BIOSYNTHESIS OF POLYHYDROXYALKANOATES BY *BACILLUS* SP.

BY
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BIOSYNTHESIS OF POLYHYDROXYALKANOATES BY *BACILLUS* SP.

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BY

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DEDICATED TO MY PARENTS

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Biosynthesis of polyhydroxyalkanoates by *Bacillus* sp.**" submitted by ***Desetty Rohini Devi*** was carried out under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Materials obtained from other sources have been duly acknowledged in the thesis.

Dr. S. K. Rawal
(Research Guide)

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ABBREVIATIONS

BSA	Bovine serum albumin
bp	Base pairs
Ci $mmol^{-1}$	Curie per milli mole
EDTA	Ethylene diamine tetra acetic acid disodium salt
g	grams
xg	Relative centrifugal force
g L^{-1}	grams per litre
h	hour(s)
IPTG	Isopropyl β-D-thiogalactoside
kb/kbp	Kilobase pairs
kDa	Kilo Daltons
μ L^{-1}	Micrograms per liter
μCi	Microcurie(s)
mg	milligrams
mM	millimolar
MCS	Multiple cloning sites
min	Minute(s)
O/N	Overnight
PEG	Polyethylene glycol
PHB/P(3HB)	Poly-3-hydroxybutyrate
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
s	Seconds
U μL^{-1}	Units per microlitre
v/v	volume / volume
w/v	weight / volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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ABSTRACT

Approximately 140 million tonnes of synthetic polymers are produced worldwide each year. These nondegradable plastics accumulate in the environment at a rate of millions of tons per year. Recently problems concerning the global environment and solid waste management have created much interest in the development of biodegradable plastics that still retain the desired physical and chemical properties of conventional synthetic plastics.

Polyhydroxyalkanoic acids (PHAs) are a class of aliphatic polyesters produced by many bacteria and archaea in response to various environmental conditions. PHAs are generally regarded as a carbon and energy reserve, the accumulation of PHA increases in some bacteria when growth is limited by a nutrient other than carbon (e.g., nitrogen or phosphate), while in other bacteria it readily accumulates during unrestricted growth. The most important property of PHAs is their complete biodegradability for which they have been drawing considerable industrial interest as candidates for biodegradable and/or biocompatible plastics for a wide range of applications such as biodegradable packagings, films and fibres, latex coatings, adhesives, as biomedical disposable material, as osteosynthetic materials, in surgery and tissue engineering.

Bacillus thuringiensis is endospore forming Gram-positive soil bacterium that produces crystal proteins (*cry* proteins), which are toxic to many species of insects. Though the accumulation of PHAs has been first identified in *Bacillus megaterium* in 1926 by Lemoigne, the molecular characterization of the genes encoding for PHB synthesis have been studied very recently. Also the studies in this species have resulted in a new class of PHA synthases (Class IV) and recognition of new genes *pha*P, *pha*Q, and *pha*R.

The present study reports the isolation of *Bacillus thuringiensis* R1 that accumulates PHB utilizing cheaper carbon sources, isolation, characterization and heterologous expression of an ~5 kb gDNA fragment that is responsible for the accumulation of PHA.

The main features of the present thesis are:

- Isolation, identification and characterization of PHA producing *Bacillus thuringiensis* R1
- Physical characterization of PHA from *Bacillus thuringiensis* R1
- PHA gene locus from *Bacillus thuringiensis* R1: Isolation, cloning, sequencing and expression in *E. coli*
- Sequence analysis of the ~5.0 kb genomic DNA fragment of *Bacillus thuringiensis* R1 harboring the PHB biosynthetic gene locus.

1. Isolation, identification and characterization of PHA producing Bacillus thuringiensis R1

Soil samples were collected from different locations on the National Chemical Laboratory, Pune campus and the bacteria were screened for PHA production by Nile Red staining and polymer extraction by cell lysis. Based on biochemical characteristics, the PHA+ isolate was characterized and identified as the endospore forming, Gram-positive *Bacillus thuringiensis,* and designated as the strain R1.

Time course studies showed that maximum cell mass formation and the accumulation of the polymer coincided with the beginning of the stationary phase (36 hrs) of the cell growth cycle. GC analysis of the *Bacillus thuringiensis* R1 cells revealed that the cells supported synthesis of the PHB homopolymer when supplied with a single carbon source. Among the different carbon sources used glycerol supported maximum PHB accumulation (34.18% of the dry cell mass) followed by table sugar (28.23%), molasses (23.06%) and fructose (19.76%). Under biphasic growth conditions PHB accumulation increased from 34.23% to 64.10% of dry cell mass. *Bacillus thuringiensis* R1 is found to accumulate PHB when grown on a single carbon source while PHB-V was accumulated when glycerol and propionic acid were used in the medium as the carbon sources.

2. *Physical characterization of PHA from Bacillus thuringiensis R1*

Bacillus thuringiensis R1 cells were grown in basal medium supplemented with glycerol and the polymer extracted by cell lysis was used to study the physical characteristics of the PHA.

PHA granules isolated from the *B. thuringiensis* R1 cells observed under scanning electron microscope showed stable spherical configuration with an average diameter of 5 microns. Physical characterization of PHA by ^{1}H NMR and FTIR revealed the PHA to be PHB. ^{1}H NMR of the purified PHB showed peaks typical to the isotactic PHB homopolymer. The GPC analysis revealed that the polydispersity index (Q) (defined as Mn/Mw), number average molecular weight (Mn), weight average molecular weight (Mw) were 1.77, 5.8526×10^4 and 1.0385×10^5 respectively. DSC and TGA analysis showed that the polymer degraded rapidly between 225°C and 270°C with a peak at 261°C. The melting temperature and the enthalpy of fusion of the polymer were 165.6°C and 84.1 J/g respectively.

3. *PHA gene locus from Bacillus thuringiensis R1: Isolation, cloning, sequencing and expression in E. coli*

Two sets of primers were used to amplify the *pha*C gene from *Bacillus thuringiensis* R1. The first set of primers (*pha*C F2 & *pha*C R4) were based on the multiple sequence alignment results of 19 PHA positive bacterial strains. The second set of primers (P5 and P6) were kindly provided by Dr. Satoh who has used the same to amplify *pha*C from *Bacillus* sp. INT005. The amplicons were cloned into pGEMT vector to produce clones pRDC1 and pRDC2 from the first and second set of primers respectively. The inserts were sequenced and sequence homology search was done which showed that the insert from pRDC2 corresponded to the putative *pha*C gene and could be used as a probe to construct and screen the genomic library of *Bacillus thuringiensis* R1.

