-mcl-PHA, it is important to examine and define enzymes and metabolic pathways for the biosynthesis of scl-co-mcl-PHA.

There are a variety of methods for the screening of PHA producing bacteria. Various phenotypic detection methods based on dye binding such as Sudan black staining, Nile blue and Nile red staining combined with microscopy have been widely used. Nile red staining combined with spectrofluorometry allows rapid differentiation between mcl and scl-PHA accumulating bacteria (Wu *et al*., 2003). These methods are

highly sensitive but less specific because these reagents also react with other lipid inclusions. Fourier transform infrared spectroscopy has also been used to detect the PHA in intact cells (Misra *et al*., 2000). These two methods require the growth of bacteria under nutrient limited conditions for the formation of PHA. If such conditions are not met and PHA is not produced, then these methods will fail to identify the microorganism of its PHA–producing capability. Timm *et al*., (1994) described a southern blot hybridization method for identifying *phaC* gene belonging to different classes. Later a method for specific PCR based detection of Class II PHA synthase was described by Solaiman *et al.,* (2000), while a colony PCR protocol based on the *Ralstonia phaC1* gene was developed by Sheu *et al.,* (2000) for the detection of PHA synthesizing bacteria isolated from the environment. Screening of PHB accumulating *Bacillus* spp. by a PCR method has been reported using primers based on *B. megaterium* sequences (Shamala *et al*., 2003). We have here used a PCR and a colony hybridization technique for faster and specific identification of PHA producing *Pseudomonas* spp. directly from soil samples.

A variety of *Pseudomonas* spp. are known to produce PHAs composed of medium chain length (mcl) 3-hydroxy fatty acids with 6-14 carbon atoms from a wide range of substrates such as fatty acids, oils, sugars, alcohol and alkanes, using different metabolic pathways (Timm and Steinbuchel, 1990; Eggink *et al*., 1992; Huijberts *et al*., 1995). Three main pathways are involved in the synthesis of mcl- PHA precursors: firstly, chain elongation, in which acyl-CoA is extended with acetyl-CoA; secondly, fatty acid degradation by β-oxidation, the main pathway when fatty acids are used as substrate; and thirdly, de novo fatty acid biosynthesis, which is the main route during growth on simple carbon compounds. Two PHA synthase genes, namely *phaC1* and *phaC2,* separated by the *phaZ* gene, encoding a PHA depolymerase, are associated with the type II system which is found only in *Pseudomonas* strains (Timm and Steinbuchel, 1992; Rehm and Steinbuchel, 1999). These two synthases are the key enzyme involved in PHA biosynthesis. The PHA operon also contains genes coding for regulatory proteins such as PhaD, PhaF and PhaI (Figure 3.1). In *Pseudomonas* sp 61-3, along with the type II system, type I loci is also present (Matsusaki *et al*., 1998), which is responsible for the presence of a blend of PHB homopolymers and a random copolymer of [P (3HB-co-

3HA)] consisting of 3HA units of C4 to C12 from sugars and alkanoic acids (Abe *et al*., 1994; Kato *et al*., 1996).

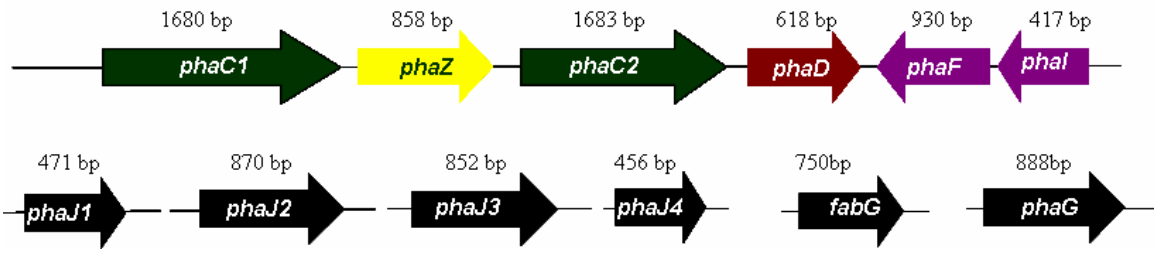


Figure 3.1 PHA biosynthetic operon of *P. aeruginosa* and other genes involved in the biosynthesis of PHA

(R)-Hydroxyacyl-Coenzyme A (CoAs), the substrate for PHA synthase may be derived from various precursors (Eggink *et al*., 1992). Enoyl-CoA, 3-ketoacyl-CoA, (S)-3-hydroxyacyl-CoA and 3-hydroxyacyl-ACP appears to be the major precursors of PHA. A list of enzymes inter-connecting the major metabolic pathways and PHA synthesis and their role is given in table 3.1

Table 3.1 Enzymes involved in PHA biosynthesis of *Pseudomonas* spp.

Enzyme	Function of the enzyme	Reference
Enoyl-CoA hydratase (PhaJ)	Catalyses the oxidation of enoyl-CoA to (R)-3-hydroxyacyl-CoA	Fukui *et al*., 1998
3-ketoacyl CoA reductase (FabG)	Converts 3-ketoacyl ACP into (R)-3-hydroxy acyl ACP	Ren *et al*., 2000
(R)-3-hydroxy acyl ACP CoA acyl transferase (PhaG)	Converts (R)-3-hydroxy acyl ACP into (R)-3-hydroxy acyl CoA	Rehm *et al*., 1998
Epimerase	Converts (S)-3-hydroxyacyl-CoA into (R)-3-hydroxyacyl-CoA	Hypothesized
PHA synthase (PhaC)	It is the key enzyme involved in polymerization.	Timm and Steinbuchel, 1992

The genes encoding these enzymes have been characterized and expressed in heterologous hosts (Fukui and Doi, 1997; Rehm *et al*., 1998; Tsuge *et al*., 2000; Hoffman

et al., 2000b; Fiedler *et al*., 2002; Tsuge *et al*., 2003). These genes are not co-localized with the polyester synthase gene, but are expected to be co-regulated. Recent research has focused on the efficient production of PHA with desirable material properties in recombinant bacteria and transgenic plants. Nine different strategies have been applied for the identification and cloning of the PHA synthase genes from bacteria (Table 3.2).

Table 3.2 Different strategies used for the identification and cloning of PHA synthase genes

Strategy	Methodology	Example (Bacterium)	Reference
A	Enzymatic analysis	*R. eutropha*	Slater *et al*., 1988
B	Hybridization using homologous gene probe	*P. putida* U	Garcia *et al*., 1999
C	Hybridization using heterologous gene probe	*Pseudomonas* sp. 61-3	Matsusaki *et al*., 1998
D	Consensus oligonucleotide derived from the multiple alignment (hybridization or PCR technique)	*P. aeruginosa*	Tim and Steinbuchel, 1992
E	Oligonucleotide derived from N-terminal or internal amino acid sequences of PHA synthase	*P. putida* BM01	Valentine *et al*., 1998
F	*In vivo* staining in PHA negative host after heterologous expression	*P. acidophila*	Umeda *et al*., 1998
G	Growth after detoxification of the media due to removal of fatty acids	*Rhodococcus capsulatus*	Kranz *et al.,* 1997
H	Genome sequence analysis and functional assignment of the PHA synthase gene.	*P. resinovorans*	Solaiman, 2000

At present the nucleotide sequences of a number of different PHA synthase genes from various bacteria have been obtained. The sequences of PHA synthase of more than 20 different *Pseudomonas* is available. Table 3.3 gives an account of *phaC1* genes cloned and assigned from *Pseudomonas* spp.

The sequences of *phaC1*, *phaC2* and *phaZ* of these species are highly homologous (Solaiman, 2000). A 70-99 % homology between the PHA synthases of

different *Pseudomonas* species can be observed. PHA synthases PhaC1 and PhaC2 of *Pseudomonas* spp., exhibit lower identities to PhbC of *R. eutropha*, which is highly specific towards scl-monomers. Zhang *et al.,* (2001) reported that the amino acid sequence of PhaC1 from different species were homologous to the extent of 79% while that of PhaC2 was homologous to the extent of 67% indicating that the sequence of PhaC1 is more conserved than that of PhaC2. In most known *Pseudomonas* spp., PhaC1 and PhaC2 share about 55-60% amino acid sequence identity (Qi *et al*., 1997). The differences between PhaC1 and PhaC2 sequences may be indicative of structural and functional differences. This may account for differences in content and composition of accumulated PHA seen when the two enzymes were separately expressed in *E. coli*. Even though there is 57% amino acid identity between PhaC1 and PhaC2 of *P. mendocina* and

Table 3.3 PHA synthases cloned from different *Pseudomonas* spp.

Bacterium	Accession number	% Identity to *phaC1* of PA01	References*
P. aeruginosa UCBPP-PA14	CP000438.1	98	Lee *et al*., 2006
P. aeruginosa PA7	CP000744.1	94	Dodson *et al*., 2007 Unpublished
Pseudomonas sp. HJ-2	AY370934.1	86	Seo *et al.,* 2003 unpublished
P. stutzeri 1317	AY278219.1	85	Chen *et al.,* 2004
P. pseudoalcaligenes HBQ06	AF336848.1	84	Zhang, *et al*., 2001
P. nitroreducens strain 0802	AF336849.2	83	Zhang, *et al*., 2001
P. resinovorans	AF129396.2	83	Solaiman, 2000
P. mendocina	DQ316602.1	83	Hein, 2002
P. oleovorans	M58445.1	82	Huisman *et al*., 1991
P. putida strain CA-3	AY714618.1	81	Lee *et al*., unpublished
P. putida U	AF150670	81	Garcia *et al*., 1999
P. putida BM01	AF042276	81	Valentine *et al.*, 1998
P. aureofaciens	AB049413.1	81	Umeda *et al*., 2001
P. fluorescens Pf-5	CP000076.1	81	Dennis, unpublished
P. mediterranea strain FBP5447	AY910768.1	81	Catara *et al.,* unpublished
P. corrugata	EF067339.1	80	Solaiman *et al.,* unpublished
Pseudomonas sp. KBOS 03	AY790327.1	80	Ciesielski *et al*., 2006
Pseudomonas sp. LDC-5	DQ444320.1	80	Shenbagarathai *et al.,* unpublished
Pseudomonas sp. 61-3	AB014758.1	79	Matsusaki *et al*., 1998

P.fluorescens PFO1	CP000094.1	79	Rhie, Unpublished
Pseudomonas sp. 3Y2	AY754343.1	79	Delamarre *et al.,* Unpublished.

** Details of unpublished work are not described in the Reference chapter*

P. stutzeri, expression studies have demonstrated that functions of these two enzymes cannot be interchangeable without affecting content and composition of the accumulated PHA (Hein *et al*., 2002; Chen *et al*., 2004). PhaC2 of *P. stutzeri* can polymerize scl-PHA and mcl-PHA both *in vivo* (homologous expression) and *in vitro* (heterologous expression) where as PhaC1 favored only mcl-3HA for polymerization (Chen *et al*., 2004). The two PHA synthases of *Pseudomonas* sp. strain 61-3 exhibit 53% identity to each other (Matsusaki *et al*., 1998) which is similar to the extent of homology between the two synthases of *P. oleovorans* (Huisman *et al*., 1991) and can polymerize scl and mcl-hydroxy acyl CoAs in homologous (Matsusaki *et al*., 1998) and heterologous systems (Park and Lee, 2004b; Nomura *et al*., 2004a). Both PHA synthases of most known PHA producing *Pseudomonas* such as *P. oleovorans*, *P. aeruginosa, P. putida*, *P. pseudoalcaligenes*, *P. nitroreducenes* or *P. resinovorans* show specificity towards mainly mcl-HA CoAs *in vivo* and *in vitro* (Huisman *et al*., 1991; Qi *et al*., 1997; Solaiman, 2000; Zhang *et al*., 2001). Polyhydroxybutyrate (PHB) synthesis using the *phaC1* or *phaC*2 of these *Pseudomonas* spp. in recombinant *E. coli* has not been reported so far.

There are also reports on the polymerization of scl-monomers by PHA synthases of these *Pseudomonas* in heterologous hosts. It has been reported that 17-26 mol% of the 3HB unit can be incorporated into the octanoate-derived polyesters accumulated by *R. eutropha* PHB-4 harboring the PHA synthase gene of *P. aeruginosa*, although *P. aeruginosa* produces mcl-PHA (Timm and Steinbuchel, 1992). PHA synthase 1 of *P. oleovorans* shows relatively high affinity towards hydroxyvaleryl CoA *in vitro*, despite the incorporation of only a small fraction of 3HV (<3 mol %) unit *in vivo* (Kraak *et al*., 1997). These experiments suggest that PHA synthesis *in vitro* might allow an even greater range of monomers to be incorporated than *in vivo* PHA accumulation. The substrate–supplying pathway for PHA synthase in host cell is important for control of the monomer composition of PHA.

E. coli has been extensively studied for recombinant production of PHA after Schubert *et al.,* (1988) reported its ability to accumulate large quantities of polymer on

introduction of *R. eutropha* PHA biosynthetic genes. *E. coli* has proven to be a suitable host for PHA production due to the advantages mentioned in 1.11.1. As explained previously, *Pseudomonas* spp. have a pool of enzymes for monomer provision, and are widely exploited to construct PHA biosynthetic pathways in recombinant *E. coli* using intermediates of fatty acid metabolism, for the production of PHA (Table 1.7). Apart from *E. coli*, researchers have developed other recombinant bacteria such as *Aeromonas, Ralstonia, Salmonella, Pseudomonas* and *Bacillus* for the production of PHA by modifying the inherent pathways or constructing hybrid pathways since these bacterial systems have many advantages. The expression of PhaC1 or PhaC2 of *Pseudomonas* sp. in *Bacillus* has not been reported yet. There is also no report on the over-expression of these two genes in homologous host. It is envisaged that over expression of PhaC1 in *P. aeruginosa* can shed light on the specificity of the enzyme in the homologous host. So we have cloned *phaC1* gene of *P. aeruginosa* for expression in *E. coli* of different genetic backgrounds, in *B. subtilis* and for over-expression in *P. aeruginosa.*

3.2 MATERIALS AND METHODS

3.2.1 Isolation of Pseudomonas spp

- Soil samples were collected from different locations in and around Mysore city.
- Test tubes containing 10 ml of *Pseudomonas* isolation broth were inoculated with 1g each of soil samples and cultured in shaker incubator for 12h at room temperature.
- Serial dilutions of the over night cultures were plated onto *Pseudomonas* isolation agar (2.1.5.5) and incubated for 24 hours at room temperature.
- The colony morphology was observed. Fluorescent greenish, bluish, yellowish or brownish colonies were inoculated to test tubes containing LB broth (2.1.5.3) and cultured in a shaker incubator for 2 hours at room temperature.
- Gram staining (2.2.6.1), motility, catalase (2.2.7) and oxidase (2.2.8) tests were carried out and the gram negative motile slender rods showing catalase and oxidase positive reactions were sub cultured and stored on LB agar plates.

3.2.2 Screening of PHA producing Pseudomonas spp.

3.2.2.1 *PCR*

PhaC1 sequences of 12 *Pseudomonas* spp. available in the data bank were aligned using Dialign 2 software programme (Figure 3.2) and primers were designed based on conserved sequences using primer 3 programme and the primer sequences are given in Table 3.4. PCR conditions used are given in Table 3.5 and PCR protocol has been described in 2.4.1.Genomic DNA isolated from different strains of *Pseudomonas* by the method described in 2.4.10 was used as template for PCR

```
gi|21689572     399  GCCGACCAAT ACCCTGTCCA ACCCGGCTGC GGTCAAACGC TTCTTCGAAA --
gi|151441       399  TCCGACCAAC ACCCTGTCCA ACCCGGCAGC AGTCAAACGC T TCTTCGAAA --
gi|17402509     399  GCCGACCAAC ACCGCCGCCA ACCCCGCGGC GGTCAAACGC TTCTTCGAGA ---
gi|13346167     399  CCCCTCCAAC AGCATGGCCA ACCCGGCGGC GGTCAAACGC TTCTTCGAGA ---
gi|45388        399  GCCGACCAAC AGCCTGAGCA ACCCGGCGGC GGTCAAGCGC TTCTTCGAGA---
gi|20086522     399  ACCCTCCAAC AGCATGGCCA ACCCGGCAGC GGTCAAACGC TTCTTCGAAA ---
gi|30721689     399  ACCAAGCAAC AGCATGGCCA ACCCGGCCGC GGTCAAACGC TTCTTCGAGA---
gi|19589601     399  GCCTTCCAAC AGCATGGCCA ATCCGGCGGC GGTCAAGCGC TTCTTCGAAA ----
gi|10835918     399  GCCCACCAAC AGCATGGCCA ACCCGGCCGC AGTCAAACGG TTCTTCGAGA----
gi|12659053       1  ---------- ---------- ---------- ---------- ----------
gi|3115089        1  ---------- ---------- ---------- ---------- ----------
gi4062966       401  CCCGACCAAC AGTGCGGCCA ATCCGGCGGC GGTCAAACGC TTCTTCGAAA ----

gi|21689572    1099  GCCTGGATGC GCCCCAACGA CCTGATCTGG AACTACTGGG TAAACAACTA ----
gi|151441      1099  GCCTGGATGC GCCCCAACGA CCTGATCTGG AACTACTGGG TCAACAACTA ----
gi|17402509    1099  GCCTGGATGC GGCCCAACGA CCTGATCTGG AACTACTGGG TGAACAACTA----
gi|13346167    1099  GCCTGGATGC GGCCGAACGA CCTGATCTGG AACTACTGGG TCAACAACTA----
gi|45388       1099  GCCTGGATGC GCCCCAACGA CCTGATCTGG AACTACTGGG TCAACAACTA----
gi|20086522    1099  GCCTGGATGC GCCCCAACGA CCTGATCTGG AACTACTGGG TCAACAACTA ----
gi|30721689    1099  GCCTGGATGC GCCCGAATGA CCTGATCTGG AACTACTGGG TCAACAACTA----
gi|19589601    1099  GCCTGGATGC GCCCCAATGA CCTGATCTGG AACTACTGGG TCAACAACTA----
gi|10835918    1099  GCCTGGATGC GCCCCAACGA CCTGATCTGG AACTACTGGG TCAACAACTA----
gi|12659053     431  GCCTGGATGC GCCCCAACGA CCTGATCTGG AACTACTGGG TCAACAACTA----
gi|3115089       14  ------- TGC GCCCCAACGA TCTGGTCTGG AACTACGTGG TGGGCAACTA----
gi4062966      1101  GCCTGGATGC GCCCTAACGA CCTGATCTGG AACTACTGGG TCAACAACTA----
```

Figure 3.2 Multiple alignments of the nucleotide sequences of the *PhaC1* gene of different *Pseudomonas* spp. using Dialign 2 (Morgenstern, 1999)

Table 3.4 Nucleotide sequences of the primers used for screening of PHA producing *Pseudomonas* spp.

Primer name	Primer sequence

Forward primer	RPC_1F_1	GTCAARCGCTTCTTCGARAC
Reverse primer	RPC_1R_1	GATSGGGCYKGTACTGGAT
Nested reverse primer	RPC_1R_2	CAGTAGTTCCAGATCAGGTC

Key to symbols R= A+G, S= G+C, K=G+T, Y=C+T

Table 3.5 PCR conditions used for screening of PHA producing *Pseudomonas* spp.

Primers	Initial denaturation	Denaturation	Annealing	Extension	Final extension
For RPC_1F_1 and RPC_1R_1	95°C 4 min	94°C 1 min	55°C 1 min	72°C 1 min	72°C 8 min
For RPC_1F_1 and RPC_1R_2	95°C 4 min	94°C 1 min	50°C 1 min	72°C 1 min	72°C 8 min

3.2.2.2 Colony hybridization

Labeling of the 700bp amplicon from *P. aeruginosa* and colony hybridization was carried out as described in 2.4.4. Serial dilutions of selectively enriched soil samples (pre incubated in *Pseudomonas* isolation broth) were made and plated on to *Pseudomonas* isolation agar (2.2.5).

3.2.3 PHA production by Pseudomonas isolates

Twenty randomly selected isolates were tested for their ability to accumulate PHA on provision of various carbon sources such as glucose and fatty acids as described in 2.2.4.1. PHA accumulation at different time intervals by the six selected isolates grown on various carbon sources was then monitored. Six of the tested isolates were selected for further investigation on the effect of acrylic acid on production of PHA as described in 2.2.4.1.

3.2.4 Analytical procedures

. PHA, extracted and quantified from dried cells by the method (2.3.2.1) of Williamson and Wilkinson (1958), is expressed as a percentage of the cellular dry weight

(% CDW). Extracted PHA was dissolved in chloroform and analyzed using FTIR (2.3.5). Gas Chromatography was carried out, by the method of Brandl *et al.,* (1988) described in 2.3.6. Identification of the PHA monomeric units was carried out using GC/MS.

3.2.5 Cloning and heterologous expression of PhaC1 genz

3.2.5.1 Bacterial strains and plasmids

The strains and plasmids used in the study are listed in Table 3.6. *E. coli* DH5α was used as host strain for general cloning. *Bacillus subtilis*, *E. coli* BL21 and *E.coli* LS1298Kan::*fadB1* were used as host strains for the synthesis of PHA. *PhaC1* gene was isolated from the *P. aeruginosa* 01. The LS1298Kan::*fadB1* was kindly provided by Eliza Mckinney of CLF Medical Technology Acceleration Program, Inc. New York, USA. The plasmid pBSPIIKS- and the strain *Pseudomonas aeruginosa* 01 were kindly gifted by Herbert P. Schweizer of Colorado State University,USA.

Table 3.6 Strains and plasmids used for cloning of *phaC1* gene in heterologous host

Strain or plasmid	Relevant characteristics
P. aeruginosa	*Prototroph*
E. coli DH5α	*supE*44 Δ*lac*U169(ǿ80- *lacZ*ΔM15)*hsdR*17 *recA*1 *endA*1 *gyrA*96thi-1 *relA*1
E. coli BL21	*hsdS gal*(λ*c* I*ts*857 *ind*1 Sam7 *nin*5 *lac*UV5-T7 gene1)
E. coli LS1298	LS1298Kan::*fadB1*
*B. subtilis*168	*trpC2*
pCE20	Apr *LacZ* Kanr Bleor *Spo* promoter
pTZ57R/T	Apr;T7 and T3 promoter; cloning vector
pBSPIIKS-	Apr; T7 and T3 promoter; broad host range cloning vector
pTPC1	pTZ57R derivative; *phaC1*Pa
pBPC1	pBSP II KS-derivative; *phaC1*Pa
pCPC1	pCE20 derivative; *phaC1*Pa

3.2.5.2 Culture conditions for E. coli

The *E. coli* strains DH5α, BL21 and LS1298Kan::*fadB1* were cultured at 37°C in LB medium (2.1.5.3). For the biosynthesis of PHA, recombinant *E. coli* strains BL21 and LS1298Kan::*fadB1* were cultured in 250 ml flasks containing 50 ml of LB medium with appropriate substrate and antibiotic (2.2.4.1).

3.2.5.3 Culture conditions for recombinant B subtilis and P.aeruginosa

Bacillus subtilis culture was revived initially in nutrient broth at 37 °C. Kanamycin (5 μg ml^{-1}) was added to the growth medium as a selective agent. Recombinant *B. subtilis* was grown in the mineral salt medium (2.1.5.2**)** for the biosynthesis of PHA with 1.5% glucose and/or 0.2% of fatty acid sodium salts. Cultivation was carried out in a rotary shaker at 200 rpm for 72 h at 37°C.

Recombinant *P. aeruginosa* was cultivated as described in 2.2.4.1

3.2.5.4 Molecular biology methods

DNA was isolated from *E. coli* by alkaline lysis method described in 2.4.7 and from *Pseudomonas spp* using the NucleoSpin extract kit (Macherey-Nagel). Oligonucleotide primers were designed for the cloning of PHA synthase 1 (*phaC1*) based on the sequence available in the data bank (Accession no ***AE004091***). The primers were so designed that the amplicon would carry a 5' stop codon (bold letters), consensus Shine-Dalgarno sequences (underlined) before the coding region and a 3' stop codon. The primers were synthesized by Sigma Aldrich, India and the sequences are given in table 3.7

Table 3.7 Primers used for the amplification of *phaC1* gene of *P. aeruginosa*

Name of the primer	Primer sequences
PAPC1F	5'**TAA**AGGAAACAGCTAATGAGTCAGAAAA CAATAA3'
PAPC1R	5'TCATCGTTCATGCACGTAGGT3'

F: forward, R: Reverse

PCR reactions for *phaC1* set of primers were cycled 35 times for 1 min at 94°C, 1 min at 40°C and 1.45 min at 72°C for denaturation, annealing and polymerization respectively. Authenticity of the PCR product was confirmed through nested PCR (using the primers RPC1F1 and RPCIR1 given in table 3.4.) and restriction digestion. The PCR amplicon was purified using gene elute kit (2.4.3) and cloned into the T-tailed vector pTZ57R/T (2.4.5). The resultant plasmid pTPC1 was used for transformation of *E. coli* DH5α (2.4.6).

3.2.5.4.1 *Checking the orientation of the PhaC1 insert*

The direction of the *PhaC1* insert with respect to l*acZ* promoter of the T- tailed vector was checked by restriction digestion of the recombinant plasmid using HincII enzyme. The position of HincII sites in the insert and the vector is shown in the figure 3.3. If the insert is in the antisense direction (Figure 3.3A) with respect to *lacZ* promoter the fragments expected to be released would be 570bp and 198bp in length. If the insert is in sense direction with the *lacZ* promoter (Figure 3.3B) the fragments expected to be released would be 980bp and 198bp in length.

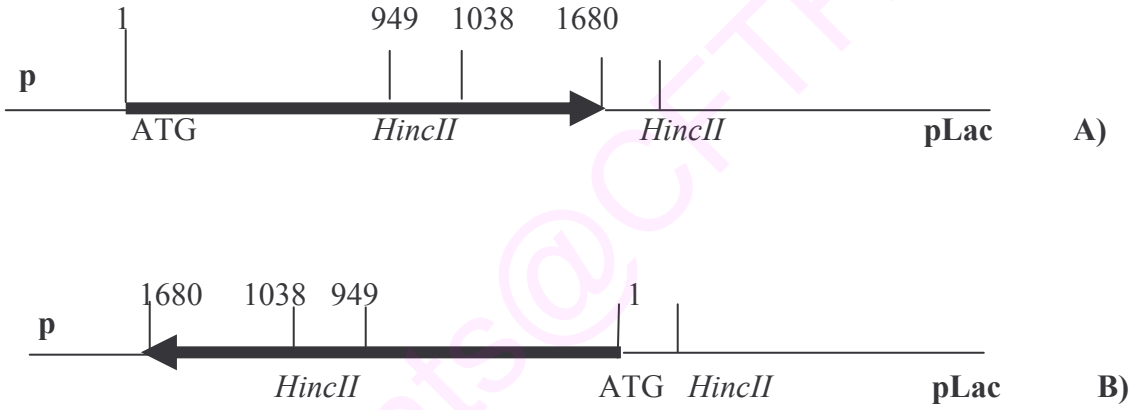


Figure 3.3 Schematic representation of the method used to determine the direction of insert with respect to *lacZ* promoter in pTZ57R vector

3.2.5.4.2 Subcloning of PhaC1 into pBSPIIKS- and pCE20

The insert released by SacI / B*am*HI from construct pTPC1 were gel eluted (using Quiagen Gel extraction kit) and ligated to B*am*HI/ S*ac*I restricted pBPS IIKS (-) and pCE20. Insert release and double digestion of the plasmids was set up as follows:

Reaction components:

PTPCI/pCE20/pBSPII KS (-)	16µl
Buffer	4µl
BamHI	1µl
SacI	1µl
Water	18µl

The components were mixed gently and incubated at 37ºC for 12 h. Agarose gel electrophoresis was carried out to check release of the insert. The insert was eluted from the gel and used for ligation. The ligation reaction was set up as follows:

Reaction components:

Double digested pCE20/ pBSPIIKS-	2µl
BamHI/ SacI insert	8µl
Ligase	1µl
Ligase buffer 10X	2µl
Water	7µl

The components were mixed and incubated at 22ºC for 12 hour. The ligated product was transformed into *E. coli* DH5 ά as described in 2.4.6. The resultant constructs pBPC1 and pCPC1 contained the *PhaC1* gene in sense orientation with respect to *LacZ* promoter (Figure 3.4) and *spo* promoter (Figure 3.4), respectively. Plasmid isolation was carried out from transformed *E. coli* and release of insert checked on agarose gels. The presence of the *phaC1* in pCE20 and pBPIIKS- was again confirmed by PCR using the RPC1F1 and RPC1R2 primers and through release of the inserted gene by restriction. pBPC1 plasmid was transformed into *E coli* strains BL21 and *fadB* mutant by the method described in 2.4.6 and *P. aeruginosa* by the method of Chakrabarty *et al*., (1975) as described in 2.4.9. *Bacillus subtilis* was transformed using pCE20 and pCPC1 by the method detailed in 2.4.11.

3.2.5.5 Expression of PHA synthase

The expression of PHA synthase in *E. coli* was monitored after induction with 0.4 mM IPTG. Crude extracts of recombinant *E. coli* were prepared by sonication (2.3.3). The supernatant was subjected to electrophoresis on a 12 % (w/v) SDS-PAGE (2.4.13). The amount of protein was determined (2.3.4) and equal quantities of protein were loaded.