Based on the Southern hybridization analysis genomic DNA from *Bacillus thuringiensis* R1 was digested with *Sac* I-*Cla* I and a sub-genomic library was constructed in pBluescriptKS+ vector. The clones were screened for PHB operon by

amplification of the *pha*C gene and colony hybridization using the insert from pRDC2 as the probe.

One positive clone (pSVA10) was obtained, this clone was found to harbor an ~5.0 kb insert based on restriction analysis. This clone was also checked for PHA accumulation, however, the accumulation of PHAs was negligible (3.0%) as compared to *Bacillus thuringiensis* R1 (34.0%). The ~5.0 kb insert in pSVA10 was subjected to automatic sequencing and the complete sequence was found to be 4787 bp. This sequence was deposited with NCBI Genbank and is available under the accession No. **DQ000291**.

4. Sequence analysis of the ~5.0 kb genomic DNA fragment of Bacillus thuringiensis R1 harboring the PHB biosynthetic gene locus.

A total of 6 ORFs coding for 6 putative genes were identified using pDRAW32 revision 1.1.61. The sequence homology search showed that the putative genes encoded by ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6 corresponded to *pha*P, *pha*Q, *pha*R, *pha*B, *pha*C and partial sequence of oxidoreductase. The sequence analysis also confirmed the absence of *pha*A (gene coding for β-ketothiolase) in the gene cluster. The putative promoters, ribosome binding sites and terminator hairpin loops for each gene were identified. Characterization of putative *pha*C (ORF 5) showed the presence of a conserved lipase box **G-Y-C-M-G-G**; Aspartic acid and Histidine residues at positions 306 **(D^{306})** and 335 **(H^{335})** that form a part of the catalytic triad needed for synthase function. Characterization of putative *pha*B (ORF 4) showed the NADPH binding motif, **V-T-G-G-A-K-G-G** that is conserved in PHA-specific acetoacetyl-CoA reductases. To increase the PHB production, plasmid pSRAB that included *pha*A and *pha*B was introduced into pSVA10 in order to synthesize monomers. The clones obtained were checked for the presence of both the plasmids based on antibiotic selection and restriction analysis. The positive clone pSVAR10 was checked for PHB production. The clone (pSVAR10) produced PHB (25.0%) of dry cell mass. This confirmed that the 4787 bp insert from pSVA10 harbored the genes for PHB synthesis from *Bacillus thuringiensis* R1.

CHAPTER 1

INTRODUCTION

Plastic materials have become an integral part of contemporary life because of their many desirable properties including durability and resistance to degradation. For the last 60 years, synthetic polymeric materials have grown up progressively forming one of the most attractive domains in material science. This success is primarily due to their low cost, their reproducibility, and their resistance to physical aging and biological attacks. However, the resistance of synthetic polymers to degrading action of living systems is becoming more and more problematic in several domains where they are used for a limited period of time before becoming wastes (Vert 2005). Approximately 140 million tonnes of synthetic polymers are produced worldwide each year. These nondegradable plastics accumulate in the environment at a rate of millions of tons per year (Anderson and Dawes 1990). Conventional plastics, formed from fossil fuels, are being joined in the market place by materials created with processes much less harmful to the environment. These materials are known as biopolymers and are produced by microorganisms, plant or animal material. Biopolymers may be defined as products which are based on renewable agricultural or biomass feed stock, capable of behaving like conventional plastics in production and utilization, but biodegradable through microbial processes (Mohanty *et al.* 2003). At a time when space for waste disposal is at a premium in many regions of the world, nondegradable conventional plastics such as polyethylene and polypropylene are filling landfills. Recently problems concerning global environment and solid waste management have created much interest in the development of biodegradable plastics that still retain the desired physical and chemical properties of conventional synthetic plastics. Biopolymers may be used alone, or in complement to conventional plastics, thus reducing the required volume of each and increasing desirable qualities of the final product. There are a variety of biopolymer materials designed for use in industry, from construction materials to plastic wraps. As mentioned by Gerngross and Slater (2000), worldwide production of plastics consumes approximately 270 million metric tons of fossil fuel each year, as a source of feedstock and energy. Although fossil fuels are readily available, biopolymer researchers are in tune with the fact that a replacement feed stock of plastic materials will be required within a relatively short time. The development of bio-based products is a promising option in reducing this widespread dependence on fossil fuels (Latere Dwan'sia *et al.* 2004).

1.1 Biopolymers

Living systems are capable of synthesis of a wide range of different biopolymers. Biopolymers occur in any organism and constitute by far the major fraction of cellular dry matter. According to their chemical structure biopolymers belong to eight classes as depicted in Table 1.1. (Pötter and Steinbüchel 2005).

1. **Nucleic acids**
   DNA, RNA
2. **Polyamides**
   Proteins and poly(aminoacids)
3. **Polysaccharides**
   Cellulose, Dextran and starch
4. **Polythioesters (PTEs)**
   Poly(3-mercaptopropionate)
5. **Polyanhydrides**
   Polyphosphate
6. **Polyisoprenoids**
   Natural rubber
7. **Polyphenols**
   Lignin
8. **Organic polyoxoesters**
   Polyhydroxyalkanoates (PHAs), polymalate and cutin

Many of these biopolymers are reserve compounds often stored in the cytoplasm as insoluble inclusions (Hoppert and Mayer 1999). The best-studied storage compounds in bacteria are PHAs.

1.2 History of Polyhydroxyalkanoates (PHAs)

That bacteria could produce polyesters was unknown to polymer chemists before 1960 and even to most biochemists and microbiologists before 1958, although their presence in bacterial cells in isolatable amounts, their chemical composition, and even that they were polymers were reported in the literature as early as 1926. The presence of Sudanophilic lipid like inclusions (Meyer 1903) soluble in chloroform (Stapp 1924) was initially observed in *Azotobacter chroococcum* a century ago. The chemical composition of similar inclusions in *Bacillus megaterium* was later identified as poly-3-hydroxybutyric acid P(3HB) by Lemoigne (1926, 1927). Due to studies on several *Bacillus* strains (Macrae and Wilkinson 1958) and phototropic bacteria (Doudorff and Stainer 1959) P(3HB) became more widely known. Wallen and Rohwedder (1974) reported the identification of polyhydroxyalkanoates other than P(3HB) especially poly-3-hydroxyvalerate P(3HV) and poly-3-hydroxyhexanoate P(3HHx). In 1983, Frindley and White reported accumulation of 95% P(3HB), 3% poly-3-hydroxyheptanoate P(3HHp), 2% of an 8-carbon hydroxyalkanoate (HA) and trace amounts of 3-other HA compounds by batch grown *Bacillus megaterium.* Around the same time De Smet (1983) and coworkers found *Pseudomonas oleovorans* to synthesize and accumulate poly-3-hydroxyoctanoate P(3HO) units and small amounts of 3-hydroxyhexanoate (HHx) units when cultivated on n-Octane (De Smet *et al.* 1983, Lageveen *et al.* 1988).