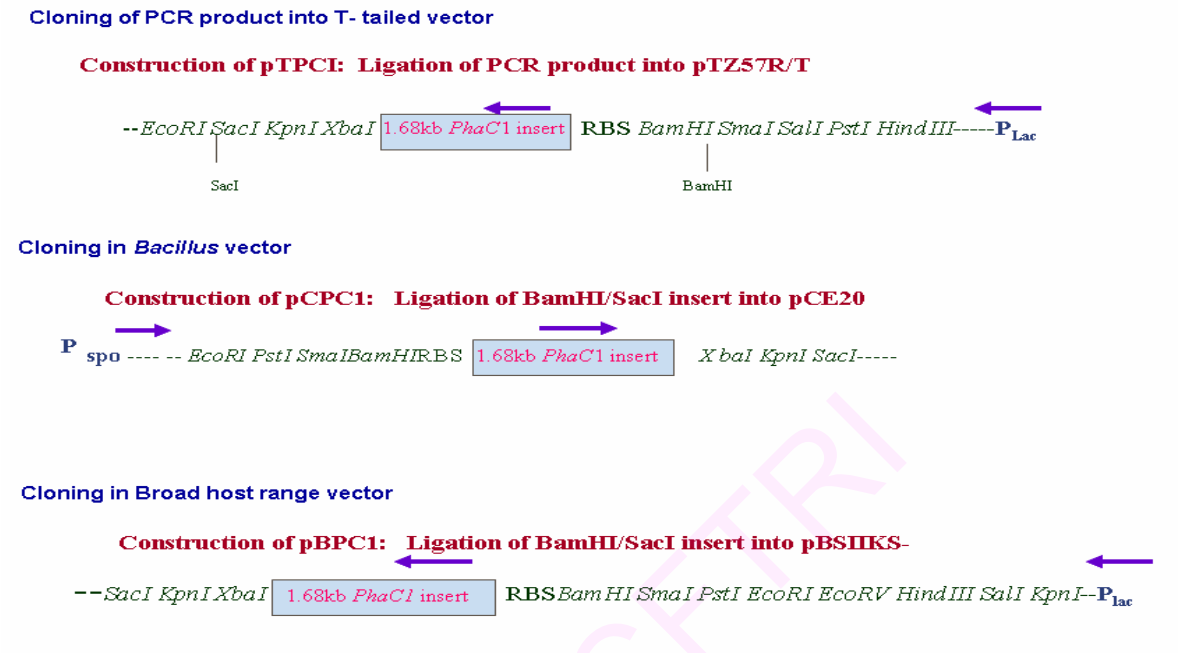


Figure 3.4 Strategy for the cloning of *PhaC1* gene in different vectors

3. 3 RESULTS

3.3.1 *Screening of PHA producer*

Pseudomonas colonies isolated on *Pseudomonas* isolation agar were screened for the presence of PHA synthase gene by PCR using the primers described in Table 3.4. The location of the primer within the *phaC* gene is given in figure 3.5. All the isolates tested reacted positively. The samples were run on 1.2 % agarose gel and the size of the amplicon was determined based on the100bp DNA marker run along with the sample. Distinct PCR products of 200bp and 700bp were obtained for the RPC1F- RPCIR1 and RPC1F - RPC1R2 primer pairs, respectively (Figure 3.6). The size of the PCR fragments accord with the length of the *phaC1* gene flanked by the primer pairs (Figure 3.5). Reamplification of the 700bp amplicon using a semi nested set of primers resulted in the production of a 200bp amplicon of expected size (Figure 3.7). The authenticity of the PCR fragment was again confirmed by restriction digestion using HincII (Figure.3.8). The expected size of the fragments on restriction with HincII were 550bp and 156bp and the results shown in figure 3.8 indicated that the fragments obtained after restriction were of expected size.

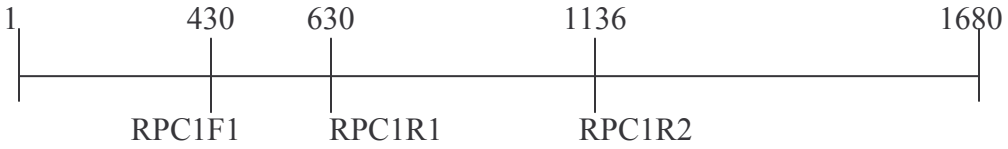


Figure 3.5 Location of primers within the *phaC1* gene used for the screening of PHA producers

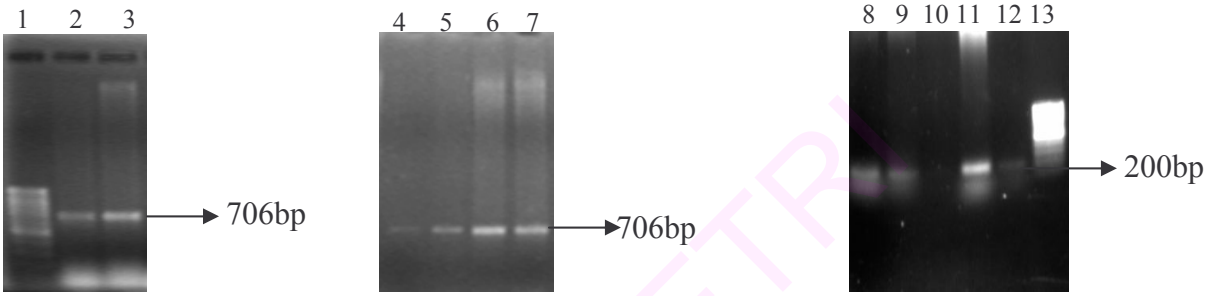


Figure 3.6 PCR detection of *phaC1* gene from different *Pseudomonas* isolates

Lane 1:100bp DNA ladder
Lane 2 & 3: 706bp amplicon

Lane 4 -7: 706bp amplicon from different isolates

Lane 8-12: 200bp amplicon from different isolates
Lane 13: 100bp DNA ladder

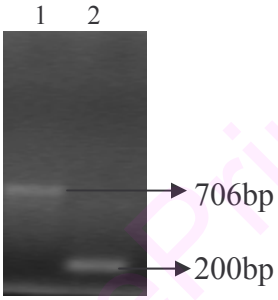


Figure 3.7 Authentication of the amplicon using seminested primer

Lane 1: 706bp amplicon
Lane 3: 200bp nested fragment

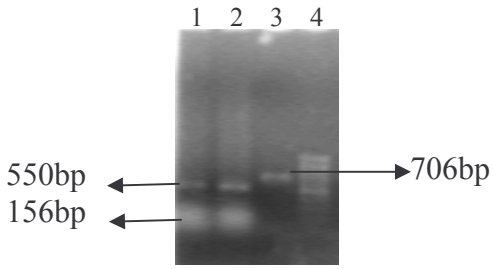


Figure 3.8 Authentication of the amplicon using HincII digestion

Lane 1&2: HincII restriction of 706bp amplicon
Lane 3: 706bp fragment (control)
Lane4: 100bp DNA ladder

After authentication, the 700bp amplicon, from *P aeruginosa* was purified and labeled with Psoralin biotin (2.4.4.3) and stored at -40 °C. This probe was used for the rapid screening of PHA producing *Pseudomonas* strains from different soil samples. Figure 3.9 shows the hybridization of *PhaC* probe with various *Pseudomonas* colonies isolated on *Pseudomonas* isolation agar from soil samples.

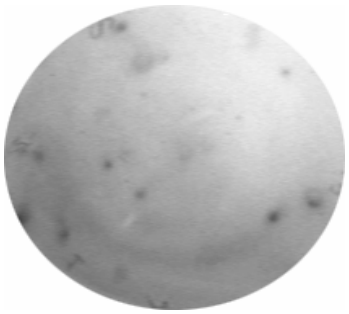


Figure 3.9 Colony hybridization using *phaC1* probe

3.3.2 *Production of PHA by different isolates and characterization of PHA*

Pseudomonas aeruginosa 01 and 20 other strains of *Pseudomonas* isolated from soil samples synthesized 3-39% CDW of polymer (Table 3.8). The polymer was deposited in granules within the cell (Figure 3.10) In the presence of glucose and under nitrogen limitation 3-hydroxy decanoate (40 to 86 mol %) was detected as the main constituent of the PHA (Table 3.8 and Figure 3.11). Most strains, synthesized a polymer consisting of approximately 50-60 mol% 3HO and 20 to 30% 3HHx during cultivation on octanoate. In most cases the polymer produced by cells grown in octanoic acid also contained 3HD; however, the molar fraction of this constituent was much lower than in the glucose-grown cells. The amount of PHA was not altered much when the cells were grown in the presence of both glucose and octanoate simultaneously (Table 3.8). GC analysis indicated that the major monomer present in the PHA of cells grown on octanoate (Figure 3.12) and on octanoate plus glucose was hydroxy octanoate (Figure 3.13). This may be due to preferential uptake of fatty acid rather than glucose by these strains. 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxy dodecanoate were minor constituents of the polymer. GCMS analysis was carried out to confirm the nature of the methyl esters observed by GC through determining the mass unit (Figure 3.14).

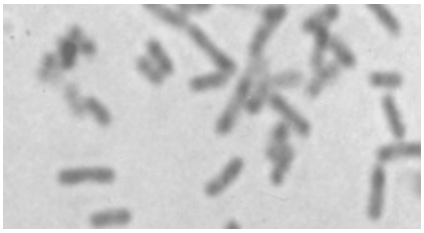


Figure 3.10 Sudan black staining of PHA granules in *Pseudomonas* isolate

Table 3.8 PHA production in different *Pseudomonas* isolates grown in the presence of different carbon sources*

Isolate	Glucose				Octanoic Acid				Glucose +Octanoic Acid			
	Biomass g/l	PHA %CDW	Major Monomer	Secondary monomer	Biomass g/l	PHA %CDW	Major Monomer	Secondary monomer	Biomass g/l	PHA %CDW	Major monomer	Secondary monomer
P1	1.23	18.8	C10	C8	1.08	22.0	C10	C8	1.28	24.0	C8	C10
P2	0.94	19.5	C10	C8	0.80	17.0	C10	C8	1.10	20.0	C8	C10
P3	1.46	9.0	C10	C8	1.00	15.0	C10	C8	1.00	15.5	C8	C6
P4	1.02	18.0	C10	C8	0.86	14.2	C10	C8	1.36	14.0	C8,C10	C10
P5	0.90	16.5	C10	C8	0.86	12.0	C10	C8	1.00	12.8	C8	C6
P6	1.06	9.4	C10	C12	1.04	13.4	C10	C12	1.15	14.0	C8	C10
P7	1.34	28.3	C8	C10	1.08	39.0	C8	C10	1.38	38.5	C8	C10
P8	0.98	22.5	C6	C8	1.18	24.0	C6	C8	1.22	24.0	C8	C10
P9	1.46	17.8	C10	C8	1.40	18.0	C10	C8	1.60	20.4	C8	C10
P10	1.10	20.0	C10	C8	1.10	17.0	C10	C8	1.30	19.5	C8	C10
P11	1.20	6.60	C10	C8	1.26	4.50	C10	C8	1.54	11.0	C8	C10
P12	1.40	31.7	C10	C8	1.02	28.0	C10	C8	1.72	29.0	C8	C10
P13	1.52	16.5	C10	C8	1.32	12.0	C10	C8	1.52	12.0	C8	C10
P14	1.46	12.6	C10	C8	0.95	4.0	C10	C8	1.05	5.40	C8	C10
P15	1.34	4.80	C10	C8	1.20	8.0	C10	C8	1.40	10.0	C8	C10
P16	1.40	9.80	C10	C8	0.89	7.0	C10	C8	1.20	9.80	C8	C10
P17	1.60	11.6	C10	C8	1.00	3.0	C10	C8	1.50	6.40	C8	C10
P18	1.00	13.7	C10	C8	0.90	15.7	C10	C8	0.95	15.7	C8	C10
P19	1.88	12.0	C8, C10	C6	1.50	12.0	C8, C10	C6	1.50	14.0	C8	C10
P20	1.72	15.1	C8	C10	1.70	23.0	C8	C10	1.73	22.0	C8	C10
PA01	1.58	25.0	C10	C8	1.50	27.0	C10	C8	1.62	27.5	C8	C10

*The values presented here are the mean of two independent experiment

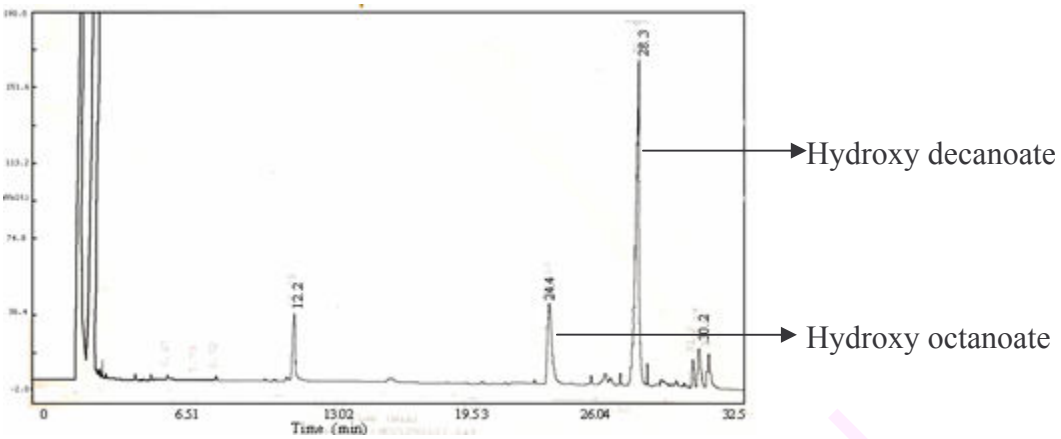


Figure 3.11 Gas chromatogram of methyl ester of PHA extracted from *Pseudomonas* sp. grown on glucose

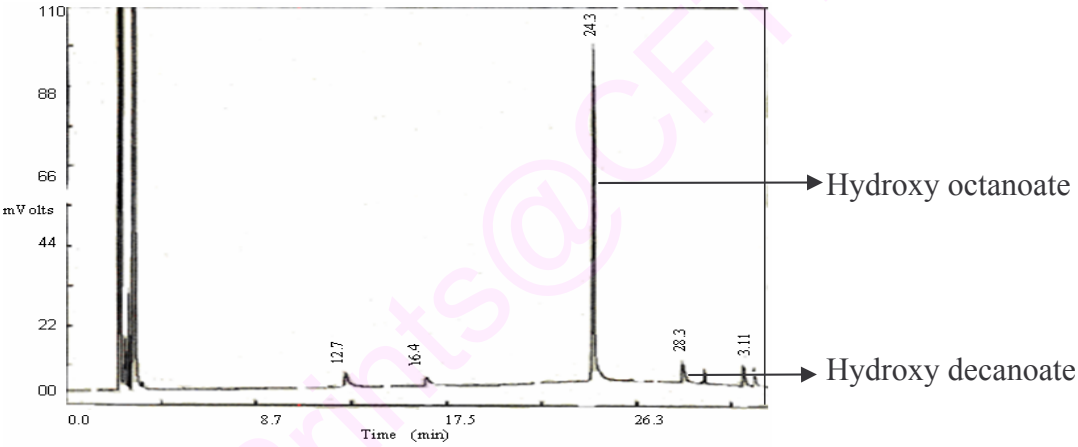


Figure 3.12 Gas chromatogram of methyl ester of PHA extracted from *Pseudomonas* sp. grown on octanoic acid

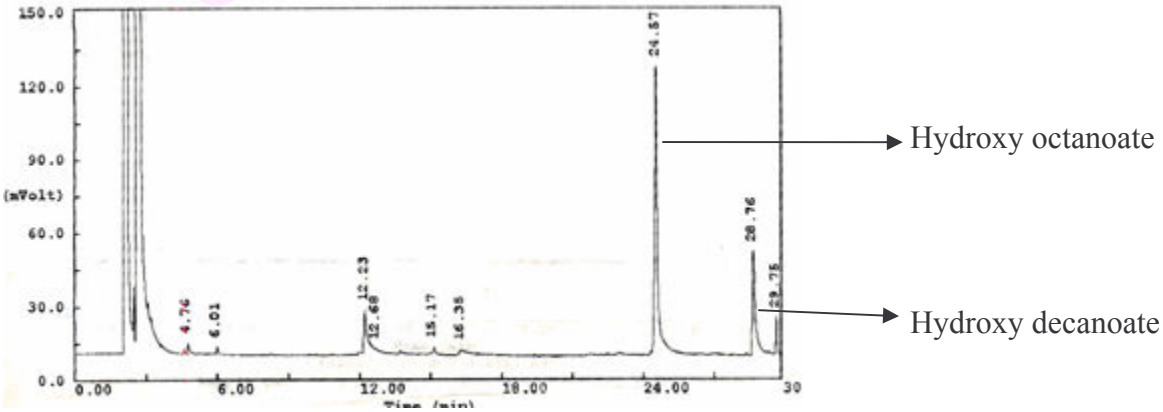


Figure 3.13 Gas chromatogram of methyl ester of PHA extracted from *Pseudomonas* sp. grown on glucose and octanoic acid

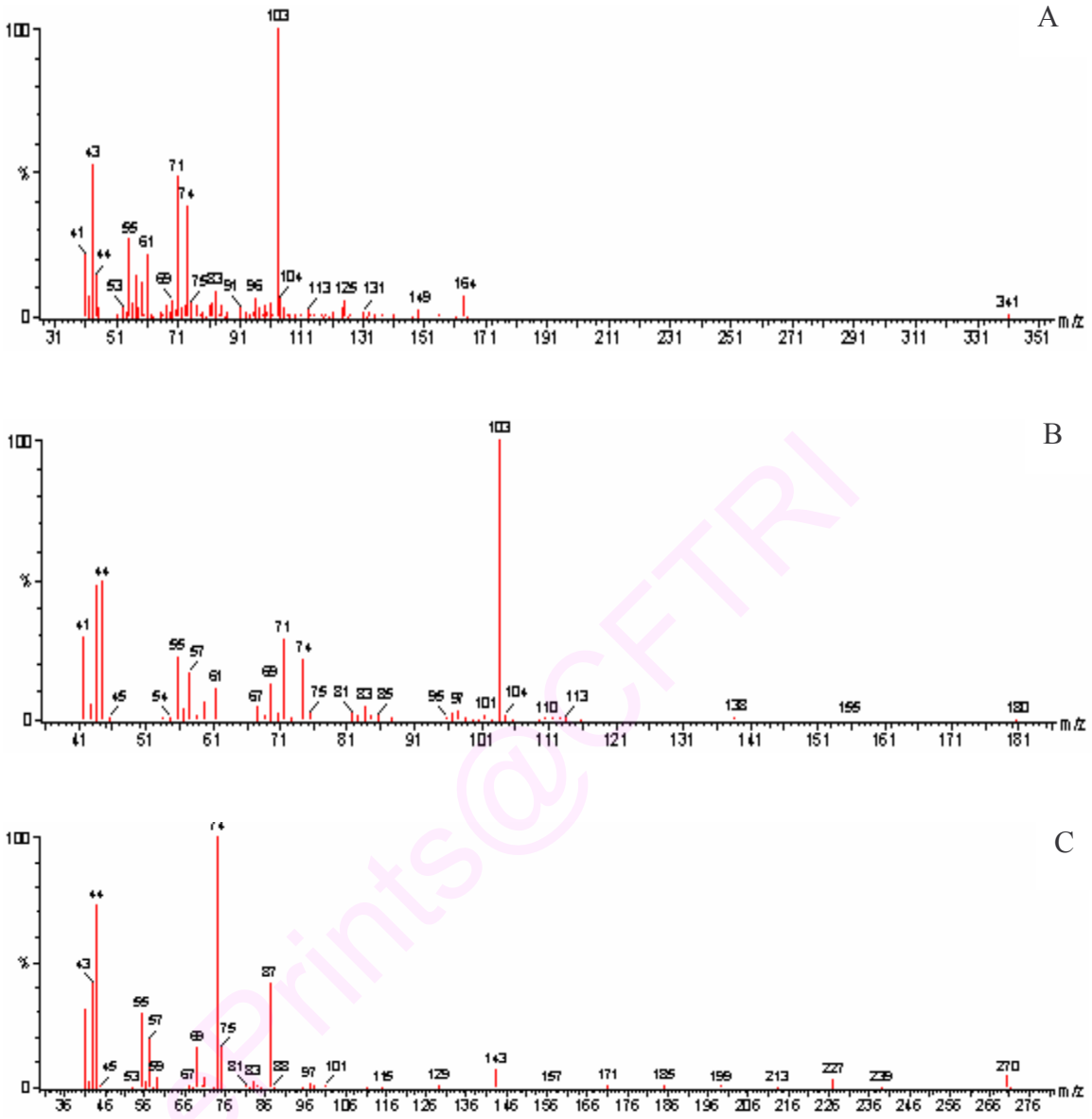


Figure 3.14 Mass spectra of different hydroxy alkanoates present in PHA extracted from *Pseudomonas* spp.

A: Octanoic acid 3-hydroxy methyl ester, B: Decanoic acid 3-hydroxy methyl ester, C: Dodecanoic acid 3-hydroxy methyl ester

Six isolates were selected based on the higher biomass and PHA content for further studies. These isolates were grown in different fatty acid alone or in the presence of glucose (Table 3.9). The amount of PHA was found to be almost similar when cells were cultivated on both glucose and decanoate but the biomass was found to be lesser in decanoate grown cells (Table 3.8). This may be due to the inhibitory effect of fatty acid on cell growth. (Figure 3.14) The isolate were grown for different periods of time in a

medium conducive to PHA production. The amount of PHA and its composition was estimated at time intervals of 12h, 24h, 48h, 72h, and 96h. No PHA accumulation was observed in these strains during the 12h incubation, even though good bacterial growth was achieved.

	Hexanoate				Decanoate			
Isolate	Biomass g/l	PHA %CDW	Major monomer	Secondary monomer	Biomass g/l	PHA %CDW	Major monomer	Secondary monomer
P1	1.0	18.8	C6	C8	1.2	22.5	C10	C8, C12
P2	0.9	19.5	C6	C8	1.0	20.0	C10	C8
P7	1.4	19.0	C6	C8	1.4	23.0	C10	C8
P8	1.0	22.0	C6	C8	1.8	36.0	C10	C8,C6
P9	0.9	16.5	C6	C8	1.6	28.0	C10	C8
P12	1.1	19.4	C6	C8	1.0	32.0	C10	C8
Pa01	1.2	16.6	C6	C8	1.3	30.0	C10	C8

Table 3.9 PHA production by selected *Pseudomonas* isolate from hexanoate and decanoate*

Least accumulation of PHA was seen at 24 h incubation in all the strains. There was a dramatic increase in PHA concentration at the 48th h which continued upto the 72ndh (Table 3.10). In some strains a fall in PHA content at the 96thh.was observed The biomass was stable at 24 h, 48 h and 72 h, but cell lysis was observed at 96 h.

Table 3.10 PHA production by selected *Pseudomonas* isolates at different growth periods*

Isolate	PHA (%CDW) in Glucose					PHA (%CDW) in Octanoate				
	12h	24h	48h	72h	96h	12h	24h	48h	72h	96h
P1	ND	4.2	20.0	18.9	20.0	ND	trace	14.0	24.6	24.0
P2	ND	6.0	26.0	22.0	18.0	ND	ND	10.6	18.8	10.3
P7	ND	trace	16.6	24.0	28.5	ND	6.6	12.2	28.3	32.0
P8	ND	trace	19.8	30.0	30.0	ND	2.6	20.4	28.9	17.8
P9	ND	trace	22.0	28.0	24.4	ND	5.5	16.6	24.0	24.0
P12	ND	4.2	18.0	22.0	16.7	ND	3.5	17.5	21.3	16.6
Pa 01	ND	3.8	16.0	28.0	19.0	ND	5.7	16.2	26.3	13.7

ND: not detectable, * The values presented here are mean of two independent experiment

Table 3.11 Effect of acrylic acid on PHA production by selected *Pseudomonas* isolates*

Isolate	Glucose		Hexanoate		Octanoate		Decanoate	
	Biomass g/l	PHA %CDW	Biomass g/l	PHA %CDW	Biomass g/l	PHA %CDW	Biomass g/l	PHA %CDW
P1	1.2	18.4	1.0	22	1.1	26	0.9	28
P2	1.3	19.0	0.9	20	1.0	24	1.0	24
P7	1.2	28.5	1.4	26	1.0	42	0.9	36
P8	1.3	22.5	1.0	25	1.1	30	1.2	32
P9	1.1	18.8	0.9	19	1.4	32	1.2	35
P12	0.9	31.0	1.0	24	1.0	26	1.0	38
Pa01	1.0	24.6	1.2	22	0.9	33	1.1	33

* The values presented here are mean of two independent experiment

3.3.3 Cloning of phaC1 gene from P. aeruginosa

PCR fragment of the expected size of 1680bp, for *phaC1* was obtained from genomic DNA of *P. aeruginosa* using the primers PAPC1F and PAPC1R. The samples were run on 1.2 % agarose gel and the size of the amplicon was determined based on the 3.0 Kb DNA marker run along with the sample (Figure 3.15). Restriction digestion and nested PCR confirmed the authenticity of the amplicon. The purified amplicon was cloned initially into the T-tailed vector pTZ57R/T to yield the plasmid pTPC1. Recombinants were selected as white clonies on IPTG/X-Gal plates. Plasmid was isolated from these clonies and release of insert after restriction digestion ascertained (Figure 3.16). The direction of the insert with respect to the *lacZ* promoter was monitored through restriction digestion. Sequencing was used to authenticate the clones. The expected fragment sizes of 980bp and 198bp, when cloned in sense direction to the *lacZ* promoter was seen on restriction digestion of pTPC1 with HincII (Figure 3.3). The results presented in Figure 3.17 shows that the size of the fragments was of the expected sizes, indicating that the insert was in the sense direction with respect to *lacZ* promoter.

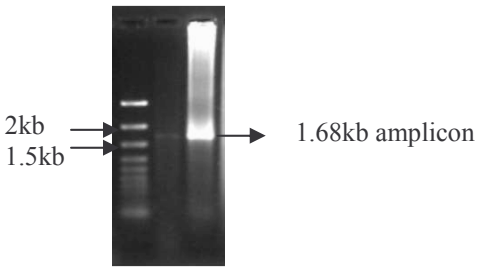


Figure 3.15 PCR amplicon of *phaC1* gene from *P. aeruginosa*

Lane 1: 3kb DNA ladder
Lane 2: Purified PCR amplicon of 1.68kb
Lane 3: 1.68 kb PCR amplicon of *phaC1*

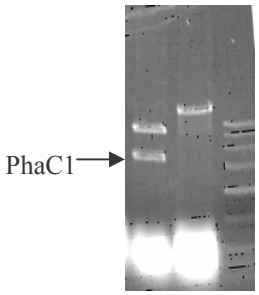


Figure 3.16 Insert release from the pTPC1 clone

Lane 1: pTPC1 digested with BamH1/SacI
Lane 2: pTPC1 digested with BamH1
Lane 3: Low range DNA ruler

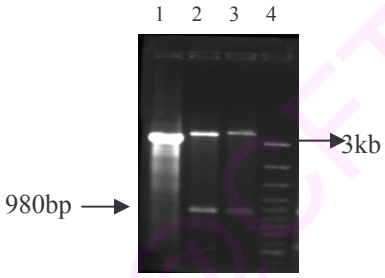


Figure 3.17 Determination of the direction of the *phaC1* insert by HincII digestion

Lane 1: pTZ57R digested with HincII, Lane 2 &3: pTPC1 digested with HincII, Lane 4: 3 kb DNA ladder

3.3.5 Subcloning of phaC1 gene into pCE20 and pBSPIIKS (-)

For the sub cloning of PhaC1 gene, the pTPC1 was digested with BamHI and SacI and the insert released was checked by agarose gel electrophoresis (Figure3.16). Insert released was found to be of the expected size and was eluted from the gel. The eluted insert was then ligated into pCE20 or pBSPIIKS (-) to obtain pCPC1 and pBPC1 and the ligated product was transformed into *E. coli* host. Plasmid isolated from the recombinant *E. coli* cells was checked by the agarose gel electrophoresis for the presence of inserted gene (Figure 3.18). The plasmids pCPC1 containing p*haC1* gene under the control of spo promoter was electroporated into *Bacillus subtilis*168. The plasmid pBPC1 was used to transform *E. coli* strains BL21 and LS1298Kan::*fadB1* and *P. aeruginosa,* which brought *phaC1* under the control of *lacZ* promoter. The transformants were checked for the presence of plasmid by resistance to antibiotic, plasmid profiling, release of insert and by PCR.

Figure 3.18 *phaC1* construct in pCE20 and pBSPIIKS(-)

Lane 1: pBPC1
Lane 2: pBSIIKS
Lane 3: pCPC1
Lane 4: pCE20

3.3.6 Expression of PhaC1

The PHA synthase gene, from *P. aeruginosa*, *phaC1,* was cloned into the broad host range vector pBSPIIKS(-) for functional expression in heterologous hosts. SDS-PAGE analysis showed a 64 kD protein band in the cell lysate of *E. coli* strain LS1298Kan::*fadB* which was not to be seen in the control strains (Figure 3.19).

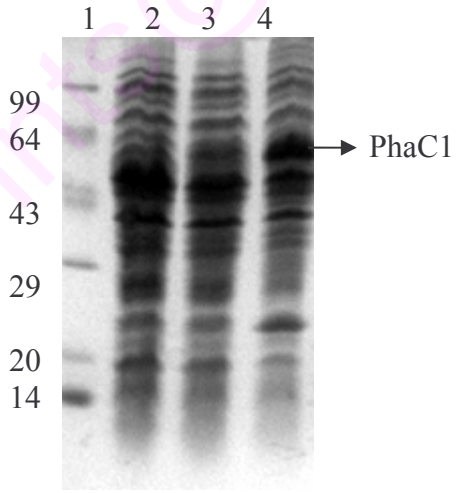


Figure 3.19 SDS-PAGE analysis of recombinant *E. coli* bearing *phaC1* gene of *P. aeruginosa*

Lane1: Standard protein molecular weight marker in kDa, lane2: Total lysate of *E coli fadB* mutant, lane 3: total lysate of *E. coli fadB* mutant bearing pBPSIIKS (-), Lane4: Total lysate of *E. coli fadB* mutant bearing pBPC1

3.3.7 Production and composition of PHA in recombinant E. coli harbouring pBPC1

Cells of *E. coli* BL21 harbouring *PhaC1* of *P aeruginosa,* grown on octanoate and decanoate accumulated 4.1% and 4.2 % PHA of cell dry weight, respectively (Table 3.12). In *E. coli fadB* mutant a greater amount of PHA accumulation was noted with all the fatty acids used as growth substrate. But there was no noted difference in the amount of PHA production between the two strains when grown on glucose. 3-hydroxy octanoate was the major PHA monomer when the recombinants were grown on octanoate while 3-hydroxy decanoate was the major PHA monomer when the recombinants were grown on glucose or decanoate (Figure 3.20). But traces of butyrate were noticed in the PHA extracted from recombinant *fadB* mutant grown on octanoate (Figure 3.20).

Table 3.12 Production of PHA by recombinant *E. coli* bearing pBPC1

Substrates	*E. coli* BL 21	*E. coli* *fadB* mutant
Glucose	2.0	1.3
Octanoate	4.1	8.3
Propionate	2.0	4.0
Decanoate	4.2	10.0

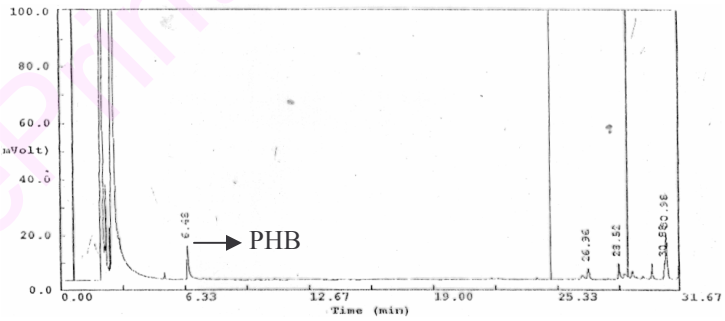


Figure 3.20 Gas chromatogram of methyl ester of PHA extracted from *fadB* mutant *E. coli* bearing pBPC1 grown on octanoate

3.3.8 Production and composition of PHA in recombinant P. aeruginosa harbouring pBPC1

Over expression of *phaC1 in P. aeruginosa* resulted in notable increase in the quantity of PHA when cells were grown on different fatty acids when compared to that of the control (cells bearing control plasmid). The change was more pronounced when cells were grown on decanoate (Table 3.13). Alteration was much higher in the composition

than in the content of PHA, when recombinants were grown on different substrates. The mol % of hydroxy decanoate in control cells when grown on glucose, octanoate decanoate were 35.8, 26 and 45 % CDW, respectively, which increased to 45, 31 and 53 % CDW, respectively, when *phaC1* gene was over-expressed in *P. aeruginosa*.

Table 3.13 Production (% CDW) and composition of PHA in recombinant *P. aeruginosa* bearing pBPC1

Substrates	pBSP II KS-	pBPC1	Monomer Composition							
			HHx	HO	HD	HA	HHx	HO	HD	HA
Glucose	33.9	33.0	6.4	19.4	45.8	28.4	5.0	20.0	55	30.0
Octanoate	25.9	29.8	15.3	25.0	26.0	33.7	17.0	22.0	31	30 .0
Decanoate	24.0	30.0	-	31.2	45.0	23.8	2 .0	27.0	53	18.0

3.3.8 Production and composition of PHA in recombinant B. subtilis harbouring pCPC1

Recombinant *B. subtilis* bearing pCPC1 was grown in the PHA medium containing glucose and different fatty acids as carbon source. *B. subtilis* 168, a non PHA producing strain, on introduction of *PhaC1* gene from *P. aeruginosa* was capable of producing PHA up to 4-10 % of CDW from glucose and fatty acids and the data is shown in the table 3.14. Considerable biomass was obtained from all the combinations of carbon sources tested. Octanoate was found to be a suitable substrate for *Bacillus* for the synthesis of PHA. The production of PHA in the recombinant *Bacillus* strain was confirmed using FTIR and further characterization of the PHA was carried out using Gas chromatography.

Table 3.14 Production and composition of PHA in recombinant *Bacillus subtilis* bearing pCPC1

Substrate	Biomass mg/ 50ml	%PHA	Major monomer	Secondary monomer
Glucose	85	4.3	3HHx	3HO, 3HB
Glucose+Hexanoate	82	6.2	3HB	3HHx
Glucose+Octanoate	64	10.0	3HB	3HO
Glucose+Nonanoate	60	4.0	ni	ni

Glucose+Decanoate	65	5.0	3HO	3HB

ni: not identified

FTIR and GC analysis of the chloroform extract of PHA from these recombinants indicated a difference between the IR spectra and chromatogram of control and recombinants bearing plasmids pCE20 and pCPC1, respectively. Commercially available PHB with an absorption peak at 1730 frequency in the IR spectrum due to the presence of C=O side group was used as a standard. PHA isolated from the the *Bacillus* recombinant bearing the pCPC1 plasmid grown in presence of glucose and octanoic acid produced a similar absorption peak at 1730 as indicated by arrows in the IR spectra (Figure 3.22). This may be taken to infer production of PHA in these recombinants. This result was confirmed on Gas chromatography of the PHA polymers thus obtained. There was no characteristic peak in the chromatogram of control *B. subtilis* bearing plasmid pCE20 while characteristic butyrate, and mcl-PHA monomers were detected during analysis of PHA extracted from recombinant bearing plasmid pCPC1 (Figure 3.21).

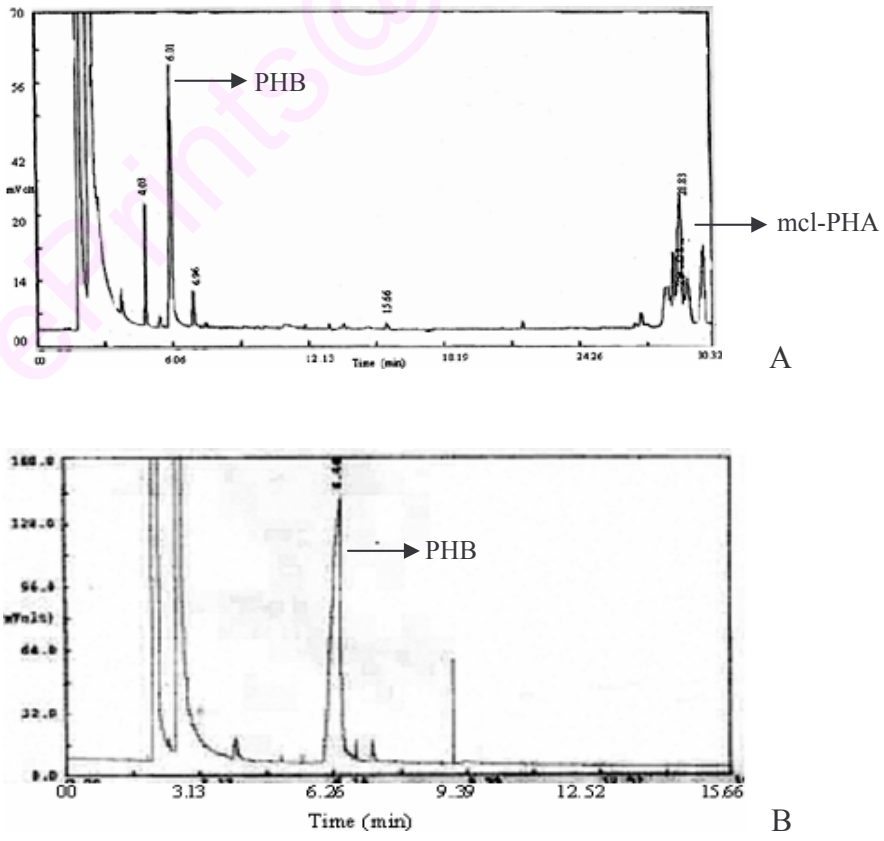


Figure 3.21 Gas chromatogram of methyl esters of PHA extracted from recombinant *B. subtilis*

A) Recombinant *B. subtilis* grown on octanoate+ glucose B) standard PHB

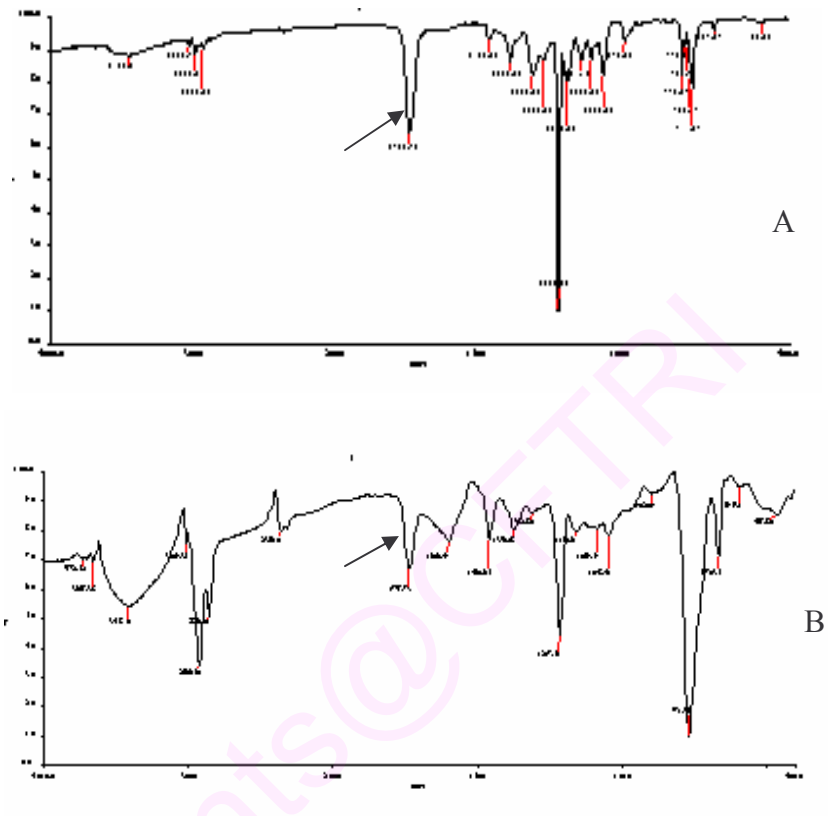


Figure 3.22 FTIR spectrum of PHA extracted from recombinant *B. subtilis*
A) Standard PHB B) *B. subtilis* grown on octanoate + glucose

3.4 DISCUSSION

In this study, isolation of different strains of *Pseudomonas* and investigation on its PHA biosynthesis ability on different carbon sources were carried out. A highly specific *phaC1* probe of 700bp of *P. aeruginosa* was developed for the rapid screening of PHA producing *Pseudomonas* spp. directly from soil. This method allowed the screening of many colonies at a time. Even though detection methods based on probes have been reported before (Timm *et al*., 1994,) this may be the first report of the use of colony hybridization for detection of PHA producing *Pseudomonas* spp.

Mcl-PHAs are accumulated by a number of gram-negative bacteria, mainly *Pseudomonas*, consisting of C6 to Cl2 hydroxyalkanoic acids. In this study all the strains

incorporated 3-hydroxydecanoyl-CoA into the polymer preferentially during cultivation on glucose. In contrast, cells grown on octanoate incorporated 3HO preferentially; 3HD and other 3-hydroxyalkanoic acids amounted to only a small fraction of the polymer. This has also been described for *P. oleovorans* (Brandl *et al.,* 1988) and for other fluorescent Pseudomonads (Timm and Steinbuchel, 1990). PHA richer in octanoate was produced by cells when grown on glucose and octanoate simultaneously. This may be attributed perhaps to the preferential uptake of fatty acid.

Competition between enzymes for precursor substrates involved in fatty acid metabolism and PHA biosynthesis may be expected. Acrylic acid is an inhibitor of fatty acid β-oxidation. It was envisaged that on addition of acrylic acid in the media of PHA producing *Pseudomonas* could deviate monomers toward accumulation of PHA. This has been investigated in *Ralstonia eutropha* (Green *et al*., 2002) and recombinant *E. coli* (Qi *et al.,*1998). *R. eutropha* which generally produces only scl-monomers, on addition of acrylic acid accumulates copolymer of scl and mcl. In the experiments reported here, we observed a significant hike in the amount of PHA produced, while its composition remained unaltered.

PHA synthase is the key enzyme in various biosynthetic pathways polymerizing the hydroxy alkanoates (Steinbuchel and Fuchtenbusch,1998; Rehm and Steinbuchel, 1999; Taguchi *et al*., 1999). Several types of PHA biosynthesis genes have been cloned and expressed from various organisms and their roles investigated (Fukui and Doi, 1997; Matsusaki *et al*., 1998; Choi *et al*., 1998; Solaiman, 2000; Zhang *et al*., 2001). In this study, a PCR cloning strategy was employed as reported early by several researchers for the functional expression of PHA biosynthetic genes. Solaiman, (2000), Matsusaki *et al*., (1998), Langenbach *et al*., (1997), Park *et al.*, (2002), and Zhang *et al*., (2001) have established the biosynthetic pathway for the production of mcl-PHA in recombinant *E. coli* using the PHA synthase gene from *P. aeruginosa*. In this study efforts were made to develop an alternate pathway for the biosynthesis of PHA using enzymes from different and hitherto untried sources with an ultimate objective of synthesizing PHA comprising polyhydoxybutyrate, polyhydroxyvalerate and mcl-hydroxy fatty acids. This work form a part of another chapter (4.3.5).

It has been previously demonstrated that recombinant *E. coli* and *E. coli* with impaired β-oxidation pathway could synthesize mcl-PHAs from fatty acids when provided with a functional mcl-PHA synthase (Qi *et al*., 1997; 1998; Ren *et al*., 2000; Park *et al.,* 2002; Ren *et al*., 2005). Initially we have expressed the PHA synthase1 from *P. aeruginosa* in *E. coli* BL21 and in a *fadB* mutant of *E. coli* and this lead to the synthesis of detectable amounts of PHA. In a *fadB* mutant of *E. coli,* β-oxidation intermediates accumulate (Park *et al*., 2005) and this may have accounted for the greater production of PHA in the *fadB* mutant observed by us. The specificity of the *P. aeruginosa* PhaC1 was found to be relaxed in *fadB* mutant of *E. coli.*

However, expression of PHA synthases of *Pseudomonas* in *Bacillus* sp. has not been hitherto reported. Earlier workers have expressed the *phaPQRBC* genes from *B. megaterium* in *Bacillus subtilis* strain (Law *et al.*, 2003). *B. subtilis* has many advantages such as short generation time, better substrate utilization, lack of pathogenicity and its capacity of secreting large amount of enzymes which can simultaneously be harvested (Law *et al*., 2003). One of the objectives of the present study was to demonstrate the functional expression of *phaC1* gene coding the PHA synthase enzyme of *Pseudomonas aeruginosa* in *B. subtilis*. Hensforth, *phaC1* gene was cloned in a *Bacillus* vector under the control of a constitutive promoter (*spo* promoter). The quantity of PHA produced by recombinant *B. subtilis* ranged from 2-10 % depended on the substrate used for cultivation. FTIR and GC analysis confirmed the production of PHA by recombinant strain. Introduction of PhaC1 alone could contribute towards PHA production, indicating the presence of other enzymes capable of providing precursor substrates (monomers) in *B. subtilis* 168. Attempts at cloning PhaJ1 or PhaJ4 genes from *P. aeruginosa* in pCPC1 plasmid for further improvement of the recombinant *Bacillus* strain was unsuccessful (data not shown).

The specificity of PhaC1 and PhaC2 of *P. aeruginosa* was studied after its expression in *E .coli.* There has been no report on the over expression of these enzymes in *P. aeruginosa*. Over expression of enzymes can result in change of quantity of PHA and its monomer composition as observed by earlier workers (Fukui *et al*., 2001). Similarly in our experiments we could observe a marginal change in the quantity of PHA and a significant change in monomer composition. The hydroxy decanoate fraction of the

PHA accumulated by the recombinant *P. aeruginosa* was increased when compared to that of the control. This indicates that the *phaC1* is active towards the polymerization of hydroxy decanoyl CoA in *P. aeruginosa*. Studies on the promoter of PHA synthase gene and the use of this gene along with other PHA biosynthetic enzymes in the development of recombinant *E. coli* strains are described in the chapters that follow.

4.0 ABSTRACT

Expression of the *Pseudomonas* enzyme [PHA synthase (PhaC1)] in *E. coli* was able to support the production of PHA (Section 3.3.4.). In this chapter, two genes *phaJ1* and *phaJ4* which code for (R)-specific enoyl CoA hydratases 1&4 (enzyme interconnecting PHA biosynthesis and fatty acid β-oxidation) were amplified by PCR from genomic DNA of *Pseudomonas* spp. (isolate no.P7 and Pa01), cloned into the T-tailed vector pTZ57R/T and sequenced. The genes *phaJ1* and *phaJ4* were sub-cloned into pBPC1 (description given in table 3.6) plasmid individually in order to analyze the effect of co-expression of these hydratase genes with the *phaC1* gene on accumulation and composition of PHA. The resultant plasmids, pBPC1J1 and pBPC1J4, containing *phaJ1* and *phaJ4* upstream of *phaC1* gene, were introduced into *E. coli* BL21 and into a *fadB* mutant of *E. coli.* The content of PHA produced in the *fadB* mutant (18-30%) was higher than that in *E. coli* BL21 (15-25%). PHA produced by the *fadB* mutant was richer in butyrate (40-50%) than that produced by *E. coli* BL21 (0.5-2.5%) as shown through GC and NMR analysis. The PHA extracted from *E. coli* bearing pBPC1J4 produced more butyrate than those bearing pBPC1J1. The difference in the composition of mcl-monomer in the polymer depended on the differences in isoforms of the hydratase enzyme and the substrate on which the recombinant strains were grown. The yield of PHA with glucose and fatty acid as substrate varied from 20-25% and this concentration was attained within 24 hours in a fermentor while prolonged fermentation (48 h) was required for shake flask cultivation.

In order to further improve yield and quality of PHA, the constructs bearing PHA synthase and enoyl CoA hydratase1 were introduced into *E. coli* JC7623 along with ketothiolase (*phaA*) and acetoacetyl CoA reductase (*phaB*) from a *Bacillus* species (Anilkumar, 2007 unpublished data). The two *Bacillus* genes were integrated in single copies into the chromosome of the *E. coli* host at the *lacZ* locus. Expression of these four proteins in *E. coli* driven under the control of *lac* promoter was visualized through SDS-PAGE. The recombinant *E. coli* (JC7623ABC1J1) grown on various fatty acids along with glucose was found to produce 28-34% PHA copolymers in the biomass from glucose and fatty acids. GC, GCMS and NMR analysis of the polymer confirmed the ability of the strain to produce scl-co-mcl-PHA copolymers. The ratio of scl to mcl PHA

varied from 22:78 to 74:26. Addition of acrylic acid, an inhibitor for β-oxidation resulted in improved production (2-10% increase) of PHA copolymer. This is a novel report of the combined use of enzymes from *Bacillus* and from *Pseudomonas* for the recombinant production of copolymer of scl and mcl-PHA in *E. coli.*

4.1 INTRODUCTION

The physical and thermal properties of PHA copolymers can be regulated by varying their molecular structure and copolymer compositions (Abe and Doi, 2002). Copolymers of PHB with PHV and mcl-PHA have better film forming and mechanical properties than homopolymers (Sudesh *et al*., 2000). The copolymer consisting of both scl and mcl 3-hydroxyalkanoate was reported to possess mechanical properties similar to those of low-density polyethylene (Doi *et al*., 1995) and has a broader spectrum of application (Wang *et al*., 2005; Chen and Wu, 2005). A few naturally occurring bacteria, such as *Aeromonas spp.* (Lee *et al.,* 2000, Chen *et al.,* 2001), *Pseudomonas* sp. 61-3 (Kato *et al*., 1996), *Bacillus* sp. INT005 (Tajima *et al*., 2003), recombinant *R. eutropha* (Luo *et al*., 2006)) and *E. coli* (Park *et al*., 2001b) are capable of producing copolymers of scl and mcl-PHA. Most natural producers, however, require prolonged fermentation for polymer synthesis and the cells are more resistant for polymer extraction and hence they are less suited for industrial production. There are different strategies for the production of copolymers in recombinant bacteria such as external substrate manipulation, inhibitor addition and recombinant gene expression. Although *E. coli* does not naturally produce PHA, this bacterium is considered to be an appropriate host for generating higher yields of the biopolymer because of its rapid growth and the ease with which it can be lysed (Li *et al*., 2007). The advantages of *E. coli* as a host strain have been discussed in 1.11.1.

The monomeric composition of a biopolymer, in native producers or a recombinants depend on the following factors 1)Host 2)PHA synthase 3)The substrate used for cultivation of bacteria 4) hydroxyacyl–CoA thioester precursor supplied to the enzyme and 5) metabolic pathway operating in the cells. PHA synthases from *A. caviae, A. hydrophila, Pseudomonas* sp. 61-3, *P. stutzeri, Bacillus* sp. INT005 and *Nocardia caronilla* are able to incorporate 3-hydroxyacyl-CoA of both scl and mcl (Hall *et al*.,

1998; Matsusaki *et al*., 1998; Chen *et al*., 2004). Experiments with *P. aeruginosa* and *P. oleovorans* PHA synthase (Timm and Steinbuchel, 1992, Davis *et al*., 2008) suggest that expression of PHA synthase in heterologous systems might allow an even greater range of monomers to be incorporated than in homologous systems. The substrate–supplying pathway for PHA synthase in host cell is hence important for control of composition of PHA. By varying the feed ratio of different substrate precursors, the carbon flux towards polymer synthesis can be altered. It has been observed that the addition of acetic acid, citric acid, propionic acid, oleic acid and amino acids such as threonine, methionine can contribute towards the formation of hydroxy valeryl CoA.

The β-oxidation pathway of *E. coli* may be engineered for PHA biosynthesis either by the inhibition of the key β-oxidation pathway enzymes **(**FadA**,** FadB) or by the amplification of enzymes (3-ketoacyl ACP reductase, Enoyl CoA hydratase, epimerase) that directly convert β-oxidation pathway intermediates to R3HA-CoAs (Park *et al*., 2005). The *fadA, fadB, fadAB* and *fadR* mutants of *E. coli* have been used widely to support mcl-PHA synthesis.

FadA codes for 3 ketoacyl-CoA thiolase which catalyses the formation of acetyl CoA from 3-keto acyl CoA. Inhibition of this enzyme may be achieved through mutation (*fadA* mutant) or addition of acrylic acid (Qi *et al*., 1998; Lu *et al*.,2003). Accumulation of intermediates (3-ketoacyl CoA) from fatty acid β-oxidation serving as a precursor for PHA biosynthesis would be expected. In a *fadA* mutant of *E. coli*, metabolic link between the PHA biosynthetic pathway and the β-oxidation pathway may be constructed using 3-ketoacyl-CoA reductase (*fabG*) which shares homology with the *phbB* gene of *R. eutrtopha* (Taguchi *et al*., 1999; Ren *et al*., 2000; Park *et al*., 2002). Co-expression of the *E. coli fabG* gene and the mcl-PHA synthase gene established a link in *E. coli* with a functional β-oxidation pathway allowing the biosynthesis of PHA (Park *et al*., 2002).

FadR gene encodes a protein that represses the transcription of genes necessary for the oxidation of long chain fatty acids while inducing the transcription of genes necessary for the fatty acid biosynthesis. Thus a mutation in *fadR* derepresses transcription of genes involved in fatty acid oxidation. Heterologous expression of PhaC1 from *P. aeruginosa* in *E. coli fadR* mutant (RS3097) grown on decanoate in the presence

of acrylic acid (0.24mg.ml-1) accumulated PHA to about 60% of cellular dry weight (Qi *et al*., 1998).

Enoyl CoA hydratase coded by *fadB* converts enoyl CoA to (S)-3 hydroxy acyl CoA. In *fadB* mutant, enoyl CoA would accumulate and be converted to (R)-3 hydroxy acyl CoA by the (R)-specific enoyl CoA hydrates or crotonase family of enzymes (Di Russo, 1990). The *fadB* mutant LS1298 equipped with the *P. aeruginosa phaC1* gene was able to synthesize PHA up to 21% dry cell weight from decanoate (Lagenbach *et al*., 1997). In bacteria such as Pseudomonads and Aeromonads, the β-oxidation and mcl-PHA biosynthetic pathways is known to be linked by a (R)-specific enoyl-CoA hydratase (Tsuge *et al*., 2003; Fiedler *et al*., 2002; Fukui *et al*., 1998). (R)-specific hydratase active with short chain length enoyl CoAs has also been found in PHA producing bacterium, *Rhodospirillum rubrum* (Moskowitz and Merrick, 1969). PHAs with diverse monomer composition have been produced in recombinant *E. coli* from fatty acids employing *PhaJ* genes of *P. aeruginosa* and *phaC2* gene of *Pseudomonas* sp. 61-3 (Tsuge *et al.,* 2003). Also the *phaJ* of *Aeromonas* sp. have been extensively used for the recombinant production of 3-hydoxybutyrate and 3-hydroxy hexanoate copolymer.

The enoyl CoA hydratases are important for the production of PHA in *Pseudomonas* spp and in *E. coli*. In this study we have cloned two of the enoyl CoA hydratases (PhaJ1 and PhaJ4) from *Pseudomonas* spp. and co expressed them along with *PhaC1* gene of *P. aeruginosa* in *E. coli* of different genetic back grounds. The hydratases expressed in the *fadB* mutant of *E. coli* with PhaC1 enabled enhanced synthesis of PHA while the composition of the PHA depended on the type of the hydratases used.

Presently the focus is on the use of metabolic engineering to generate hybrid pathways for production of copolymers of scl and mcl-PHA in recombinant bacteria. It has been found that recombinant *E. coli* with *Aeromonas* biosynthesis genes create a PHA terpolymer of 3-hydroxybutyrate, 3-hydroxyvalerate, and 3-hydroxyhexanoate while using dodecanoic acid and odd-carbon number fatty acids as carbon sources (Park *et al*.,2001b). Providing *R. eutropha phaAB* genes and *phaC* gene from *Pseudomonas* sp. 61-3 into *E. coli fadA* and/or *fadB* mutant, enabled the synthesis of PHA consisting of C4, C6, C8, and C10 monomer units (Park and Lee, 2004b). Fukui *et al*., (2002) designed a sophisticated pathway in *R. eutropha,* using *phaC* and *phaJ* from *A. caviae* and Crotonyl-

CoA reductase (Ccr) from *Streptomyces cinnamonensis*, to make P (3HB-co-3HHx) from fructose (1.5mol% of 3HHx in the polymer). A copolymer of 3HB with 5 mol% (R)-3-hydroxyhexanoate, could be produced by a recombinant strain of *R. eutropha* PHB$^-$ 4, carrying PHA synthase gene of *Aeromonas caviae,* with a high PHA content of 71–74% from soybean oil as a sole carbon source (Kahar *et al*., 2004). PHA copolymers consisting of scl and mcl (3HA) were produced by recombinant *R. eutropha* PHB−4 harboring a low-substrate-specificity PHA synthase PhaC2 from *Pseudomonas stutzeri*1317 (Luo *et al*., 2006). Qin *et al.* (2007) developed two recombinant *Aeromonas hydrophila* CQ4 (PHA synthase negative mutant of *A. hydrophila* 4AK4) strains, one with PhaC2 of *P. stutzeri* and PhaJ of *A. hydrophila*, another bearing PhbA and PhbB of *R. eutropha* and with PhaC2 of *P. stutzeri*. Both the recombinants were able to accumulate PHA copolymer containing PHB, PHHX and other mcl monomers. The modified 3-ketoacyl-acyl carrier protein synthase III gene (*fabH*) at the point of encoding amino acid 87 from *E. coli* was found to produce PHA scl–mcl copolymer from glucose when co-expressed with *A. caviae* or *Pseudomonas* sp. 61-3 PHA synthase (Nomura *et al*., 2004a). Again additional *phbA* and *phbB* from *R. eutropha* was able to enhance PHA scl–mcl copolymers production from glucose in recombinant *E. coli* (Nomura *et al*., 2004b).

Members of the genus *Bacillus* accumulate co-polymers of 3HB when grown on different substrates. Biosynthesis of 3HB-rich copolymer or terpolymer has been reported in *B. cereus* ATCC 14579 (Caballero *et al*., 1995) *Bacillus cereus* SPV (Valappil *et al*., 2007b; Valappil *et al.,* 2007c), *B. cereus* UW85 (Labuzek and Radecka, 2001) and *Bacillus* sp. INT005 (Tajima *et al*. 2003).The *Bacillus* PHA operon (McCool and Cannon, 2001; Satoh *et al*., 2002) consists of five genes *phaP*, *phaQ phaR, phaB, phaC*. The enzyme β-ketothiolase (*phaA*) that is required for polyhydroxyyvalerate (PHV) synthesis is located away from the PHA operon. PHA biosynthetic genes of *Bacillus* have not been hitherto exploited for recombinant PHA production in *E. coli*.

In the present work a novel approach for copolymer production has been used in which a hybrid pathway was designed using two monomer supplying pathways (from *Bacillus* sp. and *Pseudomonas* sp.) for P(HB-co-HV)-co-mcl PHA copolymer production in recombinant *E. coli.* The pathway employing genes for ketothiolase and reductase

(*phaA* and *phaB*) isolated from *Bacillus sp.* and genes for PHA synthase and (R)-specific enoyl CoA hydratase (*phaC1* and *phaJ1)* cloned from *P. aeruginosa.*

4.2 MATERIALS AND METHODS

4.2.1 Strains, plasmids and maintenance of culture

The strains and plasmids used in this study are listed in table 4.1.

Table 4.1 Strains and plasmids used for the expression of *Pseudomonas* and *Bacillus* genes in *E. coli*

Strain or plasmid	Relevant characteristics
P. aeruginosa	*Prototroph*
*E. coli DH5*α	*supE*44 Δ*lac*U169(ǿ80- *lacZ*ΔM15)*hsdR*17 *recA*1 *endA*1 *gyrA*96thi-1 *relA*1
E. coli BL21	*hsdS gal*(λ*c* I*ts*857 *ind*1 Sam7 *nin*5 *lac*UV5-T7 gene1)
E. coli LS1298	LS1298Kan::*fadB1*
E. coli JC7623	(F lacZ+, leu-6, his-4, ara-14, recB21, recC22, sbcB15, λ-)
pTZ57R/T	Apr;T7 and T3 promoter; cloning vector
pBSPIIKS-	Apr; T7 and T3 promoter; broad host range cloning vector
pTPJ1	pTZ57R derivative; *phaJ1*Pa
pTPJ4	pTZ57R derivative; *phaJ4*Pa
pBPC1	pBSP II KS-derivative; *phaC1*Pa
pBPC1J1	pBSP II KS-derivative; *phaC1*Pa; *phaJ1*Pa
PBPC1J4	pBSP II KS-derivative; *phaC1*Pa; *phaJ4*Pa
pBRINT-Cm	pMB1:Integration into Host Chromosome: amp, cml
PBRAB	Cml rAmp r *PhaB*+ *PhaA*
JC7623AB	JC7623 integated with pBRAB
JC7623C1J1	JC7623 bearing pBPC1J1 plasmid
JC7623ABC1	JC7623 integrant harbouring pBPC1
JC7623ABC1J1	JC7623 integrant harbouring pBPC1J1

The *phaJ1* gene was cloned from a native soil isolate of *P. aeruginosa* (P7, Chapter 3) and *phaJ4* was isolated from *P. aeruginosa* 01. *E coli* DH5α was used a host strain for general cloning. The *E. coli* strains DH5α, BL21 and LS1298Kan::fadB1 were cultured at 37°C in LB medium. *E. coli* JC7623 obtained from the Vector Collection Center, Natl. Inst. Genetics, Japan was used as the host strain for integration. Acetoacetyl CoA reductase (EU239691) and the β-ketothiolase (*phaA*) (EU239690) genes from

Bacillus sp were cloned in pBRINTcm to obtain pBRAB. (pBRAB was procured from laboratory stock, CFTRI, Anilkumar, 2007, unpublished data) Recombinant strains were maintained on semisolid LB agar with 100 $mg1^{-1}$ Ampicillin sodium salt, at 4°C and subcultured once a month.