Major impact on the research and commercial sectors for the bacterial reserve polymer was seen with the identification of HA units other than 3HB. While P(3HB) has limited applications due to its brittle nature, incorporation of other 3HAs significantly enhanced the properties of biopolymers. This finding is highlighted as a landmark that signifies the beginning of the second stage of research on PHAs. At this stage the first industrial production of copolymer P(3HB-co-3HV) took place. The emphasis now shifted to identification of all the 3HAs that could be associated with the bacterial polyesters. In 1982, Imperial Chemical Industries Ltd. (ICI) in England announced a product development program on a new type of thermoplastic polyester which was totally biodegradable and could be melt processed into a wide variety of consumer products including plastics, films and fibers (Anderson and Dawes 1990).

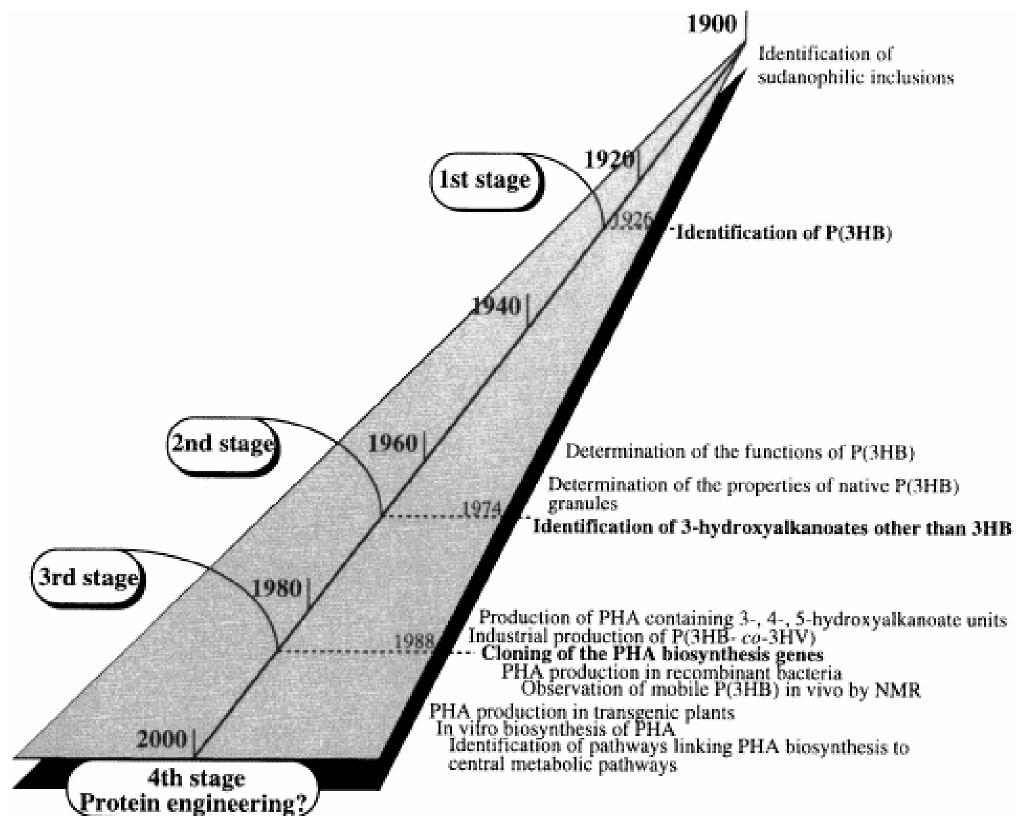
The bacterium used for this large scale production was *Alcaligenes eutrophus*, since renamed *Ralstonia eutropha* (more recently changed again to *Wausteria eutropha*) (Lenz and Marchessault 2005) and the commercial product named as "Biopol" which was a copolyester containing randomly arranged units of (R)-3-hydroxybutyrate, HB, and (R)-3-hydroxyvalerate, HV (Holmes 1988). During this period it became clear that not only Gram-negative but also a wide range of Gram-positive bacteria, cyanobacteria (aerobic photosynthetic), non-sulfur and sulfur purple bacteria (anaerobic photosynthetic), archaea can synthesize and accumulate these 3HAs. As an astounding number of approximately 150 different HAs are known to occur (Steinbüchel and Valentin 1995; Rehm and Steinbüchel 1999), a more general name comprising of all the constituents namely polyhydroxyalkanoates (PHAs) has been used to designate this family of bacterial reserve polymers.

The third stage of development in this field was the cloning and characterization of genes involved in PHA biosynthesis. Late 1970s, provided tools to successfully decipher genetic information and a molecular biology revolution ensued. By late 1980s, PHA biosynthesis genes of *Wausteria eutropha* (earlier *Ralstonia eutropha* and *Alcaligenes eutrophus*) were cloned and shown to be functionally active in *E. coli* (Slater *et al.* 1988; Schubert *et al.* 1988; Peoples and Sinskey 1989a, b). Detailed studies on *W. eutropha* revealed three enzymes *viz.* β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase to be involved in the biosynthesis of P(3HB) from acetyl-CoA and that the biosynthesis to be regulated at the enzyme level. Of the three enzymes PHA synthase was identified to play a key role in polymerization of HA monomers. To date more than 59 different PHA synthase genes have been cloned and assigned from different organisms (Rehm 2003).

The Sudanophilic bacterial inclusion identified at the beginning of the twentieth century is going into fourth stage of development, i.e. protein engineering, by which manipulation of PHA synthase and other enzymes would enable us to make environment friendly polymers in an efficient manner (Sudesh *et al*. 2000). Thus, genetic engineering combined with modern molecular microbiology provides a framework for studying plastic properties as a function of genetic and metabolic

blueprints (Madison and Huisman 1999). The development of PHA science and technology through the twentieth century is represented in Fig. 1.1.

Figure 1.1 The development of PHA science and technology through twentieth century (Sudesh *et al.* 2000)

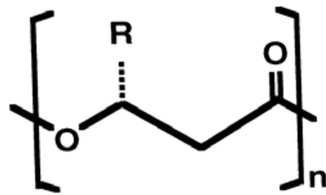


1.3 Polyhydroxyalkanoates

Polyhydroxyalkanoic acids (PHAs) are a class of aliphatic polyesters produced by many bacteria and archaea in response to various environmental conditions. PHAs are generally regarded as a carbon and energy reserve (Anderson and Dawes 1990; Lee 1996; Steinbüchel 1991). The accumulation of PHA increases in some bacteria when growth is limited by a nutrient other than carbon (e.g., nitrogen or phosphate),

while in other bacteria it readily accumulates during unrestricted growth (McCool *et al.* 1996; Braunegg *et al.* 1998). PHAs are accumulated in the cells as discrete granules, the size and the number per cell varies depending on different species. According to Byrom (1994) about 8 to 13 granules per cell having diameter range of 0.2 to 0.5 µm were observed in *Wausteria eutropha*. PHAs are deposited intracellularly in the form of inclusion bodies to levels up to 90% of the cellular dry weight. These high molecular weight molecules, typically having molecular weights on the order of 2 x 10^5 to 3 x 10^6, are composed of linear array of repeating 3-hydroxyacid monomeric units (Lee 1996). Their general formula is shown in Fig.1.2 (Steinbüchel 1991; Byrom 1994).

Fig 1.2 General formula showing the monomer repeat unit of PHA. The pendant 'R' could be substituted to get a desired PHA. For example in PHB, R=methyl group; PHV, R=ethyl group and so on.



PHAs can be biodegraded to water and carbon dioxide or methane by a large variety of ubiquitous microorganisms present in many ecosystems. This fairly easy biodegradability came as a surprise given the inertness of the water-insoluble, hydrophobic, and (partially) crystalline polymers (Jendrossek 2000).

1.3.1 Classification of PHAs

PHAs are divided into two broad groups based on the number of carbon atoms in the monomer units. They are:

a. Short chain length PHAs - $PHAs_{(SCL)}$ – consist of C_3-C_5 atoms
b. Medium chain length PHAs - $PHAs_{(MCL)}$ – consist of C_6-C_{14} atoms

This grouping is due to the substrate specificity of the PHA synthesis that only accept 3-hydroxyalkanoates (3HAs) of a certain range of carbon length (Anderson and

Dawes 1990). $PHAs_{(SCL)}$ include poly-3-hydroxybutyrate P(3HB), poly-4-hydroxybutyrate P(4HB) (Kunioka *et al*. 1988; Doi 1990), poly-4-hydroxyvalerate P(4HV) (Valentin *et al*. 1992) and poly-5-hydroxyvalerate P(5HV) (Doi *et al*. 1987). $PHAs_{(MCL)}$ include poly-3-hydroxyhexanoic acid P(3HHx), poly-3-hydroxyheptanoic acid P(3HHp), poly-3-hydroxyoctanoic acid P(3HO), poly-4-hydroxyhexanoate P(4HHx) etc. (Valentin *et al*. 1994).

1.3.2 Properties of PHAs

PHAs are a family of optically active biological polyesters, containing (*R*)-3HA monomer units (Anderson and Dawes 1990). The 3-hydroxyalkanoic acids are all in R-configuration due to the stereospecificity of the polymerizing enzyme PHA synthase. Only in the case of *Rhodococcus* sp. S-monomers were detected (Haywood *et al*. 1991). At the C-3 or β position, an alkyl group, which can vary from methyl to tridecyl is positioned. This alkyl chain can be saturated, aromatic, unsaturated, halogenated, epoxidized or with branched monomers (Abe *et al.* 1990; Fritzsche *et al.* 1990; Kim *et al.* 1991; Kim *et al.* 1992; Choi and Yoon 1994; Hazer *et al.* 1994; Curley *et al.* 1996; Garcia *et al.* 1999; Arkin *et al.* 2000). Along with the variation in the alkyl substituent, the position of the hydroxyl group is variable, and 4-, 5- and 6-hydroxyacids have been incorporated. Side chains can be modified further to get different PHAs (De Koning *et al.* 1994; Gagnon *et al.* 1994a, b). Such a vast array of variations forms the basis for diversity of the PHA polymer family. PHAs are insoluble in water and exhibit a rather high degree of polymerization ranging from 10^5 to almost 10^7 Da.

Chemical structure and monomer composition are the most important factors in determining the physical and material properties of a polymer. Most of the studies on physical and thermal properties of bacterial PHAs have been carried out with PHB and PHV. Since the presence of functional groups, epoxy-, hydroxy-, aromatic- side chains, opens up a wide range of possibilities for further chemical modification of the polymer structure, their incorporation to polymers influences the physical and material properties of PHAs (Lageveen *et al*. 1988; Fritzsche *et al*. 1990; Hori *et al*. 1994; Eggink *et al*. 1995; Casini *et al*. 1997).

The most important property of PHAs is their complete biodegradability. PHAs like PHB and PHBV are moldable thermoplastic polymers. PHB has a melt temperature of 175°C and a glass transition temperature (Tg) of 4°C (Byrom 1987; Doi 1990; Marchessault *et al.*1990; Holmes 1988). The decomposition temperature of ~200°C is rather too close to the melting temperature. However, Hahn *et al*. (1995) have shown the decomposition temperature of PHB recovered from recombinant *E. coli* to be between 250-300°C. Incorporation of 3HV units into PHB decreases the melting temperature of the co-polymer without affecting the thermal stability, thus making processing easy. The PHB recovered from recombinant *E. coli* harboring the PHA biosynthetic genes from *S. aureofacians* NRRL 2209 rapidly degraded between 250°C and 340°C with a peak at 295°C; the melting temperature and enthalpy of fusion of the polymer were 173°C and 85.0 J g^{-1} respectively (Ramachander *et al.* 2002). The melting temperature and the enthalpy of fusion of the PHB extracted from *Bacillus thuringiensis* R1 were 165.6°C and 84.1 J/g respectively and the polymer degraded rapidly between 225°C and 270°C with a peak at 261°C (Rohini *et al.* 2005).

PHB, a short chain length polymer, is optically pure i.e., it is 100% stereo-specific with D(-) configuration (Doi 1990; Steinbüchel 1991) and isotactic in nature. It is relatively stiff (Barham *et al*. 1984; Doi 1990; Holmes 1988) and highly crystalline with crystallinity ranging between 55 to 88% (Lee 1996).

The mechanical properties of P(3HB) like Young's modulus (3.5 GPA) and the tensile strength (40Mpa) are similar to those of polypropylene. Molded PHB samples do indeed show ductile behavior, but over a period of time at ambient conditions, they slowly become more brittle (Lee 1996). However, the elongation to break for PHB is 5%, which is significantly lower than polypropylene (400%). This unfavorable aging process and poor shatter strength are major drawbacks for the commercial exploitation of the PHB homopolymer. The co-polymer PHB-co-HV has much improved mechanical properties than the homopolymer P(3HB).