4.2.2 Molecular biology methods

4.2.2.1 Isolation and cloning of phaJ1 and phaJ4

Oligonucleotide primers were designed for the detection and cloning of (R)-specific enoyl CoA hydratase 1 and 4 (*phaJ1* and *phaJ4*) gene of *Pseudomonas spp,* based on the sequences available in the data bank (Accession no. AB040025, AB075927). The primers were so designed that the amplicon would carry a 5' stop codon (bold letters), consensus Shine-Dalgarno sequences (underlined) before the coding region and a 3' stop codon. The primers were synthesized by Sigma Aldrich, India and the sequences are given in table 4.2.

Table 4.2 Primers used for the amplification of *phaJ1* and *phaJ4* genes

Gene	Name of the primer*	Primer sequences
PhaJ1	PAPJ1F	5'**TAA**AGGAAACAGCTAATGCCATTCGTACCCGTAGC3'
	PAPJ1R	5'TCAGACGAAGCAGAGGCTGA3'
PhaJ4	PAPJ4F	5'**TAA**AGGAAACAGCTAATGAGCCAGGTCCAGAACAT3'
	PAPJ4R	5'TCAGCCGATGCTGATCGGCG3'.

** F: forward, R: Reverse*

The amplification programme for *phaJ1* and *phaJ4* set of primers consisted of 35 cycles for 1 min at 94°C, 1 min at 50°C and 1 min at 72°C for denaturation, annealing and polymerization respectively. Authenticity of the PCR products was confirmed through semi nested PCR and restriction digestion. The PCR amplicons were purified using the gene elute kit (2.4.3) and cloned into the T-tailed vector pTZ57R/T (2.4.5). The resultant plasmids pTPJ1 and pTPJ4 were transformed into *E. coli* DH5α (2.4.6). Presence of the insert was checked by PCR and restriction. Direction of the inserted gene with respect to *lac* promoter was checked by the method described in 3.2.5.4.1 using SalI enzyme. The clones were sequenced and assigned accession number (EU333913) for phaJ1.

4.2.2.2 Sub cloning of phaJ4/phaJ1 into pBPC1

An EcoR1/HindIII fragment bearing either *phaJ1* or *phaJ4* gene was released from pTPJ1 and pTPJ4, respectively and cloned into the plasmid pBPC1, downstream to the *lacZ* promoter (Figure 4.1). The reactions for restriction digestion and ligations were set as described in 3.2.5.4.2**.** The constructs, named pBPC1J1 and pBPC1J4 were used for transformation of *E. coli* strain, BL21 and a *fadB* mutant of *E. coli* (LS1298 Kan: *fadB1*).

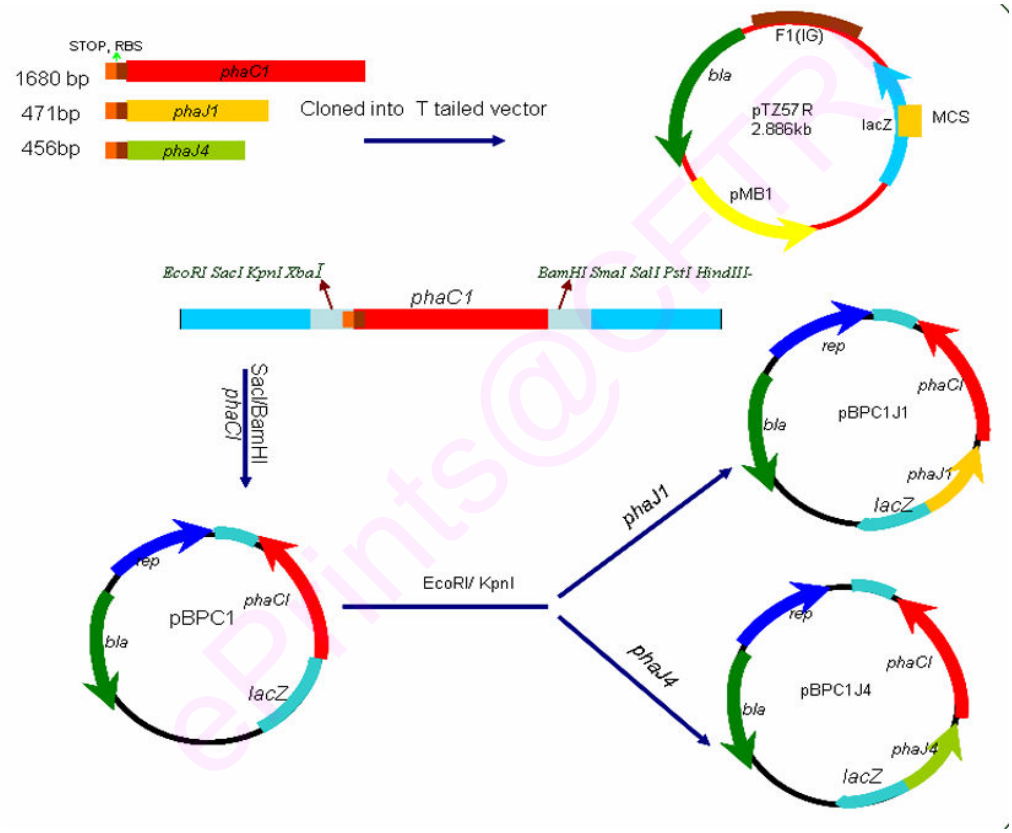


Figure 4.1 Schematic representation of cloning of *phaC1*, *phaJ1* and *phaJ4* into pBSPIIKS(-)

4.2.2.3 Transformation of E. coli JC7623

PBRBAcm was used to transform *E. coli* JC7623 using Chloramphenicol as selection agent. White colonies were selected using the X-gal-IPTG method and the presence of the two genes in the host bacterial chromosome was confirmed by PCR. The recombinant strain wherein the two *Bacillus* genes integrated into the *lacZ* gene of *E. coli*

JC7623 was named JC7623AB. This bacterium was then subjected to a second transformation using pBPC1J1 and the resultant recombinant was named as JC7623ABC1J1. Ampicillin (100 $mg1^{-1}$) was used to select these transformants. *E. coli* JC7623 transformed with pBPC1J1 and JC7623AB transformed with pBPC1 were used as controls.

4.2.2.4 Protein expression in recombinant E. coli

The expression of the two proteins in *E coli fadB* mutant and all the four proteins in *E. coli* JC76ABC1J1 was monitored on a 12 % (w/v) SDS-PAGE (2.4.13). The codon bias score of these four proteins in *E. coli* was calculated using the soft ware Codon Usage Analyzer. The molecular mass of the proteins were calculated using the software ExPASy compute pI/Mw tool.

4.2.2.5 Enzyme activity analysis

Activity of enoyl CoA hydratase was measured by assaying the hydration of crotonyl CoA at 263 nm (Snell *et al*., 2002., Park and Lee, 2003., Fukui *et al*., 1998). Cells harvested after 12h, were lysed by sonication in lyses buffer (50 mM tris pH 8, 1mM EDTA, 10mM β- mercapto ethanol and 0.5 mM PMSF). 10 µl of the clarified lysate was added to 990µl of 50 mM tris-HCl containing 25 µM crotonyl CoA (Sigma Chemicals Company) in a quartz cuvette with 1cm light path. Decrease in absorbance at 263nm was measured at 30°C. The extinction coefficient of the enoyl thioester bond is 6.7×10^3 $\mu M^{-1}cm^{-1}$. One unit of enoyl CoA hydratase activity was defined as the removal of 1µmol of crotonyl CoA per min. The specific activity of enoyl CoA hydratase was defined as the activity of enoyl CoA hydratase per milligram of protein.

4.2.3 Culture conditions for PHA production by E. coli fad mutant and E coli BL21

E. coli fadB mutant and *E. coli* BL21 were cultivated in 50 ml LB broth along with carbon substrate as detailed in 2.2.4.1. A fermentor trial with *fadB* mutant bearing pBPC1J4 was carried out as described in 2.2.4.2.

4.2.4 Culture condition for PHA production by E. coli JC7623 recombinants

JC7623ABC1J1, JC7623ABC1, and JC7623C1J1were cultivated in 50ml of PHA production medium for recombinant *E. coli* (2.1.5.4) with different combination of substrates as described in 2.2.4.1. Acrylic acid (Hi Media; 0.1g l^{-1}) was added to block β-oxidation of fatty acids at 24h of incubation.

4.2.5 Analytical procedures

PHA granules were stained using Sudan black and observed under light microscope or phase contrast microscope (2.2.6.2). PHA, extracted and quantified from dried cells (2.3.2.1) is expressed as % CDW. The monomer composition of the extracted PHA was confirmed using GC/MS (2.3.6) and NMR (2.3.7).

4.3 RESULTS

4.3.1 Cloning of phaJ1 and phaJ4

PCR fragments of the expected size of 471bp and 456bp for *phaJ1* and *phaJ4,* respectively, were obtained from genomic DNA of *Pseudomonas* sp using primers detailed in table 4.2 and separated on 1.5 % agarose gels. The amplicon size was determined using a 100bp DNA ladder run alongside the sample (Figure 4.2). Restriction digestion and nested PCR authenticated the amplicon (Figure 4.2). The purified amplicons were cloned initially into the T-tailed vector pTZ57R/T to yield the plasmids pTPJ1and pTPJ4. Plasmids isolated from the transformants were restricted for the release of insert (Figure 4.3). The direction of the insert with respect to *lacZ* promoter was checked through SalI digestion.

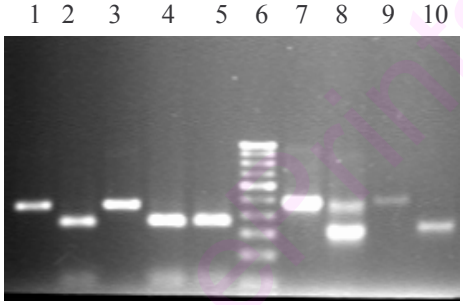


Figure 4.2 PCR, nested PCR and restriction digestion of *phaJ1* and *phaJ4* genes

Lane 1&3: 471bp amplicon of *phaJ1*
Lane 2, 4 &5: SalI digestion of 471bp amplicon
Lane 6: 100bp DNA ladder
Lane 7&9: 456bp amplicon of PhaI4
Lane 8&10: 320 bp seminested amplicon of *phaJ1*

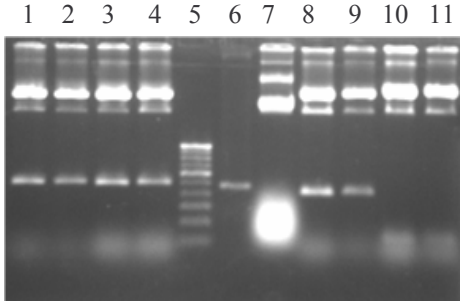


Figure 4.3 Release and direction check of the insert from pTPJ1 and pTPJ4 .

Lane 1&2: 471bp Insert released from pTPJ1
Lane 3& 4: Insert released from pTPJ4
Lane 5: 100bp DNA ladder
Lane 6: 456 bp amplicon of *phaJ4*
Lane 7: Undigested plasmid
Lane 8&9: Sal1 digestion of pTPJ1
Lane 10&11: Sal1 digestion of pTPJ4

Sequencing allowed the authentication of the clones. The sequence of PhaJ1 from the P7 isolate was found to have 98% homology with that of PhaJ1of *P. aeruginosa* as determined using the BLAST programme (Altschul *et al.,*1997).

Amino acid sequence of PhaJ1 from *Pseudomonas* isolate P7

M S Q V Q N I P Y A E L E V G Q K A E Y T S S I A E R D L Q L F A A V S G D R N P V H L D A A Y A A T T
Q F K E R I A H G M L S G A L I S A A I A T V L P G P G T I Y L G Q T L R F T R P V K L G D D L K V E L E V
L E K L P K N R V R Met A T R V F N Q A G K Q M V D G E A E I M A P E E K L S A E L A E L P P I S I G

Amino acid sequence of PhaJ4 from *P. aeruginosa*

M P F V P V A A L K D Y V G K D L G H S E W L T I D Q E R V D Q F A E C T G D H Q F I H V D P E K A A K
T P F G G T I A H G F L S L S L I P K L M E G L L V L P E G L K M A V N Y G L D T V R F I Q P V R V G S R
V R L G L T L L D V N E K N P G Q W L I K A R A T L E I E G Q E K P A Y I A E T L S L C F V

4.3.5 Co expression of phaC1 and phaJ1/ phaJ4

In order to analyze the effect of co-expression of hydratase genes with *phaC1* gene on PHA accumulation and composition of PHA, phaJ1 and *phaJ4* was sub cloned into pBPC1 plasmid individually. The resultant plasmids, pBPC1J1 and pBPC1J4, containing *phaJ1* and *phaJ4* upstream of *phaC1* gene, were transformed into *E. coli* BL21 and *LS1298* Kan::*fadB*. Expression of PhaC1 and PhaJ1/ PhJ4 based on plasmids pBPC1J1 and pBPC1J4 was confirmed by the identification of additional proteins with an apparent molecular mass of about 64 kDa, corresponding to PhaC1 and 17 kDa corresponding to PhaJ1 and phaJ4, respectively, using SD*S*-PAGE (Figure 4.4) in comparison to that of control. The calculated molecular mass of PhaJ1, PhaJ4 and PhaC1 are given in the table 4.3 along with the codon bias score (The tendency for an organism or virus to use certain codons more than others to encode a particular amino acid)

Table 4.3 Molecular mass of proteins and codon bias score

Protein	Calculated molecular weight	Codon bias score
PhaC1	62.5	0
PhaJ1	16.9	7
PhaJ4	16.7	0
PhaA	43.0	22
PhaB	29.0	20

The expressed proteins were found in the supernatant indicating the production of the enzymes in soluble form. There was no evidence for the accumulation of inclusion

bodies. This may be because of the use of *Lac* promoter and IPTG (lower levels of expression). Expression of these proteins as discrete units with their own start and stop codons may also be responsible for allowing synthesis of enzyme in soluble form. The intensity of the PhaJ1 and PhaJ4 bands were greater than the intensity of PhaC1 band. The hydratases were synthesized in larger amounts than was PhaC1, attributable perhaps to the difference in their size.

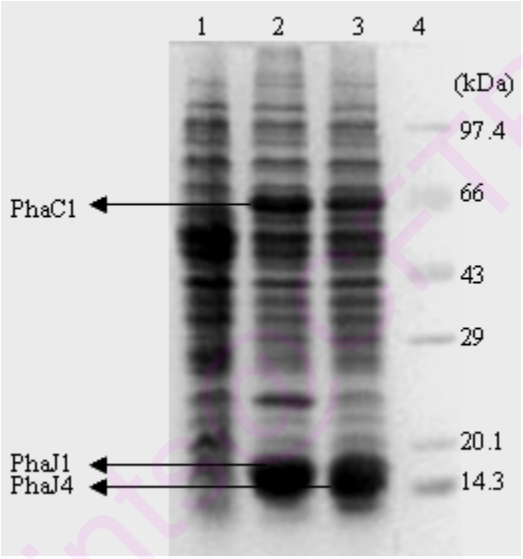


Figure 4.4 SDS-PAGE analysis of cell lysate of recombinant *E. coli* LS1298 bearing different plasmid constructs

Lane 1: Total protein from *E. coli* harbouring pBSP II KS (-) (negative control), Lane 2:Total protein from *E. coli* harbouring pBPC1J1 (PhaC1 and PhaJ1), Lane 3: Total protein from *E. coli* harbouring pBPC1J4 (PhaC1 and PhaJ4), Lane 4: Molecular mass standard protein with the masses indicated on the right.

4.3.6. Protein expression in JC76ABC1J1

The proteins in recombinant *E. coli,* incubated with the *LacZ* inducer ITPG, was observed after separation on SDS-PAGE (Figure 4.5). Expression of PhaC1, PhaJ1, PhaA and PhaB was confirmed by the presence of additional proteins with apparent molecular mass of about 64 kDa, (PhaC1), 17 kDa (PhaJ1), 41 kDa (phaA) and 27 kDa (PhaB), that were absent in the control (lane 1, Figure 4.5). This corresponds to the molecular weight deduced form the nucleotide composition of the four genes (Table 4.3).The expressed proteins were found in the supernatant indicating the production of the enzymes in

soluble form. Expression of these proteins was maximum at 0.4mm concentration of IPTG. The highest amount of protein was produced after 6 h of induction.

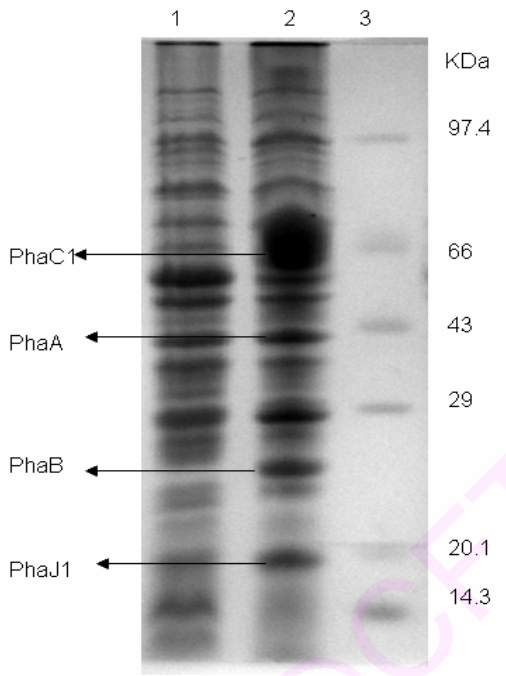


Figure 4.5 SDS-PAGE analysis of crude extract of *E. coli* JC7623ABC1J1

Lane 1: Crude extract of *E. coli* JC7623, Lane 2: Crude extract of *E. coli* JC7623ABC1J1, Lane3: Molecular mass standard protein with the masses indicated on the right

4.3.6 Enoyl CoA hydratase assay

Crude extracts from recombinant *E. coli* bearing various constructs were assayed for activity of (R)-specific enoyl CoA hydratase by the hydration of *trans* 2 enoyl CoA and the results are given in Table 4.4. Extract from the control *E. coli* strain LS1298

Table 4.4 Enoyl CoA hydratase activity of *E. coli* BL21 and *E. coli fadB* mutant harboring different plasmids

Bacterial strain	Plasmid	Specific activity (Umg^{-1} of protein)
E. coli BL21	pBSP II KS(-)	18±1
E. coli BL21	pBPC1J1	108±4
E. coli BL21	pBPC1J4	144±2
E. coli fadB mutant	pBSP II KS(-)	0
E. coli fadB mutant	pBPC1J1	101±3
E. coli fadB mutant	pBPC1J4	149.2±2

carrying the plasmid pBSP II KS (-) was inactive towards crotonyl CoA. Cell lysate derived from the strain LS1298 harboring pBPC1J1 and pBPC1J4 were very active towards the hydration of crotonyl-CoA. Cell lysate of *E. coli* harboring pBPC1J4 was active to a higher level than that from cells harboring pBPC1J1. Similar results were obtained with the strain BL21 carrying the same plasmid with the exception that the control cells showed measurable levels of native enoyl CoA hydrates activity

Higher enoyl CoA hydratase activity was detected in the supernatant of the recombinant strains (JC7623CIJ1, JC7623ABC1J1) as compared to that of the control strain (JC7623) after the addition of IPTG and further cultivation for 6h. The activities measured are given in the table 4.5.

Table 4.5 Enoyl CoA hydratase activity of *E. coli* JC7623 recombinants

Bacterial strain	Description	Specific activity (Umg-^{1}of protein)
JC7623	Control strain without plasmid	4
JC7623CIJ1	Recombinant bearing *phaC1* and *phaJ1*	107
JC7623ABC1J1	Recombinant bearing *phaA, phaB, phaC1* and *phaJ1*	105

4.3.7 Production and composition of PHA in recombinant E. coli harbouring pBPC1J1 and pBPC1J4

Biosynthesis of PHA in *E. coli* BL21 and a *fadB* mutant as a consequence of the co expression of PHA synthase1 and enoyl CoA hydratase1 or 4 from *P. aeruginosa* is seen in Figure 4.4. Data on PHA production by recombinant *E. coli* BL21 and LS1298Kan::*fadB1* grown on various substrates is summarized in table 4.6 and 4.7. The *fadB* mutant of *E. coli* harbouring pBPC1J1 and pBPC1J4 produced 20.6% and 18% of PHA from octanoate, in contrast to 10.4% and 13.5% produced by the *E. coli* BL21 strain. A similar pattern was obtained on feeding these recombinants with other substrates. The PHA was found within the cytoplasm as granules (Figure 4.6 & 4.7)

E .coli BL21, bearing either pBPC1J1 or pBPC1J4 construct, synthesized PHA with less hydroxy butyrate and more mcl-monomers (Table 4.7) But the *fadB* mutant of *E. coli* containing either pBPC1J1 or pBPC1J4 plasmid produced higher levels of PHA (Figure 4.8 and 4.9), rich in hydroxy butyrate and medium chain length hydroxy fatty acid (Table 4.6). In both cases, there was a difference in the composition of the polymer

depending on the substrate used for growing the recombinant bacterium. It was able to achieve PHA production of 22.5% CDW in 24 h using *fadB* mutant bearing pBPC1J4 in a fermentor (Table 4.8).

Table 4.6 Production and composition of PHA by recombinant *E. coli fadB* mutant bearing pBPC1J1and pBPC1J4 in shake flask culture

Substrate	PHA content (% CDW)		PHA composition (mol %)									
	pBPC1J1	pBPC1J4	pBPC1J1					pBPC1J4				
			HB	HHx	HO	HD	HA	HB	HHx	HO	HD	HA
Glucose	24.8±0.34	25.4±0.2	40	1	21	26	12	47	0	33	11	9
Octanoate	20.6±1.85	18±0.5	50	0	20	16	14	51	6	18	10	15
Decanoate	18.8±2.77	20.3±1.25	39	9	23	22	7	57	7	0	26	10

The values presented here are mean of three independent measurements. CDW: Cellular Dry Weight. HB: hydroxy butyrate, HH: hydroxy hexanoate, HO: hydroxy octanoate, HD: hydroxy decanoate and HA: higher hydroxy alkanoates.

Table 4.7 PHA production and molar ratio of PHB to mcl–PHA in *E. coli* BL21 harbouring pBPC1J1and pBPC1J4 in shake flask culture

Substrate	PHA content (% CDW)		PHB: mcl-PHA	
	pBPC1J1	pBPC1J4	pBPC1J1	pBPC1J1
Glucose	13.2±1.0	10.0±1.5	1:99	1:99
Octanoate	10.4±0.5	13.5±1.5	1:99	2:98
Decanoate	8.0±1.7	15.4±1.9	2:98	1:99

The values presented here are mean of three independent measurements. CDW: Cellular Dry Weight.

PHA produced by the *fadB* mutant of *E. coli* cells (bearing pBPC1J1 or pBPC1J4), when fed with glucose octanoate or decanoate, was rich in butyrate. There was difference even in the composition of the mcl-fractions of the PHA produced by these cells. The medium chain length fractions of PHA produced by *fadB* mutant of *E. coli* cells, bearing pBPC1J1, contained more hydroxy octanoate in contrast to the cells bearing pBPC1J4 which contained more hydroxy decanoate.

Table 4.8 PHA production by *E. coli fadB* mutant bearing pBPC1J4 in a fermentor at different growth periods

Time (h)	Biomass (mg%)	PHA (%CDW)
6	60	5.0

12	100	15.0
18	265	16.9
24	355	22.5

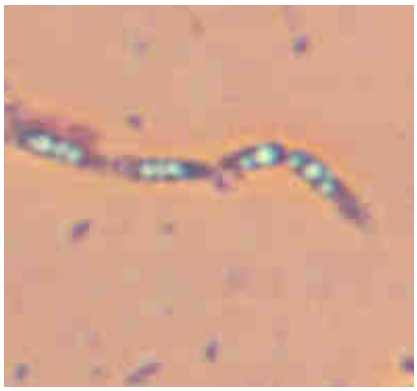


Figure 4.6 PHA granules in *fadB* mutant *E. coli* bearing pBPC1J1

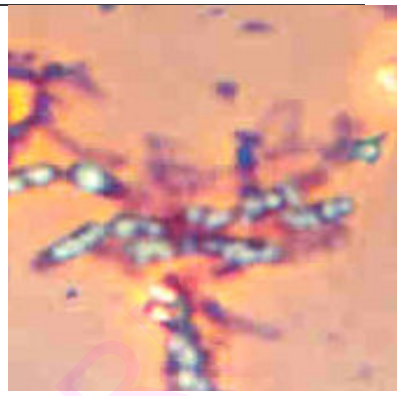


Figure 4.7 PHA granules in *fadB* mutant *E. coli* bearing pBPC1J4

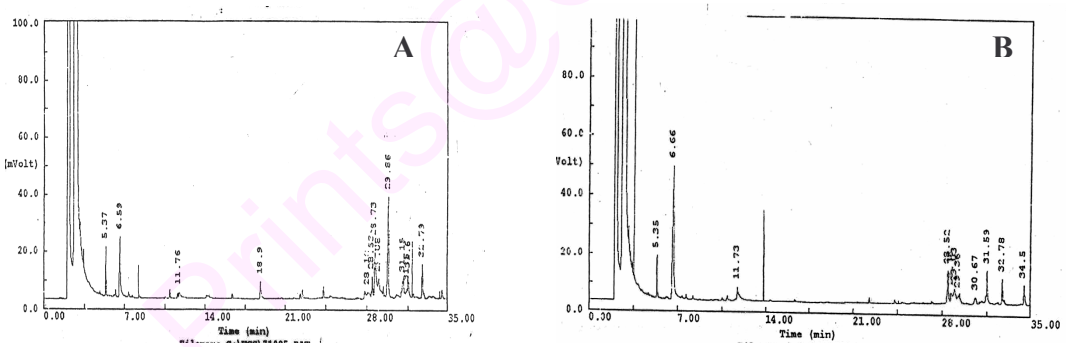


Figure 4.8 Gas chromatogram of methyl ester of PHA extracted from *fadB* mutant *E. coli* bearing pBPC1J1 and pBPC1J4 grown on octanoate
A) pBPC1J1, B) pBPC1J4

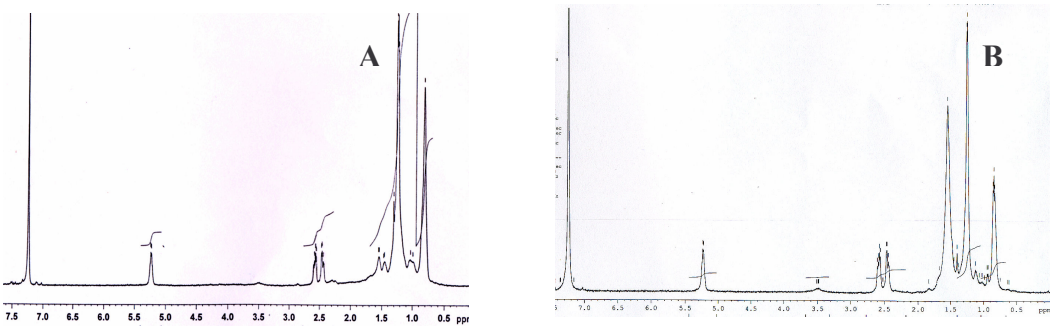


Figure 4.9 ^{1}HNMR spectra of PHA extract from *fadB* mutant *E. coli* harbouring pBPC1J1 and pBPCIJ4 grown on octanoate
A) pBPC1J1, B) pBPC1J4

4.3.8 Growth and PHA production by recombinant E. coli JC7623C1J1 and JC7623ABC1

PHA production of 8-15 % was observed in *E coli* JC7623C1J1 bearing both *phaC1 and PhaJ1* grown in different substrates. The proportion of butyrate to that of the mcl was less than 2%. In order to understand the origin of butyrate in this system, we transformed *E. coli* containing the *Bacillus* ketothiolase and reductase with only the *Pseudomonas phaC1*. The cells were found to produce 9-10 % CDW of PHA in 48h of growth in glucose. The PHA produced by these cells was rich in butyrate.

4.3.9 Growth and PHA production by recombinant E. coli JC7623ABC1J1

In *E. coli* JC7623ABC1J4 expressing four genes and grown on different fatty acid along with glucose PHA production varied from 22-33% of the biomass, Addition of fatty acids at 12 h of incubation resulted in greater production of PHA in the recombinant in comparison with those grown only in the presence of glucose. The PHA polymer was deposited as granules within the cells (Figure 4.10). The yield of PHA was greater in the presence of octanoate and hexanoate while the yield of biomass was highest in cells grown in the presence of decanoate. Growth of the recombinant and production of PHA was least in the presence of nonanoate and heptanoate.