In comparison PHA_{MCLs} are generally elastomers with low melting point (42°-58°C). These are also highly amorphous with Tg of -62°C to 26°C (Preusting *et al*. 1990; Casini *et al*. 1997; Van der Walle *et al*. 1999). Crystallinity is hampered with

the incorporation of side chains into PHA_{MCL} although saturated PHA_{MCLs} are able to crystallize due to their isotactic configuration. In general PHA_{MCLs} are semi crystalline elastomers with low melting point, low tensile strength and high elongation to break (Gross *et al*. 1989; Preusting *et al*. 1990; Gagnon *et al*. 1992) and can be used as a biodegradable rubber after cross-linking by electron beam irradiation (De Koning *et al*. 1994). Elongation to break of 250 - 350% and a Young's modulus up to 17 Mpa has been reported (Marchessualt *et al*. 1990). A comparison of PHB properties with that of other polymers is summarized in Table 1.2. The general properties and features of PHB are listed in Table 1.3.

1.3.3 Applications of PHAs

PHAs have attracted considerable industrial interest as candidates for biodegradable and/or biocompatible plastics for a wide range of applications. PHAs have been in use for various applications such as biodegradable packagings, films and fibres, latex coatings, adhesives, as biomedical disposable material, as osteosynthetic materials, in surgery and tissue engineering (Van der Walle *et al.* 2001; Lee and Choi 2001). Mcl-PHA can be used as pressure sensitive adhesive, binder in paints, coating materials, cheese coating, elastomers and in agriculture (Van der Walle *et al.* 1999), drug delivery (Williams and Martin 2002; Martin and Williams 2003), and tissue engineering (Sodian *et al.* 2000, Williams *et al* 1999). Owing to the chiral nature of the PHAs they can be used as starting material in drug industry (Van der Walle *et al.* 2001). Pötter and Steinbüchel (2005) described the possibility of using PHA granules for developing fusion proteins and construction of nanobiomaterials. These PHAs can be used as biodegradable matrixes carrying protein complexes such as insulin, antibodies, antitumour components etc.

Their applications have been extended to medical field as osteo-synthetic materials, bone plates, surgical sutures etc. P(4HB) also called as gamma-HB (GHB) has been found to have a potential therapeutic value as an anaesthetic agent since it can cross the brain barriers and induce sleep like state with cardiovascular stability (Laborit 1964; Vickers 1968; Hunter *et al*. 1971). A co-polymer of P(3HB-co-4HB) is biocompatible, biodegradable and being used in medical implants (Doi US patent 230461). Summary of the applications is given in Table 1.4.

Table 1.2 Properties of some PHAs and synthetic polymers (Poirier *et al.* 1995; Lee 1996; Sudesh *et al.* 2000)

Polymer	Melting Temperature °C	Glass-transition temperature °C	Young's modulus GPA	Elongation to break %	Tensile strength MPa
P(3HB)	180	4	3.5	5	40
P(3HB-co-3HV)*	145	-1	-	-	32
P(4HB)	53	- 48	149	1000	104
PHA_{MCL}	45-54	- 25 to - 40	-	~350	≤17
Polypropylene	176	- 10	1.7	400	34.5
Polystyrene	240	100	3.1	-	50

- P(HB-HV) copolymer containing 20 mol% C5 and 80 mol% C4 monomers

Table 1.3 Summary of the properties and features of PHAs

Properties and Features of PHB
• Thermoplastic
• Biodegradability
• Biocompatible
• Non-toxic
• Optically pure
• Isotactic
• Insoluble in water
• Highly crystalline
• Piezoelectric
• Can be produced from renewable Sources

Table 1.4: Summary of the applications of PHAs in various fields

Applications of PHAs
• Packaging films, bags and containers
• Biodegradable carrier for long term dosage of drugs, medicines, fertilizers etc.
• Disposable hygiene products like razors, diapers, female hygiene products etc.
• Raw material for chiral compounds that are enantiomerically pure chemicals and paints
• Water resistant layers and hot melt adhesives
• Ion conducting polymers
• Intravenous anesthetic
• Osteosynthetic material and bone plates
• Surgical pins, sutures, staples and swabs
• Blood vessel replacements
• Wound dressing
• Piezoelectric stimulation of bone growth and healing
• Bionanomaterials

1.4 Identification of PHA-producing organisms

Lipophilic dyes such as Sudan Black B, Nile blue A, Nile red have been traditionally used for the first line screening of PHA-containing bacteria (Schlegel *et al.* 1970; Ostle and Holt 1982; Kitamura and Doi 1994; Kranz *et al.* 1997). Typically, colonies of bacterial cells grown on agar plate are stained with the lipophilic dye dissolved in an organic solvent. Following the destaining process, cells containing lipid inclusion bodies such as PHA are identified by the retention of the dye. Variations of these techniques that allow the isolation of a viable cell were later developed (Tagaki and Yamane 1997; Spiekermann *et al.* 1999). In addition to staining of cells with lipophilic dyes, the variations also facilitated the characterization

of PHA-producing cells by instrumentation using fluorescence based methods to measure PHA levels in cells (Braunegg *et al.* 1978; Degelau *et al.* 1995; Gorenflo *et al.* 1999). Wu *et al.* (2003) could differentiate the Nile red stained scl-PHA and Nile red stained mcl-PHA based on the shift of the fluorescence maximum to differentiate the two types of polymers. A flow cytometric technique was used to differentiate microorganisms producing scl- and mcl- PHA copolymers following Nile red staining of the cells (Srienc *et al.* 2000). A rapid method to identify and differentiate cells producing scl- or mcl-PHA using Fourier transform infrared (FT-IR) spectroscopy was developed by Hong *et al.* (1999). Nucleic acid based methods for screening of PHA-producing microorganisms were developed which include Southern hybridization (Timm *et al.* 1994) and Polymerase Chain Reaction (Lopez *et al.* 1997; Solaiman *et al.* 2000; Solaiman 2002; Zhang *et al.* 2001; Hang *et al.* 2002; Sheu *et al.* 2000; Rehm 2003; Shamala *et al.* 2003) using different probes and primers.

1.5 Physiology of PHA metabolism

The effect of the growth conditions of PHA metabolism was first studied by Macrae and Wilkinson (1958) in an asporogenous strain of *B. megaterium*. They made an important observation that PHA accumulation occurred in response to an imbalance in growth brought about by nutrient limitations and the quantity of PHA accumulated increased as the carbon to nitrogen ratio increased. The biosynthesis of PHA was shown to be initiated by magnesium or sulfate deficiency (Sudesh *et al.* 2000), as well as by nitrogen, phosphate (Dawes and Senior 1973; Repaske and Repaske 1976) and/or oxygen limitations (Ward *et al.* 1977).