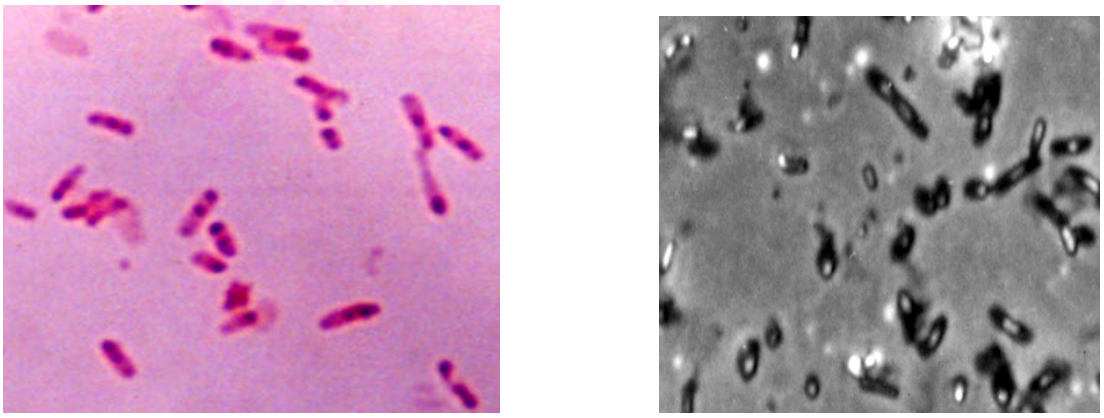
PHA produced by the *E. coli* JC7623ABC1J4 in comparison with that produced by *E coli* JC7623C1J1 contained higher amount of hydroxy butyrate. GC and GCMS data indicated that the butyrate content of the PHA ranged from 21-74%. Incorporation of butyrate was maximal when cells are grown in the presence of glucose alone and with glucose and butyrate (Table 4.9.). Medium chain fatty acids incorporated into the polymer in the presence of most of the carbon sources tested was six to twelve carbons in length with hydroxy octanoate being predominant. ^{1}HNMR data indicated that recombinant *E. coli* was able to produce hydroxyvalerate copolymer from glucose and also when valerate was provided as co carbon substrate (Figure 4.11). Addition of acrylic acid resulted in improved production of PHA in this recombinant in spite of fall in

biomass. The recombinant produced 39 and 44% PHA when fed with decanoate and octanoate respectively.

Table 4.9 Production and composition of the PHA by recombinant *E. coli* JC7623ABC1J1

Substrate	Without acrylic acid		With acrylic acid		Without acrylic acid PHA Composition (mol %)					
	Biomass Mg %	PHA %CDW	Biomass Mg %	PHA %CDW	HB	HV	HHx	HO	HD	HDD
Glucose	122±2.0	28±1.0	118±3.5	28± 1.2	74.2	3.7	0.5	14.8	3.3	3.3
Glucose+ Butyrate	134±4.0	33±0.5	126±2.0	36±1.0	70.4	0.2	1.4	14.0	14.0	-
Glucose+ Valerate	136±1.5	28±0.5	114±2.5	32±2.0	26.9	2.4	1.7	55.2	6 .0	7.0
Glucose+ Hexanoate	150±2.5	33±2.0	118±1.0	39±0.5	23.7	0	16.0	40.2	8.7	11.2
Glucose+ Octanoate	116±3.0	33±1.5	88±5.0	44±2.0	24.7	0.5	8.0	41.2	16.4	16.4
Glucose+ Decanoate	152±1.5	34±1.8	136± 3.0	39±1.0	18.0	0	4.0	10.0	64.0	3.0

The values presented here are mean of three independent measurements. CDW: Cellular Dry Weight. HB: hydroxy butyrate, HV: hydroxy valerate, HH: hydroxy hexanoate, HO: hydroxy octanoate, HD: hydroxy decanoate and HDD: hydroxy dodecanoate.



A B

Figure 4.10 Microscopic observations of PHA granules in JC7623ABC1J1

A:Light microscopy (Sudan black staining), B: Phase contrast microscopy,

The yield of PHA was greater in the presence of octanoate and hexanoate. Highest yield of biomass was obtained in the presence of decanoate. Growth and PHA production was least when grown in the presence of nonanoate and heptanoate (data not presented).The PHA produced contained good amount of hydroxy butyrate compared to that of the control. The butyrate content ranged from 21-74% of the PHA. Maximum butyrate incorporation was noted when the cells were grown in the presence of glucose and butyrate (Table 4.9). Medium chain fatty acids incorporated into the polymer were six to twelve carbons in length. Among the mcl monomers the hydroxy octanoate content of the PHA was found to be more compared to other monomers, in the presence of most of the carbon source tested. Recombinant *E. coli* was able to produce hydroxyvalerate copolymer when valerate is provided as co carbon substrate and also from glucose. Addition of acrylic acid resulted in the improved production of PHA in this recombinant even though there was a fall in the amount of biomass. Due to the inhibitory effect of acrylic acid the recombinant produced 39 and 44% PHA when fed with decanoate and octanoate respectively.

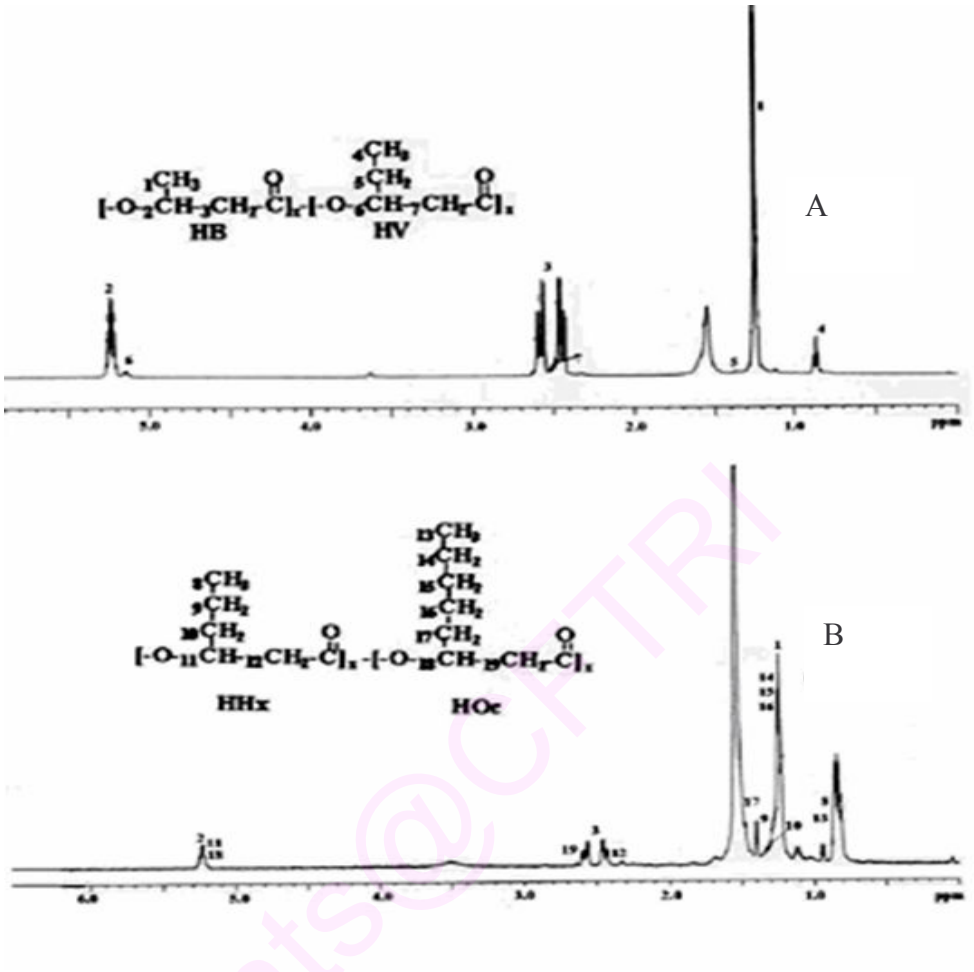


Figure 4.11 ^{1}HNMR spectra of PHA isolated from recombinant *E. coli* JC7623ABC1J1

A: Standard P(HB-co-HV),B: Recombinant *E. coli* JC7623ABC1J1 grown on glucose and butyric acid.

4.4 DISCUSSION

Recombinant *E. coli* and *E. coli* with defective β-oxidation pathway successfully synthesized mcl-PHAs from fatty acids when equipped with a functional mcl-PHA synthase (Qi *et al*., 1997; 1998; Ren *et al*., 2000; Park *et al*., 2002; Ren *et al*., 2005). Various enzymes including FadD, FadE, enoyl CoA hydratase, epimerase and 3-ketoacyl-CoA reductase are involved in the generation of R-3-hydroxyacyl CoAs, the substrate for PHA synthase from fatty acids. Because these enzymes compete with the β-oxidation pathway enzymes for intermediates, it was suggested that high level expression of these genes is necessary for the construction of a metabolic link between β-oxidation

and PHA biosynthesis. Initially the PHA synthase1 from *P. aeruginosa* was expressed in *E. coli* BL21 and a *fad B* mutant and this lead to the synthesis of detectable amount of PHA (Chapter 3).

The synthesis of PHA in *E. coli* BL21 and a *fadB* mutant of *E. coli* using the PHA synthase1 (*phaC1*) and enoyl CoA hydratase1 (*phaJ1*) or enoyl CoA hydratase4 (*phaJ4*) from *P. aeruginosa* have been demonstrated further. In a *fadB* mutant *E. coli,* β-oxidation intermediates accumulate and it is presume that the heterologous expression of (R)-specific enoyl CoA hydratase would boost the production of PHA (Park *et al*., 2005). This may account for the greater production of PHA in the *fadB* mutant observed in this study. Though earlier workers (Qi *et al*., 1997; 1998; Langenbach *et al*., 1997; Park *et al*., 2002; Ren *et al*., 2005) have demonstrated the synthesis of PHA in *fad* mutants of *E. coli,* these results differ from theirs in that we have used the PhaC1 and enoyl CoA hydratases from *P. aeruginosa* in a *fadB* mutant.

Present data indicates that the PHA produced in recombinant *E. coli* contains both hydroxybutyrate and mcl-monomers. The production of mcl-PHA alone, when *Pseudomonas phaC1* or *phaC2* was expressed in *E. coli* has been reported by earlier workers (Qi *et al*., 1997; 1998; Ren *et al*., 2000; Park *et al*., 2002). These workers did not provide the PhaC1 enzyme with a source of hydroxybutyryl CoA. The *phaC2* of *P. stutzeri* (Chen *et al*., 2004; Chen *et al*., 2006) and *Pseudomonas* sp. 61-3 (Tsuge *et al*., 2003) produced PHA containing hydroxybutyrate only when co-expressed with enzymes capable of providing hydroxy butyryl CoA such as PhaA and PhaB of *R. eutropha* or PhaJ1 of *P.* aeruginosa. The results presented here and those of others (Timm and Steinbuchel, 1992) suggest that PHA synthesis in heterologous systems might allow an even greater range of monomers to be incorporated than in homologous systems. The substrate–supplying pathway for PHA synthase in host cell is hence important for control of composition of PHA.

The *fadB* mutant cells of *E. coli* bearing pBPC1J4 accumulated more hydroxy butyrate than cells bearing pBPC1J1 in the presence of glucose and decanoate. Studies with different substrates varying in carbon chain length suggested that only the PhaJ1 among the four PhaJs from *P. aeruginosa* is active in the production of shorter chain length enoyl CoAs of C_4-C_6 (Tsuge *et al*., 2003). Since *E. coli* bearing pBPC1J4

construct accumulated PHA, rich in hydroxy butyrate, our result fail to concur with this report. The greater availability of substrates in the *fadB* mutant, as compared to that available in *E. coli* BL21, may explain the enriched butyrate content in PHA. Even though in a *fadB* mutant one of the key enzymes of β-oxidation pathway was inactivated, intermediates lacking 2, 4 or 6 carbon units were still generated (Table 4.6). Similar results were also obtained by earlier workers (Qi *et al*., 1997; 1998; Langenbach, 1997). These results suggest that enzymes having similar function to FadB exist in *E. coli* to supply those intermediates (Snell *et al*., 2002; Park and Lee, 2003; Park and Lee, 2004a). Hydroxy acyl CoA must be generated in *E. coli* from both β-oxidation and fatty acid synthesis and the enzyme thioesterase (Figure 4.12) probably interconnect the two pathways (Klinke *et al*., 1999). This may account for the production of PHA in *E. coli* from glucose. There was noted difference in the mcl-fraction of the PHA, produced by *E. coli* bearing pBPC1J1 and PBPC1J4. This may be due to the difference in the specificity of hydratase 1 and 4 towards various enoyl CoAs. PhaJ1 was found to be more active in the hydration of octanoyl CoA and PhaJ4 was more active towards the hydration of decanoyl CoA independent of the substrate used for the cultivation of the recombinant strains.

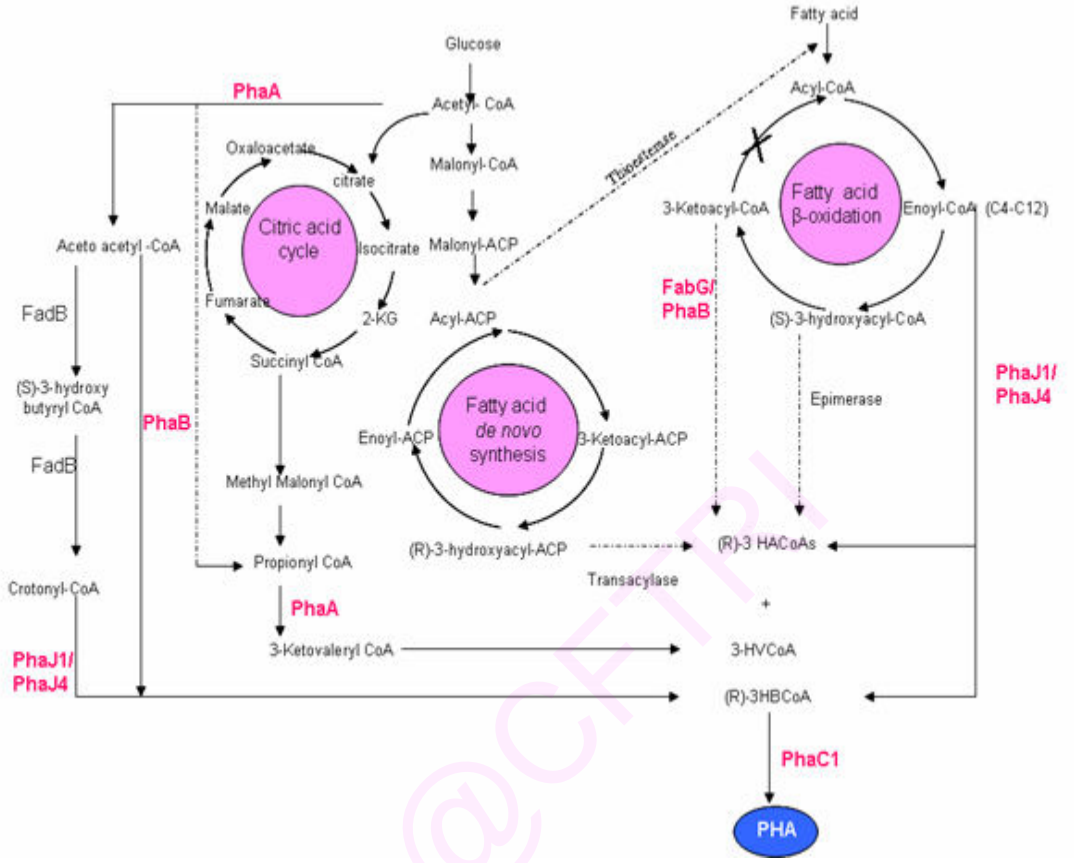


Figure 4.12 Proposed pathway of the synthesis of copolymer in recombinant *E. coli*

Postulated pathway, depicting to the synthesis of PHA, in *E. coli* from glucose and fatty acids. Enzymes that have been over-expressed during the course of this study are represented with bold characters. The cross denotes inhibition of enzyme. PhaA: ketothiolase, PhaB: NADPH dependent acetoacetyl CoA reductase, PhaC: Pha synthase, PhaJ: (R)-specific enoyl CoA hydratase, (R)-3HBCoA: (R)-3-hydroxy Butyryl Coenzyme A, (R)-3HACoA: (R)-3-hydroxy alkannoyl Coenzyme A, (R)-3HVCoA: (R)-3-hydroxy valeryl Coenzyme A. (Fukui *et al*.1997; Park *et al*. 2005; Sato *et al*. 2007; This work).

There has recently been reports on the use of hybrid pathways or modified pathways for the production of PHA copolymer in recombinant *C. necator* (Fukui *et al*., 2002; Kahar *et al*., 2004) and *Aeromonas* (Luo *et al*., 2006; Qin *et al*., 2007). *phaA and phaB* genes of *C. necator* and *phaC* gene from *Pseudomonas* sp. 61-3 when introduced into *E. coli* fadA and/or fadB mutants, could synthesize PHA consisting of C4, C6, C8, and C10 monomer units (Park and Lee, 2004). It has been reported that recombinant *E. coli* bearing the PHA biosynthetic genes from *Aeromonas* could synthesize a PHA terpolymer of 3-hydroxybutyrate, 3-hydroxyvalerate, and 3-hydroxyhexanoate when dodecanoic acid and odd-carbon number fatty acids were used as carbon sources (Park *et*

al., 2001b). Nomura *et al*. (2004) showed that the cumulative effect of two monomer-supplying pathways (fatty acid biosynthesis and β-oxidation) using fabH, 3-ketoacyl-ACP synthase and fabG, 3-ketoacyl-acyl carrier protein reductase, respectively, and a genetically engineered enzyme (fabH), resulted in the accumulation of scl–mcl copolymer in *E. coli* from non related carbon substrates. The yield of PHA obtained by all these researchers was low in recombinant *E. coli* even though production of co-polymer occurred. In the present study different pathways have been brought together to produce PHA of desired amount and type in *E. coli*. In order to attain the expression of all the four proteins simultaneously, the 4 genes were cloned down stream of the *lac* promoter. The two *Bacillus* genes were integrated into the *lacZ* gene of *E. coli* to decrease stress from additional plasmid and antibiotics and to allow cloning of multiple genes. Much higher levels of PHA containing hydroxyl butyrate, hydroxyvalerate and hydroxy fatty acid were obtained in this study than hitherto reported.

In the experiments detailed here, PHB synthesis in *E. coli* JC7623ABC1 and scl-co-mcl-hydroxy alkanoates synthesis in *E. coli* JC7623ABC1J1 were obtained. Data shown in the ^{1}H NMR spectra indicated the ability of the *E. coli* JC7623ABC1J1strain to synthesize PHB-co-HV-co-mcl-PHA which is in accordance with the data reported in the literature (Labuzek and Radecka, 2001). The classical pathway was resurrected for the production of PHB from glucose in *E. coli* cells bearing *phaABC1*genes. This pathway is probably used by most bacteria and has been extensively studied in *C. necator* (Slater *et al*., 1988). Due to the action of ketothiolase and fadB enzymes formation of crotonyl CoA from acetyl CoA and subsequent hydration of crotonyl CoA by enoyl CoA hydratase into R- 3 hydroxy butyryl CoA (Figure 4.12) has been recently proposed by Sato *et al.* (2007). A portion of the butyrate accumulated in *E. coli* may derived in a similar way as postulated by Sato *et al.* (2007) since the activity of ketothiolase and enoyl CoA hydratase were enforced. The enhanced production of 3- hydoxylbutyryl CoA in the presence of glucose in the experiments outlined in this chapter would indicate that most of this component was derived from glucose. A portion of butyryl CoA may however also be derived from the synthesis or breakdown of fatty acid.

The present study indicates the presence of hydroxy valerate in the PHA obtained from JC7623AB cells transformed with pBPC1J1. The valerate present in the PHA may

have resulted from the action of ketothiolase on propionyl CoA (Figure. 4.12). It might be postulated that propionyl CoA has been derived from succinate and the succinate concentration inside the cells was increased due to citric acid in the medium. A review of the earlier literature indicated that the beta ketothiolase was involved in the delivery of valeryl CoA to the reductase were different from those that processed only butyryl CoA (Slater *et al*., 1998). The sequence of the *Bacillus* ketothiolase cloned in this study was similar (44.5 % homology) to that reported in *C. necate*r. This could probably be the reason for the production of PHV by the recombinant even though the enzyme was from *Bacillus*.

The enoyl CoA hydratase from *Pseudomonas* used in the present study clearly could contribute to the production of mcl-PHA as shown previously (Davis *et al*., 2008) and as seen in figure 4.12. This ability was maintained in the presence of the enzymes from *Bacillus* and hence was able to obtain PHA containing hydroxybutyrate, hydoroxyvalerate and medium chain length fatty acids. The greater proportion of octanoate in the polymer produced by the recombinant bacteria attributable to the selective hydration of octanonyl CoA by the PhaJ1 enzyme used in the study. Addition of acrylic acid in the medium can inhibit the 3-ketoacyl CoA thiolase that catalyses the formation of acetyl CoA from 3-keto acyl CoA. Inhibition of 3-ketothiolase would be expected to result in the accumulation of 3- keto acyl CoA which could serve as a precursor of PHA biosynthesis. 3- keto acyl CoA reductase is the enzyme that converts 3-ketoacyl CoA into (R)-3 hydroxy acyl CoA (Figure 4.12). The acetoacetyl CoA reductase of *Bacillus* species used in the present study has sequence similarity (44% homology) to 3-keto acyl CoA reductase of *Pseudomonas* spp. This might have again enhanced the conversion of accumulated 3-ketoacyl CoA into (R) -3 hydroxy acyl CoA in the presence of acrylic acid. *phaC1* of *P. aeruginosa* which was previously reported by Qi *et al*. (1997) and Lagenbach *et al.* (1997) was used in the present study for the synthesis of mcl-PHA in *E. coli*. Our study (Davis *et al*., 2008) and that of Timm and Steinbuchel (1992) revealed that the incorporation of monomer by PHA synthase enzyme is independent on the enzyme specificity but depended on the monomer provided by various biosynthetic pathways functional in the host cell.

The present study clearly demonstrates the biosynthesis of PHA copolymer of scl and mcl from glucose and fatty acids utilizing various pathways. It has been possible to obtain PHA as high as 33-44% CDW. It would appear that in this system, PHA production can be increased to higher levels by controlling filamentation (Wang and Lee, 1997) or by providing NADPH by incorporating another enzyme. Alteration of the codon bias of the enzymes from *Bacillus* to that of *E. coli* would also allow the synthesis of larger amounts of enzymes resulting perhaps in the synthesis of higher amounts of polymer. The work collated in this chapter indicates the possibility of blending pathways for the production of copolymer in *E. coli*.

5.0 ABSTRACT

Four (R)-specific enoyl CoA hydratases (PhaJ) interconnect the β-oxidation pathway with PHA biosynthesis in *P. aeruginosa.* The use of antisense technique and hyper-expression to delineate the role of two of these enzymes, PhaJ1 and PhaJ4 forms the basis of this chapter. *phaJ1* and *phaJ4* genes were cloned in the sense as well as anti sense direction with respect to *lac* promoter in a broad host range vector. *P. aeruginosa* transformants bearing these construct showed significant variation in the quantity and monomer composition of the PHA with respect to that of the control. It has been found that *P. aeruginosa* recombinant, bearing *phaJ1* antisense construct, fed with different fatty acids, produced PHA with less hydroxy octanoate (7% reduction) with a proportionate increase in other fractions. Recombinants bearing *phaJ4* antisense construct were found to contain less hydroxy decanoate (8% decrease) and more or less equal amount of hydroxy octanoate, compared to that of the control. PhaJ1 and PhaJ4 have been hyper expressed individually in *P. aeruginosa* and co expressed, with PHA synthase1 (PhaC1) of *P. aeruginosa*. *Pseudomonas* produced PHA with more hydroxy octanoate and decanoate (6-17 % increase) respectively when PhaJ1 and PhaJ4 were hyper-expressed individually or along with PhaC1. PhaJ1 and PhaJ4, are found to be involved mainly in the production of hydroxy octanoyl CoA and hydroxy decanoyl CoA, respectively, in *P. aeruginosa.* Experiments on heterologous expression has revealed that these hydratases could contribute PHB monomers to the PHA produced (4.3).

5.1 INTRODUCTION

The β-oxidation spiral of fatty acid degradation consists of four enzymatic transformations that reduce the acyl chain length by two carbons in each cycle, resulting in the formation of acetyl-CoA as the byproduct in each cycle. The four enzymes comprising the core of the β-oxidation cycle are acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase (Figure 5.1) and the reactions proceed via enoyl-CoA, a chiral 3-hydroxyacyl-CoA and 3-ketoacyl-CoA intermediates (Poirier *et al.,* 2006).When even chain fatty acids are used as the starting material, acetyl-CoA is formed as the ultimate product of the pathway itself. When odd

chain fatty acids are used as the starting material, the final round of oxidation forms propionyl-CoA, which is converted to the more common metabolic intermediate succinyl-CoA through the action of propionyl-CoA carboxylase and methylmalonyl-CoA mutase (Halarnkar and Blomquist, 1989; Kunau, *et al*., 1995).

Enoyl-CoA hydratase catalyzes the second step in the beta-oxidation pathway of fatty acid metabolism. This enzyme facilitates the syn-addition of a water molecule across the double bond of a trans-2-enoyl-CoA thioester (Figure5.1) resulting in the formation of a β-hydroxyacyl-CoA thioester (Agnihotri and Liu, 2003). Two stereoisomers of the product are used in different metabolic pathways, and, accordingly, enzymes with different stereo specificities are involved in each pathway. The enzyme responsible for the production of the *S*-isomer is the classical enoyl-CoA hydratase (crotonase) and is involved in degradation of fatty acids in mitochondria, peroxisomes, and bacterial cells. The eukaryotic mitochondrial enzyme has a specific activity for CoA thioesters of straight chain fatty acids with a broad range of chain lengths (C4–C16) (Waterson and Hil, 1972). It is a hexamer of identical subunits composed of 261 residues in the mature form (Minami-Ishii *et al*., 1989). On the basis of sequence alignments, several conserved residues such as, Glu144 and Glu164, were identified and shown to be important for the catalytic reaction that proceeds through an acid-base mechanism (Agnihotri and Liu, 2003).

However, the enzyme responsible for the production of the *R*-isomer, (*R*)-specific enoyl-CoA hydratase (PhaJ) or hydratase 2, or D-hydratase or (*R*)-hydratase) has been identified recently in mammals, yeast, and some polyhydroxyalkanoate producing bacteria. (R)-specific enoyl CoA hydratase catalyze an *R*-specific hydrating reaction of the fatty acid β-oxidation intermediates *trans*-2-enoyl-CoAs to (R)-3-hydroxyacyl-CoAs (Hisano *et al*., 2003). Many organisms have been found to possess (R)-hydratase as a monofunctional enzyme or as an (R)-hydratase domain (hydratase 2 domain) of a multifunctional enzyme (MFE). In mammals and yeast, this enzyme occurs as a domain of the peroxisomal multifunctional enzyme type 2 (MFE-2) (Poirier *et al*., 2006).

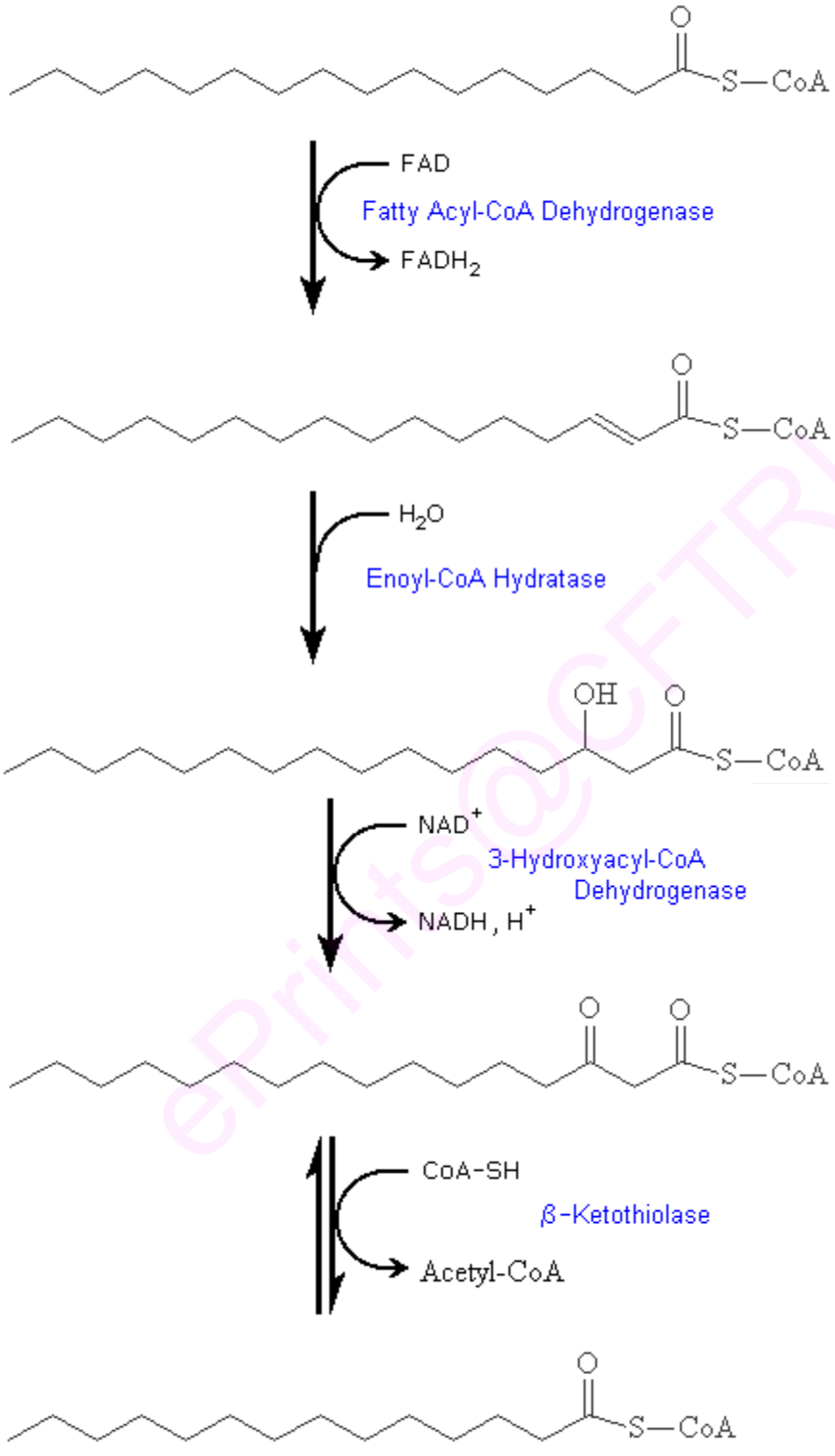


Figure 5.1 Pathway for the β-oxidation of fatty acid

(Courtesy: http://www.gwu.edu/~mpb/betaox.htm)

In mammals it is involved in the degradation of very long chain and 2-methyl-branched fatty acids and the biosynthesis of bile acids (Jiang *et al*., 1997) and in yeast it is involved in the degradation of straight chain fatty acids (Hiltunen *et al*., 1992) These eukaryotic enzymes are similar in size, composed of about 300 amino acid residues, and are highly homologous (Qin *et al*., 2000).