Physiologically, PHA was first associated with the sporulation of bacteria (Slepecky and Law 1961). It was observed that PHA is formed before the onset of sporulation, and that rapid utilization of the polymer precedes sporulation. However, PHA is not always associated with sporulation since not all spore formers make polymer. The polymer, if present was therefore thought to be a ready source of carbon and energy for the energy-demanding process of sporulation (Emeruwa and Hawirko 1973). PHA constitutes an ideal carbon-energy storage material due to its low solubility and high molecular weight, which exerts negligible osmotic pressure to the

bacterial cell (Dawes and Senior 1973). Senior and Dawes (1971) also proposed that PHA serves as a sink for reducing power and therefore could be regarded as a redox regulator within the cell. In symbiotic nitrogen fixation, PHA is found to serve as energy source as well as have a regulatory role in controlling the availability of reducing power for the operation of nitrogenase (McDermott *et al.* 1989).

A low molecular weight P(3HB) consisting of 100-200 monomer units, has been found in prokaryotes and eukaryotes, which seem to function as component of an ion channel through cell membranes (Reusch and Sadoff 1983; Reusch and Sadoff 1988; Reusch 1992). In human plasma, P(3HB) can be found associated with very-low-density lipoprotein and low-density lipoprotein, but not with high-density lipoprotein. In addition, a significant portion of P(3HB) is found to be associated with serum albumin. The lipid molecules and albumin are thought to be acting as transporters of P(3HB) through blood, with albumin being the major carrier (Reusch 1992).

1.6 Enzymes involved in PHA biosynthesis

PHB being the most widespread and thoroughly characterized of the PHAs, most knowledge on PHB biosynthesis has been obtained in *Wausteria eutropha* (Steinbüchel and Schlegel 1991). The PHB biosynthetic pathway consists of three enzymatic reactions catalyzed by three distinct enzymes. The enzymes are β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase.

1.6.1 β-ketothiolase

β-ketothiolase catalyzes the first step in P(3HB) formation. It is a member of the family of enzymes involved in the thiolytic cleavage of substrate into acyl-CoA and acetyl-CoA. This is the most thermodynamically favored reaction. But under the availability of reducing equivalents in the form of NADPH, it acts against the thermodynamically favored direction and aids in the P(3HB) biosynthesis (Masamune *et al*. 1989). These are found through out nature from higher eukaryotes to yeasts to prokaryotes and are found to be in soluble form *in vivo*.

Based on the substrate specificity thiolases are divided into two groups. First group of thiolases fall into the enzyme Class EC.2.3.1.16 and have a broad specificity for β–ketoacyl-CoAs ranging from C_4-C_{16}. While the second group, belonging to enzyme Class EC.2.3.1.9. has a narrow range of chain length specificity from C_3-C_5. The first group is involved mainly in the degradation of fatty acids and is located in the cytoplasm of prokaryotes and in the mitochondria and peroxisomes of mammalian and plant cells. The second type is considered to take part in biosynthesis of ketone bodies, steroid and isoprenoid biosynthesis, and P(3HB) biosynthesis (Madison and Huisman 1999).

In *Wausteria eutropha* two β-ketothiolases, enzyme A and enzyme B, have been discovered to take part in biosynthesis of PHA. The major difference between the two enzymes is substrate specificity. Enzyme A is a homo-tetramer of 44-kDa subunits and converts acetoacetyl-CoA and 3-ketopentanoyl-CoA to P(3HB) and P(3HV) respectively. On the contrary enzyme B, a homo-tetramer of 46 kDa subunits, has broad substrate specificity and cleaves acetoacetyl-CoA, 3-ketoheptanoyl-CoA, 3-ketooctanoyl-CoA, 3-ketodecanoyl-CoA (Madison and Huisman 1999). It was earlier thought that enzyme B is associated with the fatty acid degradation while enzyme A actively participated in P(3HB) biosynthesis. Haywood *et al*. (1988a) and Slater *et al*. (1998) have shown that enzyme B is the primary catalyzer for the P(3HB-3HV) formation.

The enzymatic mechanism of β-ketothiolase consists of biological Claisen condensation of two acetyl-CoA-moieties wherein the formation of carbon-carbon bond is catalyzed by β-ketothiolase (EC.2.3.1.9). In this enzymatically deprotonated anion derived from acetyl-CoA reacts as a nucleophile with an electrophilic acetyl-s-enzyme intermediate derived from the binding of acetyl-CoA to the active cysteine of thiolase (Masamune *et al*. 1989). Two cysteines are supposed to take part in the above reaction. With the cloning, overexpression and purification of thiolase genes of *Z. ramigera*, definitive roles of the cysteines (**Cys 89** and **Cys 378**) were determined (Davis *et al*. 1987; Masamune *et al*. 1989; Thompson *et al*. 1989) and all the thiolases reported till date seem to contain the two cysteines.

1.6.2 Acetoacetyl-CoA reductase

This is a (R)-3-hydroxyacyl-CoA dehydrogenase. Like ketothiolase, it is also a soluble protein. It catalyzes second step in the P(3HB) biosynthetic pathway by stereo-selective reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA. Two types of reductases are found in organisms (Haywood *et al*. 1988a, 1988b). First type is a NADH dependant reductase, EC.1.1.1.35, while the second type, EC.1.1.1.36, is NADPH dependant. The former is a tetramer with identical subunits of 30 kDa and plays role in the β-oxidation of fatty-acids, and in association with thiolase (EC.2.3.1.16) participates in the conversion of butyrate, crotonoate and β-HB to acetyl-CoA. The latter is a homo-tetramer of 25 kDa subunits and participates in the biosynthesis of P(3HB) by reduction of acetoacetyl-CoA to 3-HBCoA (Fukui *et al*. 1987; Ploux *et al*. 1988; Schembri *et al*. 1994). In contrast to NADPH-dependant acetoacetyl-CoA reductase which uses exclusively D(-) isomer of β-HA-CoA, the NADH-dependant enzyme uses preferentially but not exclusively L(+) isomers as substrates for the oxidative direction of catalysis (Steinbüchel and Schlegel 1991). The only known NADH-dependant acetoacetyl-CoA reductase aiding in PHB synthesis has been reported from *A. vinosum* (Leibergesell and Steinbüchel 1992) and *S. coelicolor* (Packeter and Flatman 1983).

1.6.3 PHA synthase

PHA synthases are the key enzymes of PHA biosynthesis and catalyze the stereoselective conversion of (R)-3-hydroxyacyl-CoA substrates to PHAs with the concomitant release of CoA (Rehm and Steinbüchel 1999). The first synthase gene isolated, cloned and expressed was from *W. eutropha* (Peoples and Sinskey 1989a). It prefers C_4 substrates leading to the formation of poly-β-hydroxybutyrate.

1.6.3.1 Types of PHA synthases

To date 59 PHA synthase genes have been cloned and assigned from 45 different bacteria (Rehm and Steinbüchel 1999; Rehm and Steinbüchel 2001; Rehm *et al*. 2002). With respect to the primary structures deduced from these sequences, the

substrate specificities of the enzymes and the subunit composition, four major classes of PHA synthases can be distinguished (Rehm 2003)(Table 1.4).