The eukaryotic (R)-hydratases commonly have a characteristic hydratase 2 motif, which has been defined by Qin *et al*., (2000) as [YF]-*X*(1,2)- [LVIG]-[STGC]-G-D-*X*-N-P-[LIV]-H-*X*(5)-[AS] by comparing the hydratase 2 domains of MFE-2s and several fungal and bacterial proteins. Sequence alignment by Hisano *et al*., (2003) showed that the bacterial (R)-hydratases with reference to that of *A. caviae* contain this motif with the glycine residue being replaced by Glu30. However, the major difference in enzymatic properties between eukaryotic and bacterial (*R*)-hydratases is acyl chain length substrate specificity. Eukaryotic (*R*)-hydratases, such as the *S. cerevisiae* MFE-2, have specificity for a substrate with an acyl chain length of 10 C atoms (Hiltunen *et al*., 1992). The PhaJ in *A. caviae* (Fukui and Doi, 1997) was the first characterized bacterial hydratase as a monomer supplying enzyme for PHA biosynthesis. In the case of *A. caviae*, (R)-hydratase ($PhaJ_{Ac}$) plays a critical role in supplying C4 and C6 monomer units from β-oxidation to PHA synthesis. *A. caviae* enzyme has significant sequence homology with the C-terminal region of the eukaryotic enzyme *eg.* 38.4% identity with the yeast enzyme for a limited region of 73 amino acid residues (Fukui and Doi, 1997), indicating that they are derived from a common ancestor.

The crystal structure of $PhaJ_{Ac}$ elucidated by Hisano *et al*., (2003) was reported to consist of a five-stranded antiparallel β-sheet and a central α-helix, folded into a so-called "hot dog" fold, with an overhanging segment. This overhang contains the conserved residues including the hydratase 2 motif residues. In dimeric form, two β-sheets are associated to form an extended 10-stranded β-sheet, and the overhangs obscure the putative active sites at the subunit interface. The active site is located deep within the substrate-binding tunnel, where Asp31 and His36 form a catalytic dyad. These residues are catalytically important as confirmed by site-directed mutagenesis and are possibly responsible for the activation of a water molecule and the protonation of a substrate molecule, respectively. Residues such as Leu65 and Val130 are situated at the bottom of

the substrate-binding tunnel, defining the preference of the enzyme for the chain length of the substrate. Tsuge *et al*., (2003) through site directed mutagenesis of *A. cavieae* hydratase at the positions Leu 65 (A/G) and Val130 (G) revealed that Leu-65 and Val-130 determine the length of the acyl chain substrate that may be accomodated. Single mutations altered the substrate specificity of this enzyme and the mutant enzyme was more active towards the hydration of octenoyl CoA than the wild type enzyme (Tsuge *et al*., 2003).

(R)-specific hydratase active towards short-chain-length enoyl-CoAs is also found in other PHA-producing bacteria, eg. *Rhodospirillum rubrum* (Moskowitz and Merrick, 1969; Reiser *et al*., 2000) and *Methylobacterium rhodesianum* (Mothes and Babel, 1995). In these bacteria (R)-3HB unit is supplied from two acetyl-CoA molecules via four-step reactions catalyzed by β-ketothiolase, NADH-acetoacetyl-CoA dehydrogenase, crotonase [(*S*)-specific enoyl-CoA hydratase] and (R)-specific enoyl-CoA hydratase.

YfcX, which is homologous to FadB (Crotonase), is found to be necessary for the mcl-PHA formation in a *fadB* mutant *E. coli* strain (Snell *et al*., 2002). Also, co-expression of the yfcX gene with the PHA synthase gene in a *fadB* mutant *E. coli* strain resulted in enhanced biosynthesis of mcl-PHA compared with the expression of only PHA synthase gene (Park and Lee, 2003). Recently, a new enoyl-CoA hydratase, MaoC, has been found to be important for the synthesis of mcl-PHA in a *fadB* mutant *E. coli* (Park and Lee, 2003). More recently Park and Lee, (2004a) identified five new FadB homologous enzymes in *E. coli*: PaaG, PaaF, BhbD, SceH, and YdbU, by protein database search, and examined their roles in the biosynthesis of mcl-PHA in a *fadB* mutant *E. coli* strain. Co-expression of each of these genes along with the *Pseudomonas* sp. 61-3 *phaC2* gene did not allow synthesis of mcl-PHA from fatty acid in recombinant *E. coli* W3110, which has a fully functional β-oxidation pathway, but allowed mcl-PHA accumulation in a *fadB* mutant *E. coli* WB101.

Recently an enoyl CoA hydratase with 93% homology to *A. caviae* PhaJ has been reported from *A. hydrophila* (Lu *et al*., 2004). Four (R)-specific enoyl-CoA hydratase genes, *phaJ1*Pa, *phaJ2*Pa, *phaJ3*Pa and *phaJ4*Pa, were identified in *P. aeruginosa*. The recombinant *E. coli* harboring *phaJ1*Pa or *phaJ2*Pa showed high 3-R enoyl-CoA hydratase activity with different substrate specificities specific for short-chain-length enoyl-CoA or medium-chain-length enoyl-CoA, respectively (Tsuge *et al*., 2000; Tsuge *et al*., 2003).

These four enzymes vary in their size and substrate specificities. Previous works on these enzymes have been carried out in recombinant *E. coli*. The substrate specificity of these enzymes in native host is unknown. There has been no attempt to down regulate or over-express the hydratases *in situ.*

Discovery of gene function requires inactivation in order to demonstrate the effect of the absence of gene expression on cell growth and activity. Gene inactivation by homologous recombination and transposon mutagenesis has been a widely successful strategy for studying gene function in numerous bacterial species. These modes of gene inactivation can be lethal if the gene product is essential for survival of the cell. This method is appropriate for genes that are not necessary for the cells survival. Antisense RNA provides a method of reducing transcript and protein levels without totally inactivating the targeted gene, thus providing information on the gene's possible function (Inouye, 1988; Lee and Roth, 2003).

Several types of antisense methods such as antisense oligonucleotides, antisense RNA and small interfering RNA can be used to inhibit the expression of a target gene. All antisense methods are based on the blockade and/or degradation of a target mRNA as a result of the binding of nucleic acids complementary to a subsequence of the mRNA. An antisense oligonucleotide possessing a sequence complementary to that of the target mRNA is delivered to the cell which hybridizes in a sequence-specific manner with the target mRNA and blocks mRNA translation through translational arrest or mRNA cleavage by RNase H (Good and Nielsen, 1998a; 1998b; Lee and Roth, 2003). Another method is through endogenous expression of antisense RNA through recombinant plasmids harboring antisense genes. The antisense RNA forms a duplex with the complementary mRNA sequence and blocks translation by the ribosome (Mol *et al*., 1990; Simons and Kleckner, 1988). In RNA interference, double stranded RNA is processed by the Dicer enzyme into 21–23 base pair dsRNA fragments called short interfering RNAs (siRNAs). The siRNAs are incorporated into an enzyme targeting complex, RNA-induced silencing complex (RISC). ATP dependent unwinding of the siRNA duplex generates an active complex, RISC, that targets mRNAs of complementary sequence for double-stranded RNase cleavage at the site where the antisense strand is bound. This mechanism is not to be found in prokaryotes.

Antisense technique has been widely used in yeast (DeBacker *et al* 2001), plant (Liu *et al*., 2002; Ogita *et al*., 2003, 2004) and animal systems (Mizuta, *et al*.,1999) In microbiology, antisense technology is sparingly used, although natural antisense regulation in bacteria is well known (Ranade and Poteete, 1993;Wagner and Simons 1994; Parish and Stoker, 1997; Good and Nielsen, 1998a; Ji *et al*., 1999). Inducible antisense RNAs has been used to identify essential genes in *Staphylococcus aureus* (Ji *et al*., 2002, Zheng *et al*., 2005). Ji *et al*., 2004 used a regulated antisense RNA interference technique to determine and/or confirm the mode of action of compounds directed to a specific target that exhibit antibacterial activity in *S. aureus.* The antisense approach was successfully applied to engineering phage resistance in *Streptococcus thermophilus* (Sturino and Klaenhammer, 2004) and *Lactococcus lactis* (McGrath *et al*., 2001). The expression of antisense RNA in *Clostridium acetobutylicum* to alter enzymatic pathways in anaerobic bacterial cells and hence improve the fermentation of various sugars into commercial solvents, such as acetone and butanol has been studied by Tummala *et al*., (2003). Bouazzaoui and LaPointe (2006) used antisense technique to modulate glycosyl transferase gene expression and the consequences for the molecular mass of exopolysaccharides produced by *Lactobacillus rhamnosus.* Recent studies have shown that antisense RNA based strategies also can be used to regulate the biosynthesis of global regulatory proteins (Tchurikov *et al*., 2000; Srivastava *et al*., 2000; Kemmer and Neubauer, 2006). Antisense techniques have not been hitherto used for the study of PHA synthesis.

This chapter reports the study of the role of two (R) hydratases from *P. aeruginosa* using two strategies: over expression and antisense expression.

5.2 MATERIALS AND METHODS

5.2.1 Plasmids strains and culture conditions

The strains and plasmids used in the study are listed in table 5.1. *E. coli* DH5α was used as host strain for general cloning. *P. aeruginosa 01* was used for over-expression and antisensing studies. Cultivation conditions for *E. coli* DH5α and *P. aeruginosa* has been described in earlier chapters (2.2.4.1).

Table 5.1 Strains and plasmids used for the over expression and antisense expression of PhaJ1 and PhaJ4

Strains and plasmid	Relevant characteristics
E. coli DH5α	*supE*44 Δ*lac*U169(ǿ80- *lacZ*ΔM15)*hsdR*17 *recA*1 *endA*1 *gyrA*96thi-1 *relA*1
P. aeruginosa 01	Prototroph
pBSP II KS	Ap^r; T7 and T3 promoter; broad host range cloning vector
pBPJ1	pBSP II KS(-)derivative; $phaJ1_{Pa}$
pBPJ4	pBSP II KS(-)derivative; $phaJ4_{Pa}$
pBPJ1A	pBSP II KS(-)derivative; $phaJ1_{Pa}$ antisense to lacZ promoter
pBPJ4A	pBSP II KS(-)derivative; $phaJ4_{Pa}$ antisense to lacZ promoter
pBPC1J1	pBSP II KS(-)derivative; $phaC1_{Pa}$; $phaJ1_{Pa}$
PBPC1J4	pBSP II KS(-)derivative; $phaC1_{Pa}$; $phaJ4_{Pa}$

5.2.2. Antisense and sense construct of PhaJ1 and PhaJ4

HindIII/ KpnI fragments, released from pTPJ1 and pTPJ4, were cloned into the pBSPIIKS-vector, in the antisense orientation with respect to the *lacZ* promoter. HindIII/ EcoRI fragment from pTPJ1 and pTPJ4 were cloned into the same vector in the sense orientation with respect to the promoter for hyper-expression. The constructs resultant from the sub cloning of *phaJ1* and *phaJ4* into pBSPIIKS-, in both sense and antisense direction, with respect to the *lac* promoter, were named as pBPJ1, pBPJ4, pBPJ1A and pBPJ4A respectively (Figure 5.2). These constructs were transformed in to *P. aeruginosa* by the method of Chakrabarty *et al.* (1975) (2.4.9).

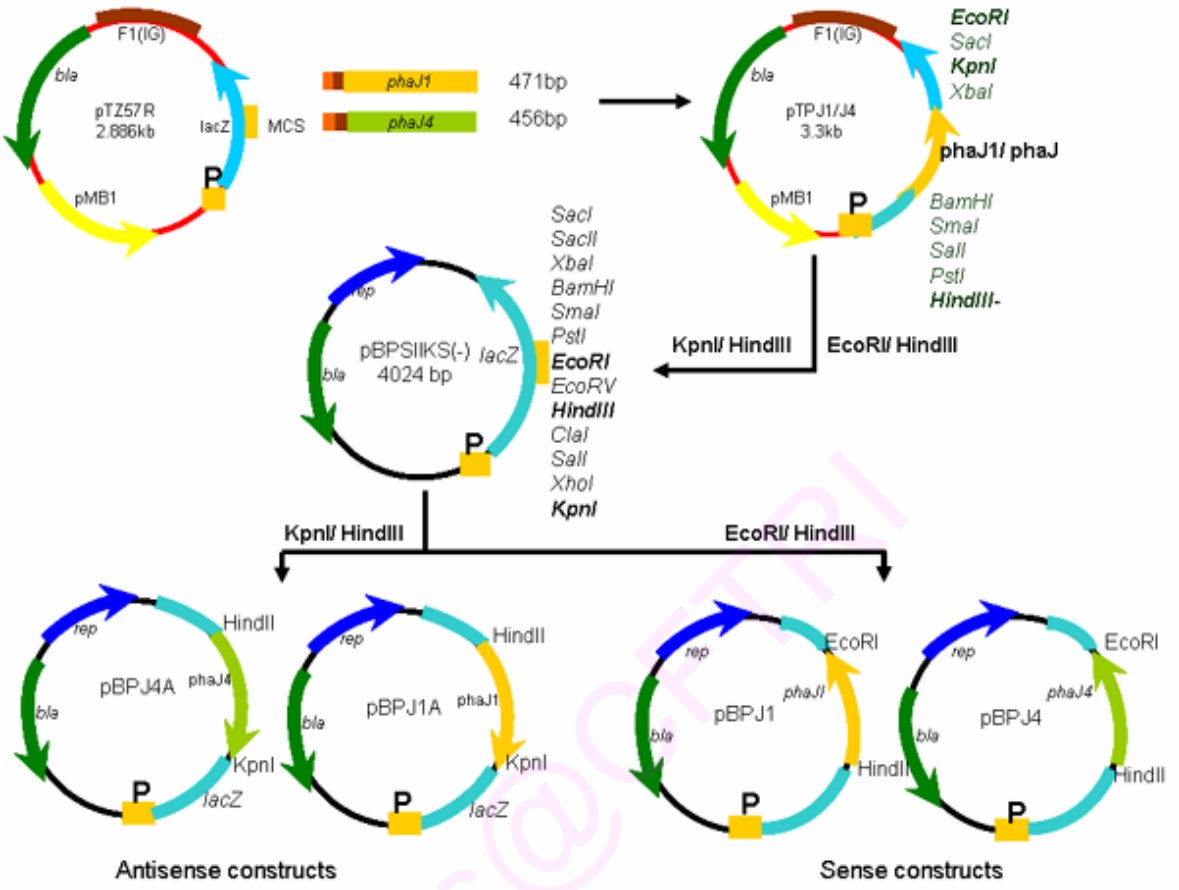


Figure 5.2 Schematic representation of the designing of sense and antisense constructs of *phaJ1* and *phaJ4*

5.2.3 Protein expression in recombinant P. aeruginosa

Pseudomonas transformants were grown in minimal media with octanoate/decanoate as carbon source and IPTG (0.4 mM) was used as an inducer of *lacZ* after 8 h of growth. Cell pellets harvested after 24 h, were washed in carbonate buffer (0.25 M, pH 8.5). Cell lysates were prepared by sonication of the cell pellet in a protease inhibitor cocktail [100 mM EDTA, 100 mM β-mercapto ethanol and 10 mM Phenyl Methyl Sulfonyl Fluoride (PMSF)]. The supernatant was subjected to electrophoresis on a 15% (w/v) SDS-PAGE (2.4.13). The amount of protein was determined by a dye binding method using Coomassie Brilliant Blue G250 (2.3.4). Equal quantities of protein were loaded on SDS-PAGE gels. The gel image was captured and the relative intensities of the bands were recorded in BioRad gel documentation unit.

5.2.4 Enzyme activity analysis

Activity of enoyl CoA hydratase was measured by assaying the hydration of crotonyl CoA at 263 nm as described in 4.2.2.5. Three independent experiments were carried out and the average value with standard deviation is presented.

5.2.5 Growth and PHA production

Recombinant *P. aeruginosa* bearing the sense construct, the antisense construct or the construct bearing synthase and hydratase were grown in the PHA production media containing glucose and fatty acids. Carbenicillin was used as a selective agent. Culture conditions were as described in 2.2.4.1. The yield and composition of PHA in these transformants after IPTG induction was compared with that of the control *P. aeruginosa* [bearing the plasmid pBSP II KS (-)]. Three independent experiments were carried out and the mean value and standard deviation is presented.

5.2.6 Analytical techniques

Extraction of PHA and characterization of the polymer using GCMS was carried out by the methods described in 2.3.2 and 2.3.6, respectively.

5.3 RESULTS

5.3.1 Cloning of PhaJ1 and PhaJ4 in sense and antisense direction

The cloning of PhaJ1 *and PhaJ4* into pTZ57R was carried out as already described in chapter 4 (4.3.1). Direction of the inserts was checked using restriction digestion and PCR. The inserts were then released and cloned into pBPSPII KS (-) using appropriate enzymes. The presence of the insert was checked by PCR (Figure 5.3) using the primers described in Table 4.2.

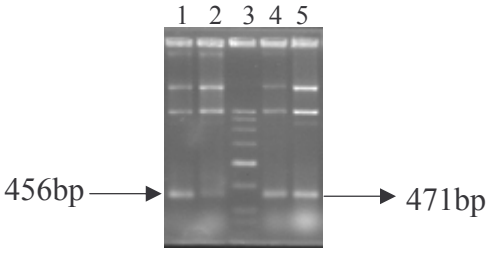


Figure 5.3 Authentication of the sense and antisense construct of *phaJ1* and *phaJ4* using PCR

Lane 1: 456bp amplicon of phaJ4 from pBPJ4, Lane 2: 456bp amplicon of phaJ4 from pBPJ4A, Lane 3: 100bp DNA ladder, Lane 4: 471bp amplicon of phaJ1 from pBPJ1, Lane 5: 471bp amplicon of phaJ1 from pBPJ1A.

5.3.2 Protein expression.

A higher level of expression of PhaJ1 and PhaJ4 was observed in *P. aeruginosa* bearing the sense construct, while a reduction in the amount of these proteins was noted in cells harbouring the antisense construct (Figure 5.4). The relative intensity of the bands corresponding to PhaJ1/PhaJ4, measured from the gel image (Biorad, Chemidoc XRS) is given in Table 5.2. The intensity of the band corresponding to the PhaJ4/PhaJ1 protein (~16 kD) was greater by 7% and 9% than that of the control while a 3%and 7% decrease was noted in cells bearing the antisense construct (in comparison to that of the control).

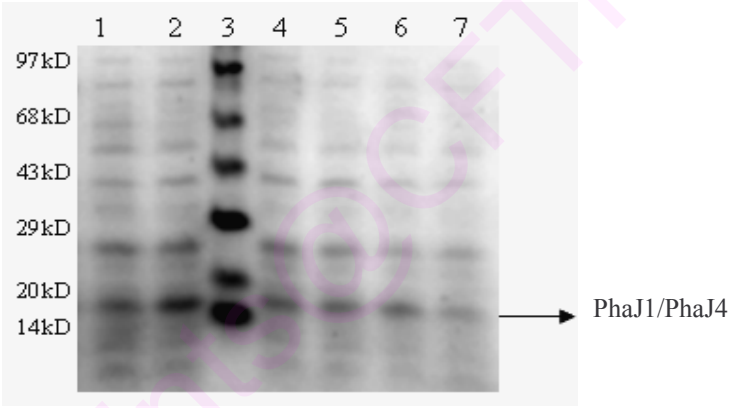


Figure 5.4 SDS-PAGE analysis of recombinant *P. aeruginosa* harbouring sense and antisense construct of *phaJ1* and *phaJ4.*

Lane 1: Total protein from *P. aeruginosa* harbouring pBSPIIKS-, Lane 2: Total protein from *P. aeruginosa* harbouring pBPC1J4, Lane 3: Molecular mass standard protein, Lane 4: *Total* protein from *P. aeruginosa* harbouring pBPJ1, Lane 5: Total protein from *P. aeruginosa* harbouring pBPJ4, Lane 6: Total protein from *P. aeruginosa* harbouring pBPJ1A, Lane 7: Total protein from *P. aeruginosa* harbouring pBPJ4A

Table 5.2 Relative intensities of protein bands corresponding to PhaJ1/PhaJ4

Plasmid/Constructs	Relative intensity of band corresponding PhaJ1and PhaJ4
pBSPIIKS-	37
pBPJ1	49
pBPJ1A	34
pBPJ4	44

pBPJ4A	30

5.3.3 Hydratase activity assay

Cell free extracts from recombinant *P. aeruginosa* bearing various constructs were assayed for activity of (R)-specific enoyl CoA hydratase by the hydration of *trans* 2 enoyl CoA and the results are given in Table 5.3. The specific activity of the extract from *P. aeruginosa* harboring the pBSPIIKS- (control) was 25.4 (Umg-^{1}of protein). The specific activity of extracts from *P. aeruginosa,* bearing the plasmids pBPC1J1, pBPC1J4, pBPJ1 and pBPJ4, ranged from 43, 59,157 and 102, respectively. The highest specific activity was found in the extracts of *P. aeruginosa* bearing the plasmid pBPJ1 (157 U). There was considerable reduction in the specific activity of these two hydratases when the genes, coding the enzymes, were antisensed and the specific activity measured in extracts of *P. aeruginosa* bearing the constructs pBPJ1A and pBPJ4A was 7 and 10 respectively.

Table 5.3 Enoyl CoA hydratase activity of *P. aeruginosa* harboring different plasmids

Plasmid	Specific activity(Umg-^{1}of protein)
pBSP II KS(-)	25.5± 2.2
pBPC1J1	43.0±4.0
pBPC1J4	59.0±2.0
pBPJ1	157.0±2.5
pBPJ1A	7.0±0.5
pBPJ4	102.0±2.8
pBPJ4A	10.0±0.5

Activities in the cell free extracts from cells grown in LB broth for 24 h at 37°C. All values presented here are average of three independent experiments

5.3.4 Production and composition of PHA in P. aeruginosa bearing pBPC1J1, pBPC1J4, pBPJ1 and pBPJ4

We have analyzed the effect of over-expression of *phaJ1* and *phaJ4* in *P. aeruginosa* individually and in combination with *phaC1*. Yield of PHA was estimated from the *P. aeruginosa* bearing different plasmid constructs (data presented in table 5.4).Over-

expression of these genes in *P. aeruginosa,* fed with fatty acids and glucose, resulted in increased PHA content in comparison with that of the control. Increase in the amount of PHA was observed particularly in cells bearing pBPC1J1 and pBPC1J4 when grown on different substrates.

Table 5.4 PHA production (% CDW) by *P. aeruginosa* harboring different plasmids grown on various carbon sources

Substrates	pBSP II KS	pBPJ1	pBPJ4	pBPC1J1	pBPC1J4	pBPJ1A	pBPJ4A
Glucose	33.9±1.5	37.8±2.6	36.9±2.6	37.8±1.4	40.7±1.4	30.0±0.5	31.9±2.6
Octanoate	25.9±1.6	34.0±0.5	30.0±1.0	31.7±1.7	34.6±2.2	18.9±1.4	21.8±2.4
Decanoate	24±2.0	27.6±3.41	32.5±1.8	35.8±0.9	34.0±1.2	17.0±0.2	16.0±0.5

All values presented here are average of three independent experiments. Cells were grown for 72 hour in PHA production medium with glucose, octanoate or decanoate as sole carbon sources. IPTG (0.4mM) was added as inducer. Carbenicillin (200 mg l^{-1}) was used for selection. pBSP II KS(-) was used as the control plasmid. CDW: Cellular dry weight

Results of experiments of over-expression of PhaJ1 and PhaJ4 individually and in combination with PhaC1 in *P. aeruginosa* on the composition of PHA are presented in Table 5.5. There was greater accumulation of hydroxy octanoate or hydroxy decanoate in the PHA of cells over-expressing *phaJ1* and *phaJ4,* either individually or in combination with the PHA synthase. This pattern of accumulation was more pronounced in cells harbouring pBPC1J4 wherein PHA richer in hydroxy decanoate was synthesized. Over-expression of the hydratase 4 and PHA synthase1 in *Pseudomonas* resulted in the accumulation of PHA rich in hydroxy decanoate while there was greater accumulation of hydroxy octanoate in the polymer when hydratase1 and synthase were over-expressed.

Table 5.5 Composition of PHA extracted from *P. aeruginosa,* bearing various plasmid constructs, grown on different substrates

	PHA Composition (mol %)											
	HHx	HO	HD	HA	HHx	HO	HD	HA	HHx	HO	HD	HA
Constructs	Glucose				Octanoate				Decanoate			
pBSP II KS(-)	6.4	19.4	35.8	38.4	15.3	25.0	26.0	33.7	0.0	31.2	45	23.8
pBPC1J1	7.4	34.9	42.0	15.7	10.0	41.0	19.0	30.0	2.8	47.9	24.0	25.3
pBPC1J4	8.0	24.0	48.0	20.0	17.0	19.0	37.0	27.0	0.0	20.0	49.0	31.0
pBPJ1	6.5	15.6	36.6	41.3	21.4	40.0	20.4	19.8	0.0	30.2	39.7	30.1

pBPJ4	7.1	21.5	34.5	36.9	23.0	20.0	37.0	20.0	0.0	29.8	55.6	14.6
pBPJ1A	6.5	17.6	35.6	40.3	21.4	18.4	20.4	39.8	0.0	20.2	39.7	40.1
pBPJ4A	7.1	20.5	35.5	36.9	23.0	28.0	19.0	30.0	0.0	39.8	35.6	24.6

pBSP II KS(-) was used as the control plasmid. HH: hydroxy hexanoate HO: hydroxy octanoate, HD: hydroxy decanoate and HA: higher hydroxy alkanoates.

5.3.5 Production and composition of PHA in recombinant P. aeruginosa bearing the antisense constructs

The specific activity of enoyl CoA hydratases in the extracts of *P. aeruginosa* bearing the antisense constructs was reduced to one third of that in the control (Table5.3). A fall in the intensity of protein bands, corresponding in size to that of the hydratases, was observed in SDS-PAGE gels of extracts of cells bearing antisense constructs. *P. aeruginosa* bearing the pBPJ1A construct, when fed on fatty acid, produced 7% less PHA than the control (Table 5.4). There was reduction in the molar fraction of hydroxy octanoate and increase in that of the higher alkanoates in cells fed with fatty acids used in the experiment (Table 5.5). The hydroxy octanoate fraction of the polymer, produced by control cells, was 25% and 27%, respectively, from octanoate and decanoate and this decreased to 18% and 20%, respectively, in cells bearing pBPJ1A (Table 5.5). Reduction in the hydroxy octanoate content of the PHA indicated that PhaJ1 enzyme is specific for the production of hydroxy octanoyl CoA monomers towards PHA biosynthesis. In this experiment a slight decrease in the molar fraction of decanoate was also observed. There was an enhancement in the molar fraction of higher alkanoates in the PHA produced by the cells harbouring pBPJ1A, grown in any of the carbon substrates tested and particularly in cells fed on decanoate (8% increase).

There was no significant variation in the amount of PHA in *P. aeruginosa* harbouring the pBPJ4A construct when fed with octanoate and glucose, while an 8% reduction of PHA content was noted in cells fed with decanoate in comparison to that of control *Pseudomonas* bearing the pBSP II KS(-) plasmid (Table 5.4). The monomer composition of PHA produced in cells, grown in glucose, bearing the pBPJ4A was the same as that of the control. The composition of PHA was altered when cells were grown in the presence of fatty acids (in contrast to the composition of PHA produced be cells grown

in glucose). The PHA produced contained less hydroxy decanoate and more hydroxy octanoate when cells were grown in the presence of octanoate or decanoate. The molar fraction of decanoate in the polymer decreased by 6-7% and a proportionate increase in the low molecular weight monomers was observed when cells were grown in the presence of octanoate (Table 5.3, Figure 5.5). A slight decrease in the content of higher alkanoates monomer in the PHA was also noted. These results indicated that enoyl CoA hydratase 4 is more active towards the hydration of decanoyl CoA (Figure. 5.5) in *P. aeruginosa* during PHA synthesis.

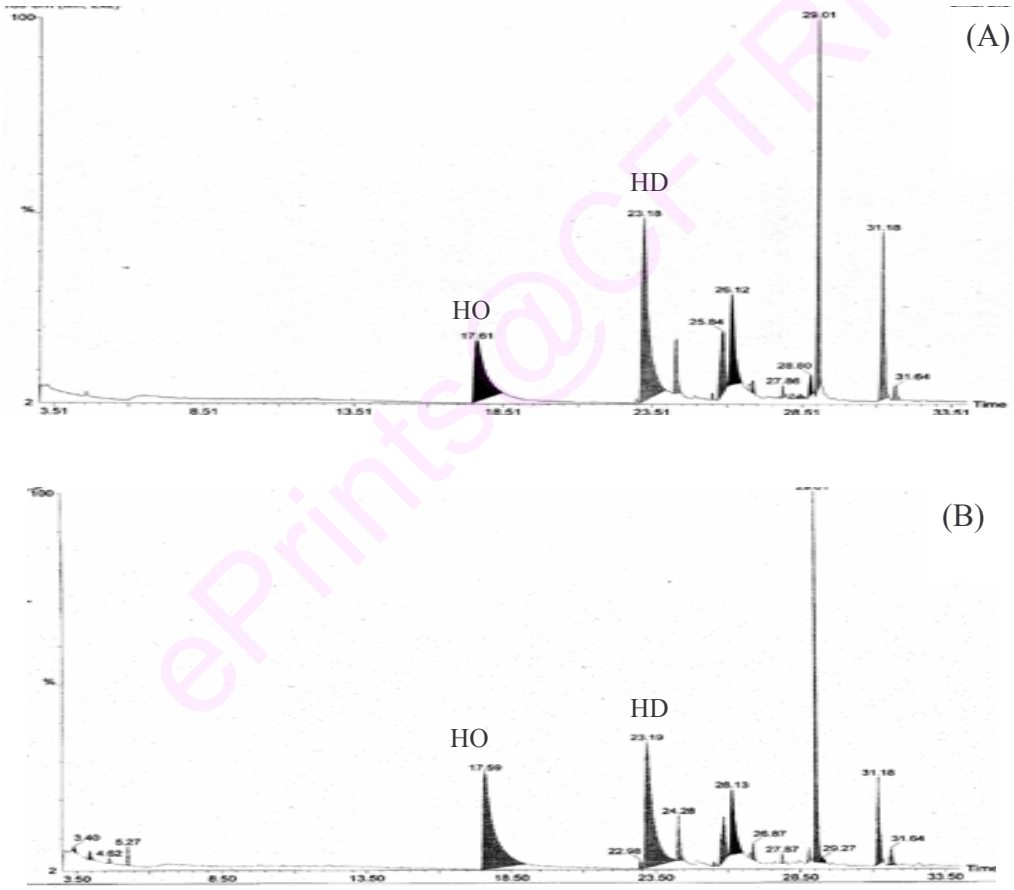


Figure 5.5 Gas chromatograms of methyl esters of PHA extracted from *P. aeruginosa* harbouring pBPJ4A grown on decanoate

A) *P. aeruginosa* harbouring pBPIIKS(-), B) *P. aeruginosa* harbouring pBPJ4A

5.4 DISCUSSION

In bacteria such as Pseudomonads and Aeromonads, the β-oxidation and mcl-PHA biosynthetic pathways is known to be linked by a (R)-specific enoyl-CoA hydratase (Tsuge *et al*. 2003; Fiedler *et al*. 2002; Fukui *et al*. 1998). (R)-specific hydratase active on short chain length enoyl CoAs is also found in PHA producing bacterium, *Rhodospirillum rubrum* (Moskowitz and Merrick 1969). Recently it has been found that the enoyl CoA hydratase from *A. caviae* could contribute to PHB formation in *E. coli* from glucose (Sato *et al*. 2007). PHAs with diverse monomer composition have been produced in recombinant *E. coli* from fatty acids employing *phaJ* genes of *P. aeruginosa* and *phaC2* gene of *Pseudomonas sp 61-3*. PhaJ and its isoforms in *P. aeruginosa* are important enzymes in the construction of microbial plastic factories whose exact role in the native bacterium is unknown. So the major aim of the present study was to investigate the specificities of two of these enzymes, PhaJ1 and PhaJ4, in *P. aeruginosa*.

One approach towards altering the quality and quantity of PHA produced involves increasing the number of copies of genes that are identical or homologous to those already expressed on the host chromosome. This approach resulted in marginal change in the quantity of PHA accumulated with a significant difference in composition (Fukui *et al*. 2001; Choi *et al*. 2003).This strategy has been applied to study the role of different enzymes involved in PHA biosynthesis in *A. hydrophila* (Han *et al*. 2004). In this study PhaJ1 and PhaJ4 were over-expressed individually and along with PhaC1 to study the contribution of monomers for the synthesis of PHA by these enzymes in the native bacteria. It is clear from the data that over-expression of hydratase lead to two fold or more increase in its activity compared to that of control (Table 5.3). The enhancement in activity was more pronounced when either PhaJ1 or PhaJ4 was over-expressed individually rather than when co-expressed with PhaC1. This may be attributed to the sharing of a common resources pool, when the two enzymes were co expressed. PhaJ1 or PhaJ4 when co-expressed with PhaC1, the PHA content increased significantly rather than when these genes were expressed individually. This may be attributed to the synergistic effect of higher PHA synthase activity and enoyl CoA hydratase activity. The native PHA synthase activity was unable to support the polymerization of monomers contributed by the over-expression of either PhaJ1 or PhaJ4 alone. Han *et al*. (2004) reported a similar effect when

PhaJ of *A. hydrophila* was over-expressed alone and co-expressed with PhaC in *A. hydrophila*. This results indicate significant differences in composition of PHA when the two hydratases under study were expressed individually or together with PhaC1. Fukui *et al*. (2001) and Choi *et al*. (2003) working with the homologous over-expression of enzymes from *Aeromonas punctata* and *R. eutropha*, respectively, observed significant changes in the composition of PHA in relation to that of the native bacteria. We have observed that a higher octanoate fraction in PHA is promoted by increased PhaJ1 activity while higher decanoate fraction in PHA is promoted by increased PhaJ4 activity (Figure. 5.6). Since PhaJ1 provides more octanoate and PhaJ4 provides more of decanoate towards the synthesis of PHA, it may be inferred that the availability of other intermediates is reduced.

Antisense RNA mediated regulation in bacteria is common and acts as selective gene silencer, either by blocking translation or by inducing degradation of the target molecule. Antisense RNA technique has been used for the identification and characterization of essential genes and down-regulation of gene expression in *Staphylococcus aureus* (Good and Nielson 1998a; 1998b; Ji *et al*. 1999; Ji *et al*. 2001) but has not been hitherto used for the study of PHA synthesis. Earlier workers have studied the

four hydratases over-expressed in *E. coli* (Tsuge *et al*. 2000; Tsuge *et al*. 2003).

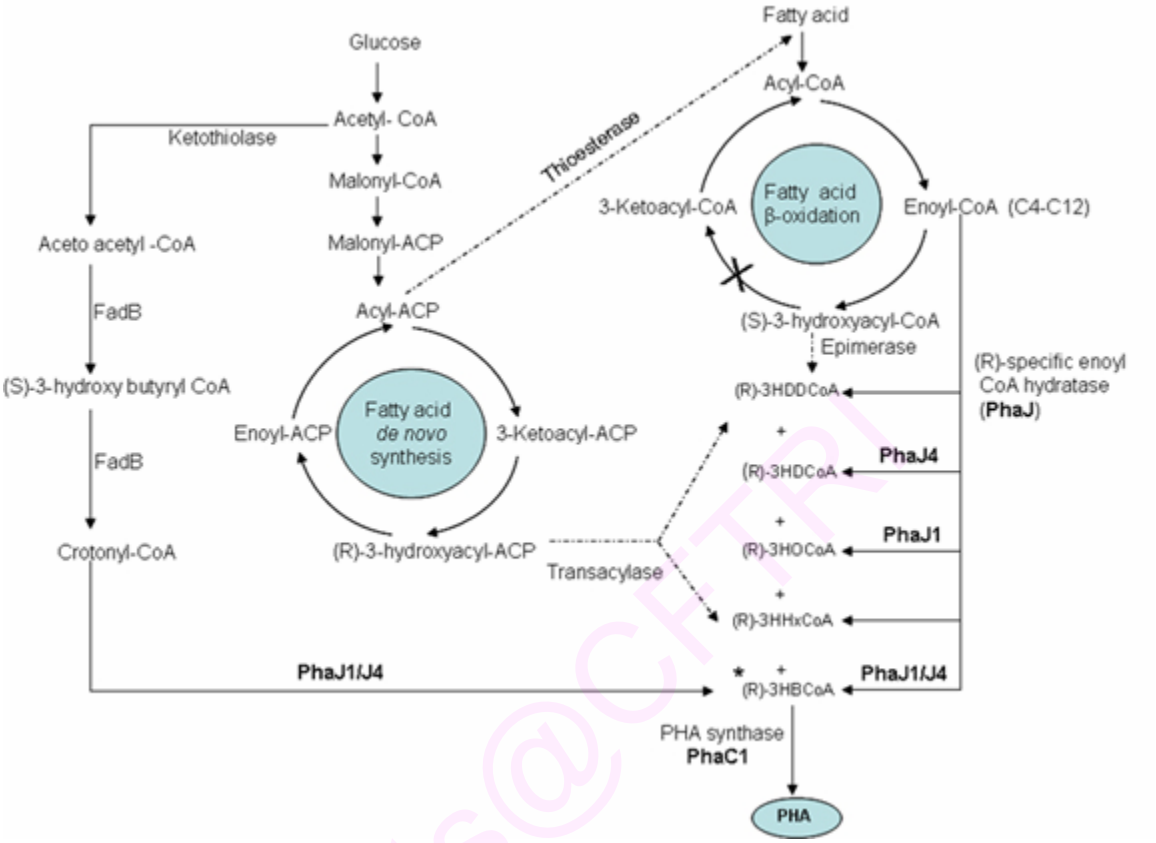


Figure 5.6 Postulated pathway of PHA synthesis in *Pseudomonas* sp. from glucose and fatty acids

Enzymes that have been over or under-expressed during the course of this study are represented with bold characters. The cross denotes deletion of enzyme (*fadB* mutant). The star indicates synthesis observed only in *E. coli.* (R)-3HBCoA: (R)-3-hydroxy Butyryl Coenzyme A, (R)-3HHxCoA: (R)-3-hydroxy hexanoyl Coenzyme A, (R)-3HOCoA: (R)-3-hydroxy octanoyl Coenzyme A, (R)-3HDCoA: (R)-3-hydroxy decanoyl Coenzyme A, (R)-3HDDCoA: (R)-3-hydroxy dodecanoyl Coenzyme A. (Fukui et al.1997; Park et al. 2005; Sato et al. 2007; This work)

According to Tsuge *et al*. (2003) PhaJ1 exhibited highest activity towards C4 substrate and also had high activity towards the C6 substrate. In contrast, PhaJ2, PhaJ3 and PhaJ4 all exhibited greatest activity for the C8 substrate. The PhaJ of different *Aeromonas* sp show maximum activity for the C6 substrate in the native bacterium and on expression in *E. coli* (Fukui *et al*. 1999). Attempts have not been made so far to down regulate the hydratases either in *situ* or elsewhere. Therefore, antisense technique has used to study substrate specificity of the two enzymes in *P. aeruginosa*. This study demonstrate clearly that it is possible to decrease the amount of hydratases significantly by expressing them in antisense orientation, and the activity of these enzymes decreased about one third with respect to that

of the control. Reduction in the amount and activity of these hydratase affect the content and composition of PHA produced by the recombinant *P. aeruginosa* bearing the anti sense construct. The change in composition was more pronounced when fatty acids rather than glucose was fed as carbon source. *P. aeruginosa* bearing *PhaJ1* antisense construct produced PHA with less hydroxy octanoate and those bearing *PhaJ4* antisense construct produced PHA with less hydroxy decanoate, independently of the fatty acid used for growing these recombinants (Table 5.5). Over-expression and anti sense expression data are equally supportive of the finding that PhaJ1is specific towards the hydration of octanoyl CoA and PhaJ4 is specific towards the hydration of decanoyl CoA in *P. aeruginosa*. This data is further corroborated with the data obtained when these enzymes were expressed in *E. coli.* This study provides a method for the alteration of the composition of PHA through the use of different hydratases in combination with other enzymes in the PHA biosynthetic pathway.

6.0 ABSTRACT

The full length promoter (-416), and two deletion fragments (-300 and -200) of *phaC1 g*ene of *P. aeruginosa* were cloned by PCR using primers designed based on the sequences available in the data bank. These fragments were fused with β-galactosidase gene in the pMP220 vector (a promoter less vector with β-galactosidase reporter gene), transformed into *P. aeruginosa* and the activity of the reporter gene was assayed under different growth conditions. The full promoter and the two deletion constructs induced β-gal expression in the presence of hexanoic acid, octanoic acid, nonanoic acid, decanoic acid and glucose under nitrogen limitation. Decanoic acid was the strongest inducer while hexanoic acid was the weakest. The full length promoter was able to drive higher levels of expression of the reporter gene than did the -300 and -200 promoter fragments.

Regulation of the expression of the two PHA synthase genes *PhaC1* and *PhaC2* and their specificities was studied in *P. aeruginosa* transformed with constructs expressing antisense RNA to the promoter and the corresponding Shine Dalgarno sequence. Changes were observed in the composition of the PHA produced by recombinants bearing the promoter antisense constructs. These changes depended on the substrate on which the recombinants were grown. *P. aeruginosa* recombinant grown in the presence of decanoate or glucose and bearing the antisense construct to the *phaC1* promoter accumulated polyhydroxyalkanoates with less hydroxy decanoate than that of the control strain. Recombinants cultivated in the presence of decanoate or octanoate and bearing the antisense constructs to the *phaC2* promoter accumulated PHA with less hydroxy octanoate in comparison to that of the control. These results indicated that both the *phaC1* and *phaC2* genes are differentially regulated in the presence of different carbon substrates. This study also suggests that PhaC1 and PhaC2 preferentially polymerises hydroxy decanoate and hydroxy octanoate, respectively, in *P. aeruginosa*.

6.1 INTRODUCTION

In cells, genes consist of a long strand of DNA which is associated with regulatory regions, transcribed regions (coding regions) and/or other functional sequence regions (Pearson, 2006; Pennisi, 2007). These regulatory elements are promoter region, operator region, activator region and terminator region. The genes of eukaryotic organisms can

contain non-coding regions called introns that are removed from the messenger RNA in a process called splicing. In prokaryotes, introns are less common and genes often contain a single uninterrupted stretch of DNA, called a *cistron*, that codes for a product. Prokaryotic genes are often arranged in groups called operons with promoter and operator sequences that regulate transcription of a single long RNA. This RNA contains multiple coding sequences. Each coding sequence is preceded by a Shine-Dalgarno sequence that ribosome recognize (Lewin, 2007).

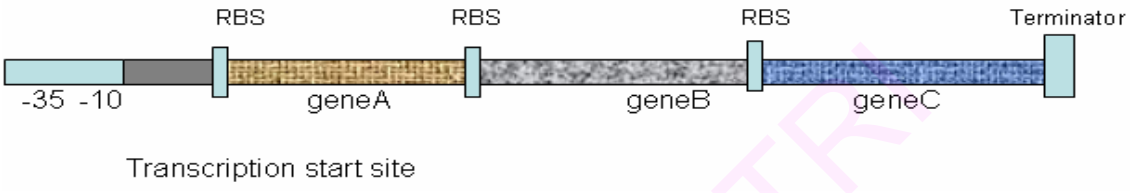


Figure 6.1 Structure of a prokaryotic operon

Promoters are sequences that precede transcriptional start sites and determine recognition by RNA polymerase to initiate transcription. There are two regions which are important in the definition of a prokaryotic promoter (the -35 region and the -10 region also known as the Pribnow box).

- The -35 region:- This sequence is centered about 35bp upstream of a bacterial gene. It functions in the initial recognition of a gene by RNA polymerase. The co*nsensus* sequence is TTGACAT.
- The Pribnow box (-10 region):- This has the consensus sequence TATAAT and is centered about 10bp before the start of a bacterial gene ie there are about 12bp between the -35 region and the Pribnow box.

Most bacterial genes are regulated at the level of transcription and expression from different classes of promoters is under the control of RNA polymerase with different sigma (σ) factor. The sigma subunit of RNA polymerase confers specificity in promoter sequence recognition and is essential for transcription initiation (Ishihama, 2000). In gram negative bacteria σ^{70} (sigma70) factor recognizes a large number of promoters especially those controlling expression of the house keeping genes. Other sigma factors having homology to σ^{70} such as σ^{28}, σ^{38}, σ^{24} and σ^{32} (Lonetto *et al*., 1992) also belong to the same family. The sigma factor σ^{54} is an entirely different one which recognizes highly conserved sequences where transcription initiation is always dependent on additional transcriptional factors

(Cases *et al*., 2003). All the above mentioned sigma factors have been found and characterized in *P. putida* and *P. aeruginosa* (Cuevas and Marques, 2004). About 149 σ^{70} dependent promoters have been identified from *P. aeruginosa* and *P. putida*. The consensus sequence of the -35 and -10 elements in *P. aeruginosa* is TTGACA and TATAAT, respectively (Cuevas and Marques, 2004). The consensus DNA sequence motifs in σ^{54} dependent promoters are TGGCACG, and TTGCTA, respectively, that are found at -12 and -24 sequences.

The PHA locus of *Pseudomonas* consist of two PHA synthases and a PHA depolymerase as well as three adjacent genes *phaD*, *phaF* and *phaI* whose exact functions are yet unclear but seems to be involved in regulation of PHA biosynthesis (Prieto *et al*.,1999; Klinke *et al*., 2000). In *P. aeruginosa* two transcriptional start sites were identified upstream of *phaCl*: one at the position -12 and -24, which is preceded by a nucleotide sequence (GGGCN,TGCA) resembling the *E. coli* consensus sequence for σ^{54} dependent promoters and the other at a position -35 and -10, which is preceded by a sequence (CTGAAANGGTAGT) resembling the *E. coli* consensus sequence for a σ^{70}dependent promoter. A nucleotide sequence at -35 and -10 (GTGAAANTATAAC) highly similar to the σ^{70} dependent promoter was also identified upstream of *phaC2* (Timm and Steinbuchel, 1992). PHA biosynthesis in *P. aeruginosa* requires an intact RpoN (σ^{54}) gene when gluconoate or octanoate is used as carbon source. This was proven by an experiment using rpoN mutant *P. aeruginosa* which failed to synthesize PHA in all the conditions tested (Hoffmann and Rehm, 2004).

Transcriptional analysis of genes involved in PHA biosynthesis in the presence of gluconate and octanoate showed similar results where *phaC1* plus *phaZ* as well as *phaC2* plus *phaD* were transcribed as single operons (Timm and Steinbuchel, 1992). Heterologous expression and *in vitro* studies revealed that the two enzymes PhaC1 and PhaC2 of *P. aeruginosa* exhibit similar properties resulting in similar extent of PHA accumulation, similar composition and molecular mass and these enzymes may be interchanged (Qi *et al*., 1997; Rehm, 2003; Qi *et al*., 2000). But the level of expression and specificity of these two synthases vary in a few *Pseudomonas* spp. Prieto *et al*., (1999) through constructing a recombinant *P. oleovorans* strain carrying a *phaC1::lacZ* reporter system demonstrated that *phaC1* gene was expressed efficiently in the presence of octanoic acid while its expression repressed when glucose or citrate was used as carbon source. The gene organization in *P.*

oleovorans is similar to *P. aeruginosa,* but there exist substantial differences in the sequences of the promoter of the PHA operon in these two bacteria, which may be responsible for different regulation patterns of *P. oleovorans*.

Most studies regarding the specificity of different PHA synthases from various *Pseudomonas* spp. were carried out by heterologous expression or by *in vitro* analysis using purified enzymes (Huisman *et al*., 1991; Tim and Steinbuchel, 1992; Qi *et al*., 1997; Langenbach *et al*., 1997; Qi *et al*., 2000; Solaiman *et al*., 2000, Chen *et al*., 2004). Even though PHA synthases of *P. oleovorans* and *P. aeruginosa* are known for its specific activity towards mcl-monomers there are reports on the incorporation of scl-monomers by these enzymes (Tim and Steinbuchel 1992; Kraak *et al*., 1997). In these experiments enzyme was exposed to an environment entirely different from that of its native state. These experiments proved that the ability of PHA synthase to accumulate different hydroxy alkanoates monomers depends on the availability of the monomer which ultimately depends on various monomer supplying pathways operating inside the cell. There is no report on the down regulation of these enzymes in homologous host.

In the previous chapter (5.2), different antisense techniques to study gene function has been discussed. In eukaryotes, RNAi seems to be a common mechanism in controlling gene expression in nearly all organisms and eukaryotic gene silencing by RNAi has become a standard procedure in research. In contrast, only a few antisense RNA based regulatory mechanisms are known in prokaryotes, which control mainly the biological function of accessory genetic elements like phages, transposons and plasmids (del Solar *et al*., 1998).

Most bacterial antisense RNAs are encoded at genetic loci other than those of the target genes. The Ribosome binding site is most likely to be susceptible to antisense inhibition (Figure 6.2). In the bacterial transposon IS10, RNA-OUT is an antisense RNA for the tnp transcript. RNA-OUT inhibits translation of the tnp-encoded transposase by sequestering its ribosome binding site (Ma and Simons, 1990). In *E. coli* four small RNA molecules including DicF, MicF, DsrA, and OxyS have been suggested to regulate gene expression through the formation of imperfect duplexes. OxyS inhibits translation of two target genes, the rpoS and fhlA. OxyS might pair with a short sequence overlapping the fhlA Shine-Dalgarno sequence to block ribosome binding (Zhang *et al*., 1998; Altuvia *et al*., 1998; Argaman and Altuvia, 2000). Good and Nielsen (1998b) reported that peptide

nucleic acids targeted to functional and accessible sites in ribosomal RNA can inhibit translation in an *E. coli* cell-free transcription / translation system. Previous studies have indicated that short methyl phosphonate oligonucleotides targeted at SD sequence inhibits growth of permeable *E. coli* cells (Jayaraman *et al*., 1981).

In this chapter a study determining the specificity of two PHA synthases of *P. aeruginosa* after down regulation of the expression of the enzymes using antisense RNA targeted to their respective ribosome binding sites is presented. Reporter fusions of 3 deletion derivatives of PhaC1 promoter were also made to locate the cis acting elements, involved in regulation of the expression of the gene by different substrates.

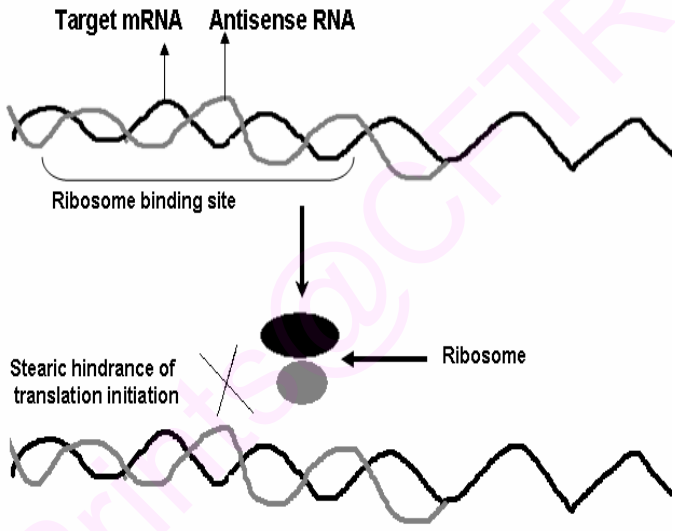


Figure 6.2 Mechanism of antisense inhibition of gene expression at the SD sequence region

6.2 MATERIALS AND METHODS

6.2.1 Strains and Plasmids used

Strains and plasmids used and their description are given in table 6.1. pMP220 and pRK2013 were kindly provided by Dr. Paola Visca and Federica Tiburzi, Università degli Studi di Roma Tre Dipartimento di Biologia, Viale Marconi, Roma, Italy

Table 6.1 Bacterial strains and plasmids used in the study of *phaC1* and *phaC2* promoters

Strain or plasmid	Relevant characteristics
P. aeruginosa	Refer Table 3.6
E. coli DH5α	-do-
pTZ57R/T	-do-
pBSP II KS(-)	-do-
pRK2013	Helper plasmid ColE1 replicon, Km^r Mob^+Tra^+
E. coli MC400	*araD139 rpsL150 relA1 flbB5301 deoC1 pstF25 rbsR* _(*lacZYA-argF*) *U169*, Strr 7
pMP220	Broad host range, low copy number promoter probe vector, IncP replicon, *laZ* Tc^r Tra
pTPro1F	416 bp fragment encompassing the entire *phaC1* promoter, along with withSD sequence and start codon of the *phaC1* cloned in pTZ57R/T
pTpro1M	300bp fragment of *phaC1* promoter, along with SD sequence and start codon of the *phaC1* cloned in pTZ57R/T
pTpro1S	200bp fragment of phaC1 promoter, along with SD sequence and start codon of the *phaC1* cloned in pTZ57R/T
pTPro2F	303 bp fragment encompassing the entire *phaC2* promoter, along with SD sequence and start codon of the *phaC2* cloned in pTZ57R/T
pTPro2S	200 bp fragment of *phaC2* promoter, along with SD sequence and start codon of the *phaC2* cloned in pTZ57R/T
pBPro1A	200bp Sac1/HndIII fragment of pTpro1S ligated to pBSPIIKS(-) in the antisense orientation to *lacZ* promoter
pBPro2A	200 bp Sac1/HndIII fragment from pTpro2S ligated to pBSPIIKS(-) in the antisense orientation to *lacZ* promoter
pMPPro1F	416 bp EcoRI/ PstI fragment from pTPro1F ligated upstream to the *lacZ* reporter gene in pMP220
pMPPro1M	300bp EcoRI/ PstI fragment from pTPro1M ligated upstream to the *lacZ* reporter gene in pMP220
pMPPro1S	200bp EcoRI/ PstI fragment from pTPro1S ligated upstream to the *lacZ* reporter gene in pMP220

6.2.2 Cloning of *phaC1* and *phaC2* promoters

Oligonucleotide primers were designed for the cloning of upstream flanking regions of *phaC1* and *phaC2* genes of *P. aeruginosa,* based on the sequences available in the data bank. For 5' deletion analysis of the *phaC1* promoter, 3 forward oligonucleotides were synthesized, the sequence of which were chosen to be conducive to priming of the coding strand from positions -400, -300 and-200 relative to the ATG translation start codon of the *phaC1* gene. A reverse primer annealing to the coding strand at the start codon of *phaC1* gene was used for PCR in combination with each of the forward primers. The amplification primers were designed to conserve the start codon of the *phaC1* gene and the original RBS.

Primers were also synthesized for the amplification of *phaC2* promoter. The expected product sizes and the position of the primers are shown in figure 6.3. The primers were synthesized by Sigma Aldrich and the sequences are indicated in table 6.2. Various 5' deletions of the *phaC1* and *phaC2* upstream flanking regions were PCR amplified using genomic DNA of *P. aeruginosa* as template by the method described in 2.4.1 and using the conditions given in table 6.3. The PCR products were authenticated by performing semi nested PCR.

Table 6.2 Nucleotide sequences of the primers used for amplification of different portions of *phaC1* and *phaC2* promoters

PhaC1 promoter	Primer sequences
C_1PF_1	GAGTCGGTCGTCAAGCTGAT
C_1PF_2	CAGACTGCCAGACCAGACAT
C1PF3	CCGATGCTTCTGCCAGCTTG
C_1PR	CATCGGCAACGCTCCATTGT
$PhaC_2$ promoter	
C_2PF_1	CCTCGTCCGTTCCTGCCCAA
C_2PFN	AGTCAATGTTCCGCAACGG
C_2PR	CATGGACCACACTCCGTCGT

Table 6.3 PCR conditions used for the amplification of *phaC1* and *phaC2* promoters

Primers	Denaturation	Annealing	Extension
C_1PF_1&C_1PR	94˚C for 1 min	58˚C for 1 min	72˚C for 1 min
C_1PF_2&C_1PR	94˚C for 1 min	55˚C for 1 min	72˚C for 1 min
C1PF3 & C_1PR	94˚C for 1 min	55°C for 30 s	72˚C for 1 min
C_2PF_1&C_2PR	94˚C for 1 min	60˚C for 1 min	72˚C for 1 min
C_2PFN&C_2PR	94˚C for 1 min	58˚C for 1 min	72˚C for 1 min

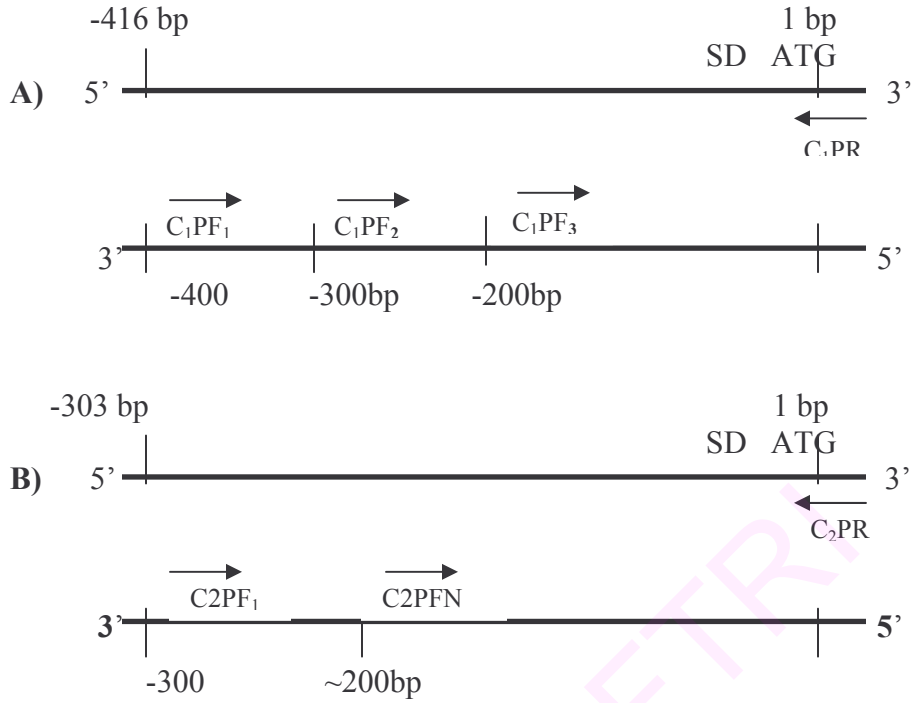


Figure 6.3 Positions of the primers used for PCR amplification of *phaC1* and *phaC2* promoter

The amplified promoter fragments were purified (2.4.3) and cloned in pTZ57R vector (2.4.5). The resultant plasmids pTPro1F, pTPro1M, pTPro1S, pTPro2F and pTPro2S were transformed into *E. coli* DH5α. Presence of the insert was checked by PCR and restriction. Direction of the inserted gene with respect to *lac* promoter was checked by the method described in 2.4.8.

6.2.3 Construction of PhaC1 promoter β-gal fusions

PstI/EcoR1 fragments released from the deletion constructs pTPro1F, pTPro1M and pTPro1S were sub cloned into the promoter probe vector pMP220 in an orientation with the 3'end of the promoter adjacent to the start codon of *lacZ* gene (Figure 6.4). These promoter reporter fusion constructs named pMPPro1F, pMPPro1M and pMPPro1S were transformed into *P. aeruginosa* by tri parental mating and the activity of the reporter gene was assayed under different growth conditions.