Class I and class II PHA synthases comprise enzymes consisting of only one type of subunit (PhaC) with molecular masses between 61 kDa and 73 kDa (Qi and Rehm 2001). According to their *in vivo* and *in vitro* substrate specificity, class I PHA synthases (e.g. in *Wausteria eutropha*) preferentially utilize CoA thioesters of various (R)-3-hydroxy fatty acids comprising 3 to 5 carbon atoms, whereas class II PHA synthases (e.g. in *Pseudomonas aeroginosa*) preferentially utilize CoA thioester of various (R)-3-hydroxyfatty acids comprising 6 to 14 carbon atoms (Amara and Rehm 2003; Slater *et al*. 1992; Slater *et al*. 1988; Schubert *et al*. 1988; Peoples and Sinskey 1989b).

Class III PHA synthases (e.g. in *Allochromatium vinosum*) comprise enzymes consisting of two different type of subunits: (a) the PhaC subunit (molecular mass of approx. 40 kDa) exhibiting amino acid sequence similarity of 21-28% to class I and II PHA synthases and (b) the PhaE subunit (molecular mass of approx. 40 kDa) with no similarity to PHA synthases. These synthases prefer CoA thioesters of (R)-3-hydroxy fatty acids comprising 3 to 5 carbon atoms (Liebergesell *et al.* 1992; Yuan *et al.* 2001).

Class IV PHA synthases, a new class of synthases (e.g. in *Bacillus megaterium*) resemble the class III PHA synthases, but PhaE is replaced by PhaR (molecular mass approx. 20 kDa) (McCool and Cannon 2001).

Extensive comparision of the 59 PHA synthases from various bacteria revealed that these enzymes exhibit similarity (8-96% identical amino acids). With respect to amino acid sequence regions with stronger similarity, six conserved blocks could be identified, whereas the N-terminal region (approx. 100 amino acids relative to class I PHA synthases) is highly variable. Overall, eight amino acid residues are identical in all the known PHA synthases, suggesting an important role for these residues in enzyme function (Rehm 2003).

Recently a PHB synthase from an extremely halophilic archaebacterium was identified and characterized, which might constitute a new class of synthases (Hezayen *et al.* 2002). This enzyme was stable up to 60°C and still exhibited approx. 90% of maximum enzyme activity (Rehm 2003).

1.6.3.2 Genetics of PHA synthases

The PHA synthase genes and genes for other proteins related to metabolism of PHA are often clustered in the bacterial genomes (Rehm and Steinbüchel 1999; Rehm and Steinbüchel 2001). The organization of the PHA synthase genes in different classes is represented in Fig. 1.3.

In *Wausteria eutropha* genes for class I PHA synthase (*pha*C), β-ketothiolase (*pha*A) and NADPH-dependent acetoacetyl-CoA reductase (*pha*B) constitute the operon (Steinbüchel and Schlegel 1991; Slater *et al*. 1992; Slater *et al*. 1988; Schubert *et al*. 1988; Peoples and Sinskey 1989a). Besides the frequently found genetic organization of *W. eutropha* some bacteria show a different order but atleast, the synthase gene is co-localized with other PHB biosynthesis genes (Rehm and Steinbüchel 2001).

All Pseudomonads, which accumulate medium-chain-length PHAs possess two different *pha*C genes encoding class II synthases which are separated by the structural gene *pha*Z encoding intracellular depolymerase. In addition, downstream of the synthase gene arrangement, the *pha*D gene (encoding a structural protein with unknown function) is collinearly located, followed by the genes *pha*I and *pha*F, which are transcribed in the opposite direction and code for structural and regulatory proteins (Rehm 2003).

In all bacteria possessing class III PHA synthase, *pha*C and *pha*E are directly linked in their genomes and most probably constitute a single operon. The class IV synthase genes are found in bacteria belonging to the genus *Bacillus* and comprise *pha*R and *pha*C, which are separated by *pha*B (McCool and Cannon 2001; Satoh *et al.* 2002).

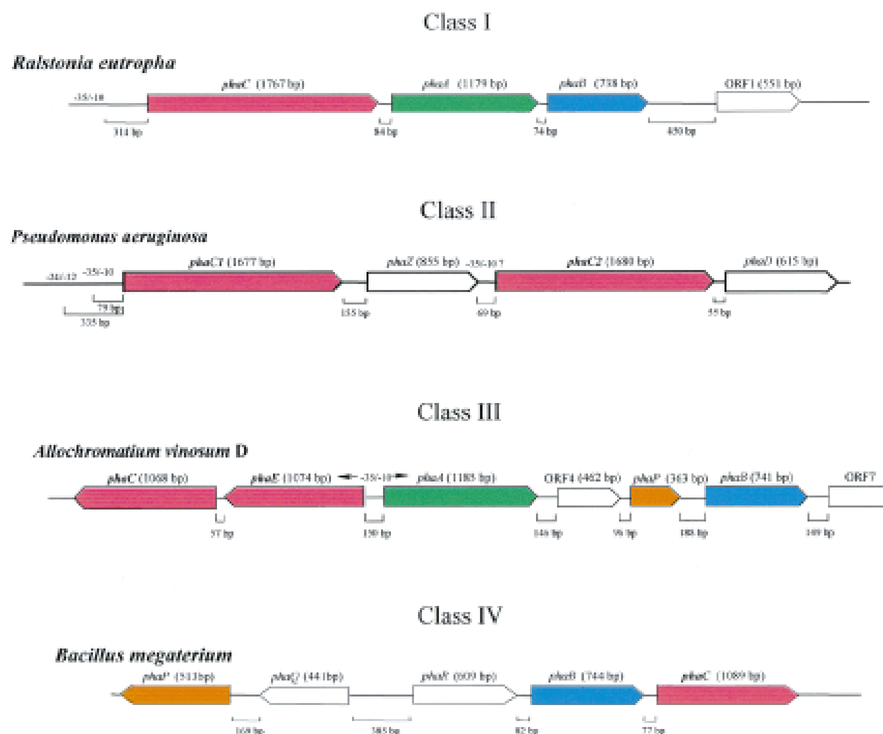
1.6.3.3 Structural features of PHA synthases

Though the evidence for secondary structure composition of PHA synthases has been obtained by predictions considering the multiple sequence alignment studies, the tertiary structure of PHA synthases has not been resolved by X-ray diffraction analysis (Rehm 2003). The studies revealed that PHA synthases are mainly composed

Table 1.4 Four different classes of PHA synthases (Rehm 2003)