6.2.4 Antisense construct of phaC1 and phaC2 promoter

In order to make antisense constructs of these promoters, SacI/HindIII fragment of pTProC1 and pTProC2 were cloned into the broad host range vector pBPSKS- in the antisense direction with respect to the *lacZ* promoter (Figure 6.5). The constructs were named as pBProC1A and pBproC2A and transformed into *P. aeruginosa.*

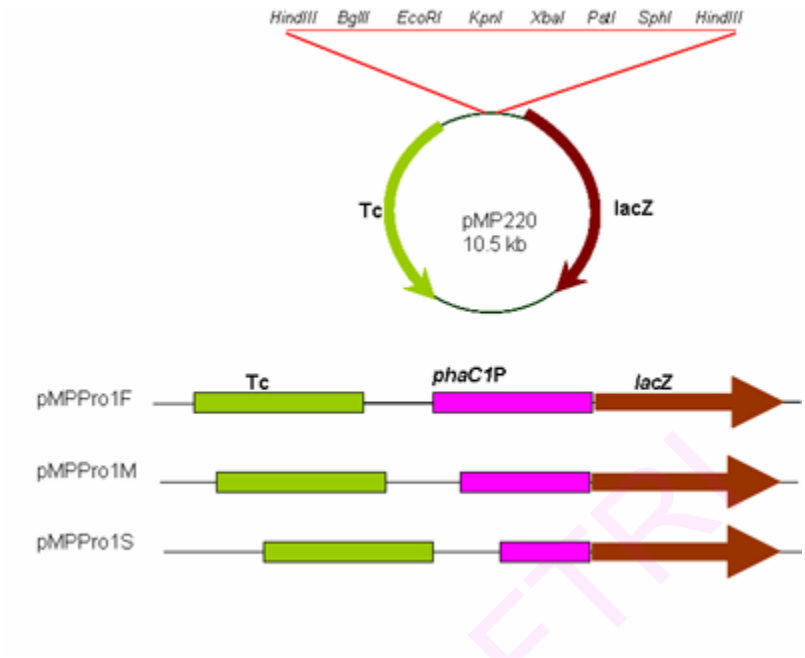


Figure 6.4 Fusion of *phaC1* promoter with reporter gene

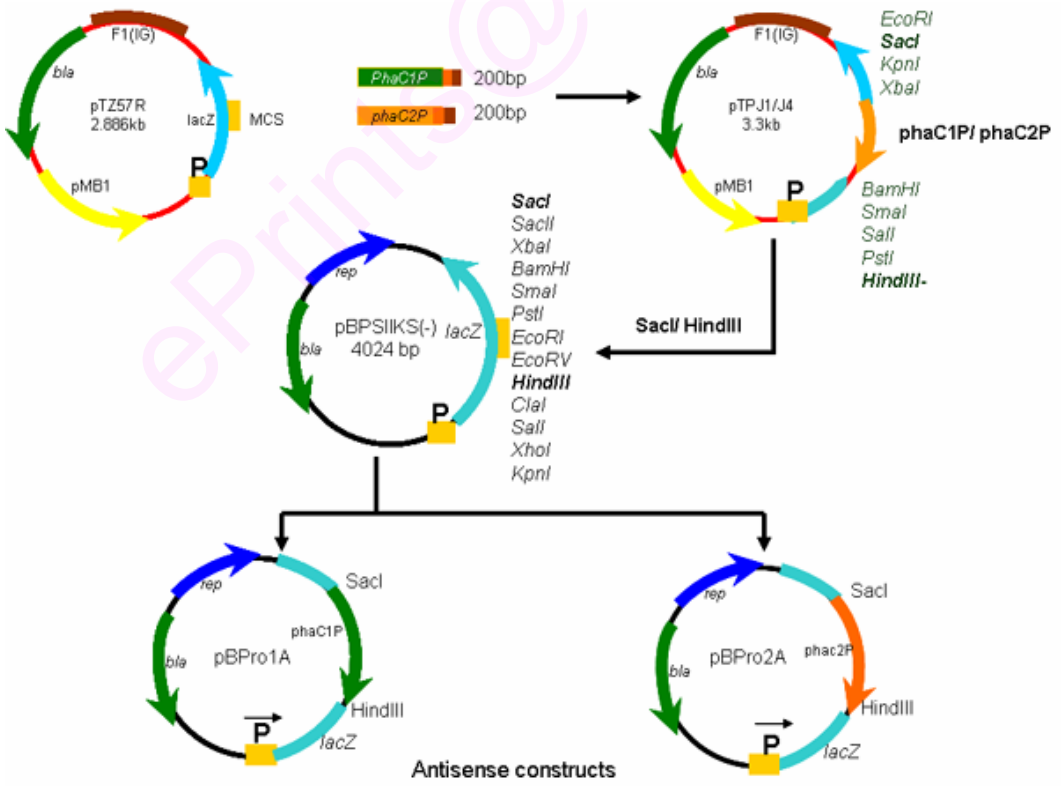


Figure 6.5 Designing of the antisense constructs: pBPro1A and pBPro2A

6.2.5 Tri Parental Mating (Goldberg and Ohman, 1984)

1. Overnight cultures of the following strains were prepared in LB broth using the appropriate antibiotics:
   A. Bacteria with the plasmid to be transferred, 30ºC.
   B. *E. coli* MC 400 containing the helper plasmid pRK2013, 30ºC with 25 µg/ml kanamycin.
   C. *P. aeruginosa* strain, the recipient, 42ºC.
2. 0.1ml of C was transferred into 2ml of LB broth, and 0.1ml of A and B were added.
3 This bacterial mixture was filtered using a 0.45 µm nitrocellulose filter. Filter was removed and the filter was placed cell side up on an LB agar plate. The plates were incubated overnight at 30ºC.
4. Bacteria from the plates were resuspended in 2 to 5 ml of sterile saline. 1/100 dilution of this suspension was made in saline. 100 µl of undiluted and 1/100 dilutions were spread on selection plates. The plates were incubated for 1 to 2 days at 37ºC or until distinct colonies are seen.
5. Colonies were streaked again on selection plates to ensure purity and presence of antibiotic marker.

6.2.6 β-galactosidase assay

P. aeruginosa strains harboring pMP220 derived plasmids were grown for 12-24 h at 37°C in mineral salt medium (2.1.5.2) containing tetracycline (100µg/ ml) with appropriate carbon source for measurements of *LaZ* reporter gene activity. Two types of assays determining the activity of the enzyme were performed.

A) Qualitative assay

Mineral salt agar was melted, tetracycline(100µg/ ml) and neutralized sterile solutions of fatty acids (2g/L media) or glucose (20g/L) were added and plated. The medium was allowed to solidify and 30µl of X-gal (4 chloro-3-bromo-indolyl galactopyranose) was spread over the medium. Overnight grown cultures were streaked on to agar plates and incubated at 37°C for 24 h and the development of blue colonies was observed**.**

B) Quantitative assay (Zuber and Losick, 1983; Burkard *et al*., 2007)

100 µl of the culture aliquots were added to 900µl of Z buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 50mM β-mercaptoethanol, pH7) in a test tube.

20 µl of toluene was added to permeabilize the cells. After removal of toluene by incubation at 37°C for 45 min the β- galactosidase reaction was started by adding 200 µl of *o*-nitro-phenyl-β-D-galacto pyranose (ONPG, 4mg/ml in Z buffer) at 28°C to the samples and incubated till yellow colour developed. The reaction was stopped by adding 500 µl of 1M Na_2CO_3. OD was measured at 420 and 550nm. Enzyme activity was normalized to the A600 of the bacterial suspension and expressed in Miller units.

$$1 \text{ Miller Unit} = \frac{1000[Ab_{420}-(1.75Ab_{550})]}{t \; v \; Ab_{600}}$$

Where,

Ab_{420}: is the absorbance of the yellow ortho nitro phenol, Ab_{550}: is the scatter from cell debris which when multiplied by 1.75 approximates the scatter observed at 420nm, t : reaction time in min, v :Volume of culture assayed in milliliters, Ab_{600}-reflects cell density. Two independent assays were carried out in duplicates.

6.2.7 Growth and PHA production

Recombinant *P. aeruginosa* bearing the antisense construct was grown in the PHA production medium containing glucose and fatty acids. Carbenicillin was used as selective agent. Culture conditions were as described in 2.2.4.1. The yield and composition of PHA in these transformants after IPTG induction was compared with that of the control *P. aeruginosa* (bearing the plasmid pBSP II KS (-)). Three independent experiments were carried out and the mean value and standard deviation is presented.

6.2.8 Analytical techniques

Extraction of PHA and characterization of the polymer using GCMS was carried out by the methods described in 2.3.2.1 and 2.3.6, respectively.

6.3 RESULTS

6.3.1 Cloning of PhaC1 and PhaC2 promoter

PCR fragments of the expected size of 416bp, 300bp, 200bp for *phaC1* promoter and 300bp and 200bp for PhaC2 promoter, respectively, were obtained from genomic DNA of *P. aeruginosa* using the primers (given in table 6.2) and separated on 1.5 % agarose gels. The amplicon size was determined using a 100bp DNA ladder run alongside the sample

(Figure 6.6). Nested PCR authenticated the amplicon. The purified amplicons were cloned initially into the T-tailed vector pTZ57R/T to yield the plasmids pTPro1F, pTPro1M, pTPro1S, pTPro2F and pTPro2S. Plasmids isolated from the transformants were subjected the release of insert (Figure 6.7). The direction of the insert with respect to *lacZ* promoter was determined by PCR using M13 sequence primer (Figure 6.8).

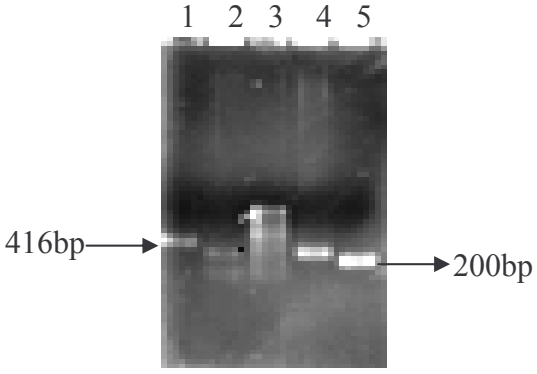


Figure 6.6 PCR amplification of *phaC1* and *phaC2* promoters

Lane1: 416bp amplicon of PhaC1promoter
Lane2: 300bp amplicon of PhaC1promoter
Lane3: 100bp DNA ladder
Lane4: 300bp amplicon of PhaC2 promoter
Lane5: 200bp amplicon of PhaC2 promoter

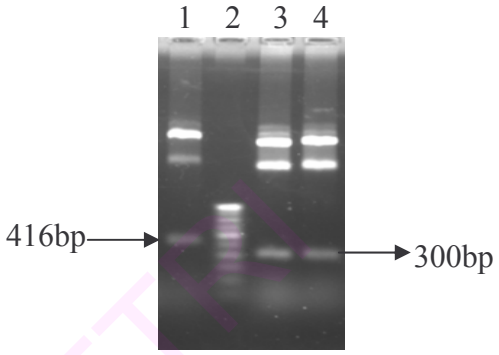


Figure 6.7 SacI/HindIII digestion of different promoter constructs

Lane1: SacI Hind III digestion of pTPro1F
Lane2: 100bp DNA ladder
Lane3: SacI Hind III digestion of pTPro1M
Lane4: SacI Hind III digestion of pTPro2F

Figure 6.8 Determination of orientation of the insert with respect to *lacZ* promoter by PCR using M13 sequence primers

Lane 1: PCR using C_1PF_1&C_1PR
Lane 2: PCR using $M_{13}F$ & $M_{13}R$
Lane 3: PCR using $M_{13}F$ & C_1PF_1
Lane 4: PCR using $M_{13}F$ & C_1PR
Lane 5: 100bp DNA marker
Lane 6: 100bp DNA marker
Lane 7: PCR using C2PF1 & C2PR
Lane 8: PCR using $M_{13}F$ & $M_{13}R$
Lane 9: PCR using $M_{13}F$ &C2PF1
Lane 10: PCR using $M_{13}F$ &C2PR

From figure 6.8 it is clear that amplification occurred in PCR with the $M_{13}F$ and gene specific reverse set of primers indicating that *phaC1* and *phaC2* promoters had been cloned in antisense direction to the *lacZ* promoter. Later the insert was mobilized into the broad

host range vector in antisense direction to the *lacZ* promoter by directional cloning (Figure 6.5). In order to analyze the different environmental conditions on the activation of PhaC1 promoter, the deletion constructs of phaC1 promoter were fused with β-galactosidase gene in a promoter probe vector. The presence of the insert was confirmed by PCR and restriction. After authentication the clones were used for transformation of *P. aeruginosa*.

6.3.2 Assay of β-galactosidase activity

Activity of β-galactosidase under the control of *phaC1* promoter was determined by plate assay using X-gal and by quantification of the enzyme activity in miller units using ONPG. Derivatives of pMP220 carrying all the deletion segments exhibited strong induction of β-galactosidase activity under nitrogen limitation and in the presence of fatty acids or glucose as seen in figure 6.9 and Table 6.4. *P. aeruginosa* strain carrying *phaC1* promoter *lacZ* fusion showed a blue phenotype when cultured in minimal medium with X-gal indicator and in the presence of different carbon sources. In the absence of carbon substrate under nitrogen limitation the activity of the β-gal was very less (Figure 6.9). Induction of β-gal was maximum in the presence of decanoate and minimum in the presence of hexanoate. Both qualitative and quantitative analysis showed almost similar results. Promoter region -416 conferred greater induction of β-gal expression than -300 and -200 in the presence of most carbon sources tested. Promoter region -300 confered highest activity of β-galactosidase in the presence of hexanoate and nonanoate. All the 3 deletion segments drove β-galactosidase to the same level of expression in the presence of octanoate. Promoter region -250 conferred the lowest activity to β-galactosidase in the presence of all the carbon sources tested except nonanoate.

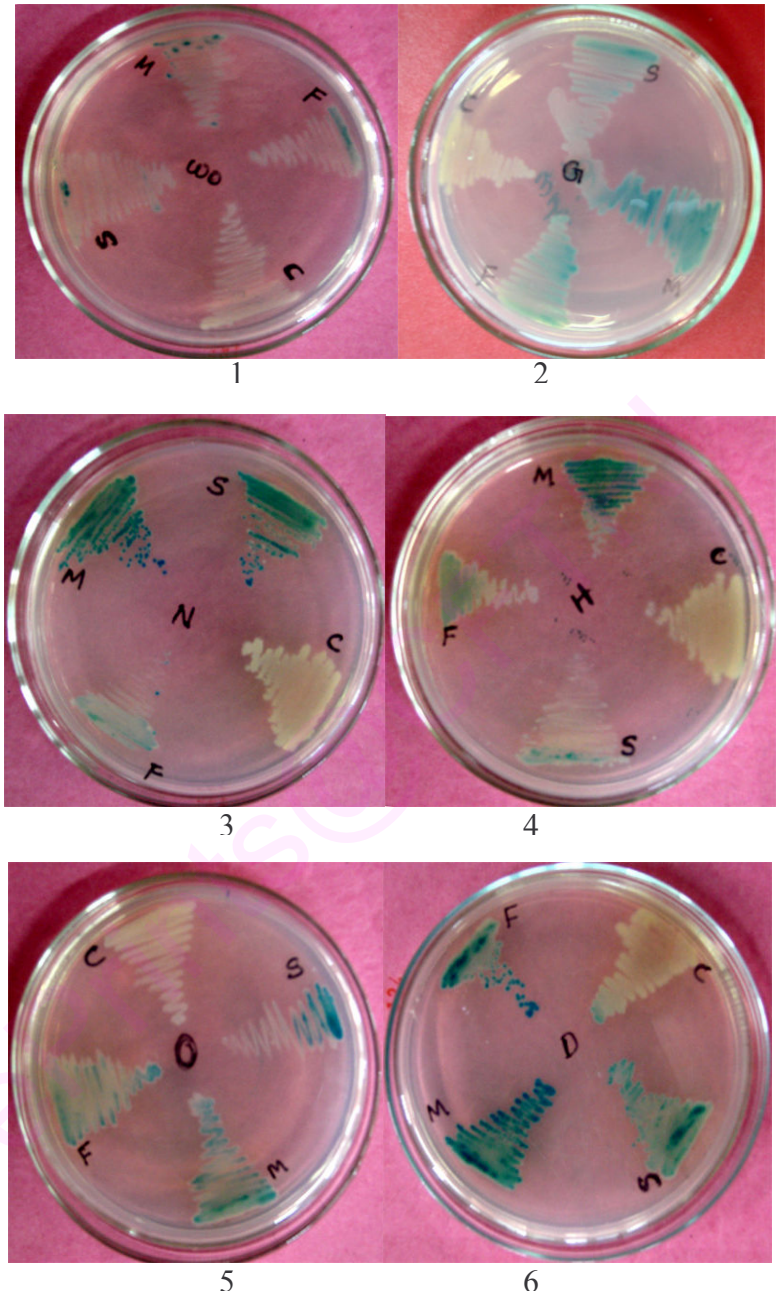


Figure 6.9 Assay of β-galactosidase activity driven by *phaC1* promoter fragments

Recombinant *P. aeruginosa* bearing different promoter deletion β-gal fusions were grown on mineral salt agar at 37°C for 24 h with X-gal, tetracycline and in the presence of different carbon substrate. 1 (wo)-without substrate, 2 (G)-glucose, 3 (N)-nonanoic acid, 4 (H)-hexanoic acid, 5 (O)-octanoic acid, 6 (D)-decanoic acid, C- *P. aeruginosa* bearing control plasmid, F- *P. aeruginosa* bearing pMPPro1F, M- *P. aeruginosa* bearing pMPPro1M, S- *P. aeruginosa* bearing pMPPro1S

Table 6.4 Effect of different carbon sources on *phaC1* promoter::*lacZ* activity

Substrates	LacZ activity in *P. aeruginosa* bearing different deletion of *phaC1* promoter::*lacZ* fusions		
	pMPPro1F	pMPPro1M	pMPPro1S
Glucose	2126	2022	1449
Hexanoate	2000	2316	1719
Octanoate	2437	2426	2493
Nonanoate	2779	3294	2896
Decanoate	4033	4012	2796

β-galactosidase activity is expressed in Miller units. The values are mean of three independent determinations. The mineral salt medium was supplemented with different carbon substrate, tetracycline and grown for 24h at 37°C

3.4 Production and composition of PHA in P. aeruginosa bearing the anti sense constructs

PHA production in *P. aeruginosa* bearing the antisense construct grown in minimal media in the presence of glucose, octanoate and decanoate are given in table 6.5. The amount of PHA in cells, grown in glucose and bearing the pBProC_1A and pBProC_2A constructs, was low in comparison with that present in cells bearing the control plasmid {pBSP II KS (-)} without any insert. This reduction in content of PHA was more pronounced in cells bearing pBProC1A construct (11% reduction) than those bearing the pBProC2A (4% reduction) when grown in the presence of glucose (Table 6.5). Similar results were obtained when decanoate was used as carbon substrate. The PHA content was 24% CDW in cells bearing control plasmid when grown in the presence of decanoate and this decreased to 17% CDW in cells bearing antisense construct of *phaC1* promoter. The amount of PHA in cells, grown in octanoate and bearing pBProC_1A and pBProC_2A constructs, was less in comparison with that present in cells bearing the control plasmid [pBSP II KS (-)]. The decrease was greater in cells bearing pBProC_2A construct than those bearing pBProC_1A construct.

Table 6.5 Production of PHA in *P. aeruginosa* bearing the promoter antisense construct

Substrates	pBSP II KS	pBPro1A	pBPro2A
Glucose	33.9±1.5	22.0±1.6	29.0±2.6
Octanoate	25.9±1.6	19.0±0.5	17.0±1.0
Decanoate	24.0±2.0	17.6±1.2	22.0±1.0

GC analysis indicated that the monomer composition of PHA altered in cells bearing the antisense constructs of both the *phaC1* and *phaC2* promoters in the presence of all the carbon sources tested in comparison to that of cells bearing the control plasmid. Recombinant bearing pBProC1A accumulated PHA with less hydroxy decanoate and more hydroxy octanoate in the presence of glucose and decanoate in the medium (Table 6.6). The hydroxy decanoate content of PHA produced by cells bearing the control plasmid was 47% and 76% from glucose and decanoate and this decreased to 38% and 46%, respectively in cells bearing pBPro1A. A hike in hydroxy octanoate content of PHA was noted in the cells bearing pBPro1A in the presence of the all the three carbon sources tested. There was reduction in the hydroxy octanoate as well as hydroxy hexanoate content of PHA in cells bearing pBPro2A as compared to that of control in the presence of glucose and octanoate. There was no significant change in the hydroxy dodecanoate molar fraction of the polymer in the cells bearing either of the antisense constructs and grown in the carbon substrates used in this experiment.

Table 6.6 Composition of PHA extracted from recombinant *P. aeruginosa* bearing antisense constructs: pBPro1A and pBPro2A

Constructs	PHA Composition (mol %)											
	HHx	HO	HD	HA	HHx	HO	HD	HA	HHx	HO	HD	HA
	Glucose				Octanoate				Decanoate			
pBSP II KS(-)	11	30	51	8	16	56	22	6	0	21	76	3
pBPro1A	3	49	38	8	11	78	8	3	0	28	46	26
pBPro2A	2	20	48	20	10	66	18	6	0	11	80	9

6.4 DISCUSSION

The recognition and binding of a transcription factor to its DNA responsive element (promoter or enhancer) is the basis for the initiation and regulation of gene expression in all the cells (Scott, 2000). In *P. aeruginosa* and various other microorganisms, promoters have been experimentally identified or postulated based on the sequence upstream of the *pha* and *phb* synthetic genes. A few regulatory proteins such as PhaR, PhaS, PhaF, etc. have been identified in PHA producing bacteria. The requirement of intact RpoN for PHA accumulation from gluconate or octanoate provided evidence that RpoN might be involved in regulation of PHA biosynthesis genes in *P. aeruginosa* (Hoffman and Rehm, 2004). PHA biosynthesis is regulated at enzymatic level and molecular level. Activation of *pha* gene expression may occur due to specific environmental signals, such as nutrient starvation or metabolic intermediates or inhibition of metabolic enzymes of competing pathways. Overall, regulation of PHA biosynthesis genes at the molecular level in *P. aeruginosa* has been only studied to a limited extent.

The environmental conditions that influence the expression driven by the *phaC1* full length promoter and deletion fragments were studied initially. Promoter reporter fusion construct was employed in the present investigation to study the effect of different fatty acids and glucose on PhaC1 expression. Prieto *et al*., (1999) studied the environmental factors affecting the *P. oleovorans* PhaC1 gene expression using a β- gal reporter system and suggested that PhaC1 expression could be induced by octanoic acid in the medium and was repressed by glucose. From our results it may be concluded that the presence of carbon substrates and nitrogen limitation are equally important for the activation of phaC1 promoter. Previously it was reported that intracellular carbon to nitrogen ratio is one of the key factors activating the enzymes involved in PHA biosynthesis (Haywood *et al*., 1988). In this study activation of *phaC1* promoter was observed both in the presence of glucose and fatty acids in contrast to the result of Prieto *et al*., (1999), who noted activation of *phaC1* promoter only in the presence of fatty acids. It is also clear from our experiment that the chain length of carbon substrate plays an important role in the activation of PhaC1. The highest level of activation of *phaC1* promoter was observed in the presence of decanoic acid, the largest sized fatty acid used.

Glucose and decanoate activated the smaller PhaC1 promoter fragments to a lesser degree than they did the full length promoter. Activation of the different promoter

fragments by octanoate, nonanoate and hexanoate was similar. This could be taken to indicate that an enhancer element for glucose and decanoate is present upstream of -300 region. Such enhancer elements have not been reported hitherto. -200 fragment appears to be sufficient enough to drive the expression of *phaC1* gene even in the presence of glucose and was particularly activated by nonanoate and also by octanoate and decanoate. The extent of activation of the three promoter fragments by nonanoate in comparison to that by glucose was greatest with -200 and -300 fractions than with the full length promoter. This would indicate that the activation element for nonanoate resides within the -300 fragment.

Even though the PhaC1 and PhaC2 enzymes of *P. aeruginosa* and those of other *Pseudomonas* have been exploited widely for the biosynthesis of PHA in recombinant *E. coli* and other bacteria (Qi *et al.,* 1997; Qi *et al*., 1998; Fiedler *et al*., 2002) the specific role played by these two enzymes in the native host have not been studied hitherto. In the present study antisense constructs of PhaC1 and PhaC2 promoter along with SD sequences have been used to study the specificity of these two enzymes towards different monomers. Inhibition of gene expression by antisense RNA targeted against SD sequences has been reported earlier (Good and Nielsen, 1998; Zhang *et al*., 1998; Altuvia *et al*., 1998; Argaman and Altuvia, 2000). In this study we could demonstrate clearly, that the amount of PHA decreased and its composition changed significantly in *P. aeruginosa* bearing the PhaC1 and PhaC2 promoters in antisense orientation with respect to that of the control. This may be due to the inhibition of expression of these enzymes by the binding of antisense SD sequences targeted towards these genes. There was reduction in the PHA content in cells bearing either of the antisense constructs when grown in the presence of all the carbon sources tested. This indicates that activation of both the enzymes are independent of the carbon substrate used. But the level of expression of these two genes may be different under various environmental conditions. From the compositional analysis it is clear that there is reduction in the decanoate content of PHA in the cells bearing the antisense construct of PhaC1 promoter when grown in the presence of glucose, decanoate and octanoate. The finding that PhaC1 is specific towards the polymerization of decanoyl CoA is further supported by the data of over-expression of *phaC1* gene in *E. coli* and *P. aeruginosa* (3.3.8).

CONCLUSIONS AND FUTURE PERSPECTIVES

The results of the present investigation on 'Studies on the molecular mechanism of polyhydroxyalkanoates biosynthesis in *Pseudomonas* species' may be summed up as follows:

1) A highly specific *phaC1* probe was developed for the rapid screening of PHA producing *Pseudomonas* spp. from soil samples by colony hybridization. This method allowed the screening of many PHA producing *Pseudomonas* isolates directly from soil samples avoiding the tedious procedure of DNA isolation. These isolates were found to produce 18-33% cellular dry weight (CDW) of PHA. Hydroxy octanoate and hydroxy decanoate were the major monomers found in the PHA produced by the different isolates. Addition of acrylic acid in the PHA production medium of *Pseudomonas* resulted in increased PHA content. Further screening of natural isolates that differ in their profile of PHA accumulation may be useful for the development of microbial plastic factories.

2) *PhaC1* gene of *P. aeruginosa* was cloned and expressed in *E. coli* and *Bacillus subtilis.* This led to the accumulation of PHA by these recombinants. The PHA production was found to be low in recombinant *B. subtilis*, which may be enhanced by cloning of different monomer providing enzymes and optimizing the growth parameters.

3) The over-expression of *phaC1* in *P. aeruginosa* resulted in the production of PHA with more hydroxy decanoate. This indicated that the *phaC1* is active towards the polymerization of hydroxy decanoyl CoA in *P. aeruginosa*. The over-expression system may be used to produce enzyme for crystallographic studies and those with altered properties.

4) (R)-specific enoyl CoA hydratase 1 and 4 (*phaJ1* and *phaJ4*) were cloned and sequenced from a soil isolate of *Pseudomonas.* and from *P. aeruginosa,* respectively. These two enzymes were expressed along with *phaC1* of *P. aeruginosa* in *E. coli* of different genetic backgrounds. Production of 20-25% CDW of PHA, containing both short chain length (scl) and medium chain length (mcl) monomers was achieved in recombinant strains of *fadB* mutant of *E. coli*. Efficient channeling of PHA precursors from inherent or newly constructed metabolic pathways will further improve PHA content and composition.

5) A hybrid pathway for the synthesis of PHB-co-Hydroxy valerate (HV)-co-mcl-PHA copolymer was developed in *E. coli*JC7623 using two genes from *Pseudomonas* (*phaJ1* and *phaC1*) and two genes from a *Bacillus* sp. [β-ketothiolase (*phaA*) and NADPH dependent-acetoacetyl CoA reductase (*phaB*)]. This recombinant was capable of producing 28-34% (CDW) of PHA. Addition of acrylic acid, an inhibitor of β-oxidation resulted in improved production (3-11% increase) of PHA copolymer by this recombinant strain. PHA production can be further improved in this strain by controlling filamentation or by providing NADPH through the incorporation of relevant enzyme. Alteration of the codon bias of the enzymes from *Bacillus* to that of *E. coli* may also allow the synthesis of higher amounts of polymer. It may be concluded that successful blending of pathways involved in the biosynthesis of PHA leading to copolymer production could be possible in *E. coli.*

6) Antisense technique and over expression was used for the study of the specificity of two hydratases in *P. aeruginosa*. Our study suggested that the PhaJ1 and PhaJ4 found in *P. aeruginosa* use hydroxy octanoyl CoA and hydroxy decanoyl CoA as substrates respectively in *Pseudomonas sp.* for the production of PHA. This study provides a method for the alteration of the composition of PHA through the use of different hydratases in combination with other enzymes in the PHA biosynthetic pathway. Antisense techniques and over-expression methods may be used towards investigation of the role of various other enzymes involved in the synthesis of PHA.

7) The regulation of expression of *phaC1* gene in the presence of different carbon sources was investigated using a reporter gene. Decanoic acid and hexanoic acid were found to be the strongest and weakest inducers of *phaC1* promoter, respectively. The differences in the specificity of the two PHA synthases of *P. aeruginosa* was studied using antisense RNA, targeted towards the Shine Dalgarno sequences of the respective genes. This experiment revealed that the substrate specificity of PhaC1 and PhaC2 are different, which mainly polymerizes hydroxy decanoyl CoA and hydroxy octanoyl CoA, respectively, in *P. aeruginosa.* Much more work especially on transcriptional factors is certainly required to elucidate the regulatory network of PHA accumulation in *Pseudomonas.* The knowledge gained in the present investigation can certainly form the basis for further research in this area.

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Research papers

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Reeta Davis, A Chandrashekar and TR Shamala. **"**Use of the *fad B* mutant of *E. coli* bearing genes from *Pseudomonas aeruginosa* for recombinant production of Polyhydroxyalkanoates" Poster presented at 75th annual meeting of SBCI held during 8th- 11th December 2006 at JNU New Delhi.

Reeta Davis, PK Anilkumar, A Chandrashekar and TR Shamala. "Heterologous expression of the proteins from *Pseudomonas sp.* involved in the biosynthesis of Polyhydroxyalkanoates" Poster presented at Biotech- 2006 held during 26th – 28th November 2006 at CCMB Hyderabad.

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