Class	Subunits	Species	Substrate
I	PhaC ~60-73 kDa	*Ralstonia eutropha*	$3HA_{SCL}$-CoA (~C3-C5) $4HA_{SCL}$-CoA, $5HA_{SCL}$-CoA, $3MA_{SCL}$-CoA
II	PhaC ~60-65 kDa	*Pseudomonas aeruginosa*	$3HA_{MCL}$-CoA (~≥C5)
III	PhaC PhaE ~40 kDa ~40 kDa	*Allochromatium vinosum*	$3HA_{SCL}$-CoA ($3HA_{MCL}$-CoA [~C6-C8], 4HA-CoA, 5HA-CoA)
IV	PhaC PhaR ~40 kDa ~22 kDa	*Bacillus megaterium*	$3HA_{SCL}$-CoA

Figure 1.3 Organization of PHA synthases in different classes (Rehm 2003)



of variable-loop (49.7%) and α-helical (39.8%) secondary structures, whereas only 10.4% are predicted to be β-sheet secondary structures (Cuff *et al.* 1998). CD spectroscopy studies of PHA synthase from *P. aeruginosa* showed the secondary structure composition as: 10% α-helix, 50% β-sheet and 40% random coil (Rehm *et al.* 2001). This shows that the PHA synthases correspond to the missed class of proteins with respect to secondary structure prediction.

In vitro PHA synthases exist as equilibrium of monomeric and dimeric forms. However, dimerisation is found to be significantly induced in the presence of substrate or trimeric CoA analogues (3-hydroxybutyryl)$_3$-CoA respectively (Rehm *et al.* 2001; Wodzinska *et al.* 1996). It was also observed that in presence of trimeric analogues there was a reduction in enzymic lag phase and increase in the specific activity (Müh *et al.* 1999; Wodzinska *et al.* 1996). This indicates that the dimeric form is substantially more active than the monomeric form in the absence of putative primer (Wodzinska *et al.* 1996). They observed that labeled trimeric-CoA analogs were covalently bound to the dimeric form of PHA synthase as indicated by size exclusion chromatography. Immunoelectron microscopy studies on *A. vinosum* PHA synthase have shown the presence of PHA synthase complexes comprising of 10 sub-units at the surface of the PHA granule (Liebergesell *et al*. 1994; Mayer *et al*. 1996). Hence apparently *in vitro* PHA synthase consisted of 2 sub-units and is composed of 10 sub-units while associated with PHA granule *in vivo* (in *A. vinosum*).

The multiple sequence alignment studies showed the presence of six conserved blocks and eight conserved amino acid residues (Rehm and Steinbüchel 1999). All PHA synthases are found to contain a lipid box **(GX[S/C]XG)** (Steinbüchel *et al.* 1992) in which the essential active site **serine** of lipase is replaced with **cysteine** in the PHA synthase. These synthases require the active **cysteine** nucleophile for covalent catalysis. The conserved-domain-homology search strongly suggested that PHA synthases contain α/ β-hydrolase domain at the C-terminal region (Rehm 2003).

Jia *et al.* (2000) proposed an excellent structural model between residues 131-175 comprising the lipase box and the α/ β-hydrolase domain based on ClustalW alignment studies and threading algorithm studies of class III PHA synthases and lipases. This structure revealed that the conserved residues **His-331**, **Asp-302** and **His 303** are adjacent to the core structure. They also confirmed that **Cys-130** is not

involved in covalent catalysis as it is not adjacent to the core structure. However, a **Cys-149** residue at the nucleophile elbow is conserved and replacement of this residue strongly impairs catalytic activity.

A similar approach was used to build a structural model for class II PHA synthase PhaC1 from *P. aeuginosa,* which also showed significant identity with enzymes related to the superfamily of α/ β-hydrolases (Amara and Rehm 2003). The conserved and proposed catalytic residues of PhaC1 aligned with amino acid residues constituting the catalytic triad in enzymes belonging to α/β-hydrolases. A threading model of PhaC1 has been generated using alignment studies and some software packages. Inspection of the protein model of PhaC1 showed that the active site **Cys-296,** the conserved **His-480** and the **Asp-452,** presumably forming a catalytic triad, are adjacent to the core structure. These residues are conserved in all PHA synthases and are proposed to be required for catalytic activity (Qi and Rehm 2001). The active site **Cys-296** was located at the nucleophile elbow, a sharp γ-turn containing the nucleophilic residue, positioned between a β-strand and an α-helix, which is one of the most conserved features of the α/ β-hydrolases.

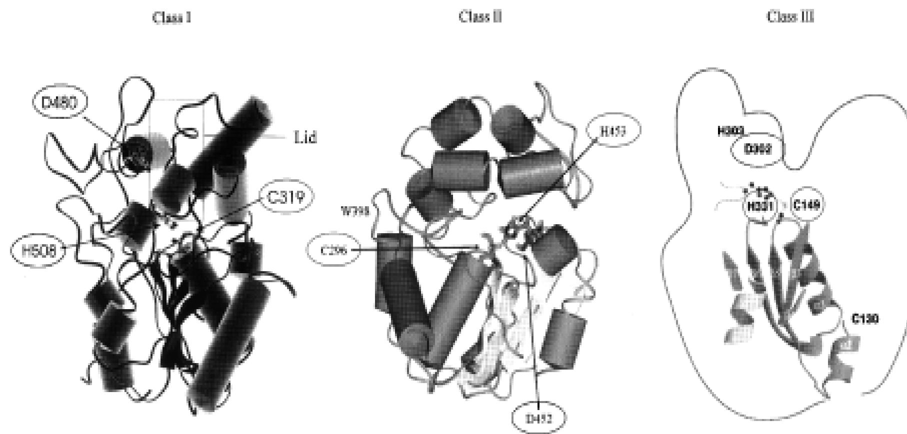
Recently a model was also generated for the class I PHA synthase from *W. eutropha* (Rehm *et al.* 2002). The protein model generated using the homology studies and software packages showed that the active site **Cys-319**, the conserved **His-508** and the **Asp-480**, presumably forming a catalytic triad, are adjacent to the core structure. The active site **Cys-319** was located at the nucleophile elbow, a sharp γ-turn containing the nucleophilic residue, positioned between a β-strand and an α-helix, which is one of the most conserved features of the α/ β-hydrolases. The proposed threading models of class I, class II and class III synthases are depicted in Fig. 1.4.

1.6.3.4 Catalytic mechanism of PHA synthases

PHA synthase studies involving various sulfahydryl inhibitors have suggested that these are sulfahydryl enzymes (Griebel *et al*. 1968). Ballard and coworkers (1987) proposed a model with two thiol groups being involved in locating HA monomers. It was later modified by Kawaguchi and Doi (1992) stating that water may act as a chain transfer agent. The modified model was contradicted based on the assumption

that the PHA synthase is not capable of transferring to a new chain (Gerngross and Martin 1995; Su *et al*. 2000). However, the proposed model was purely hypothetical.

Fig. 1.4 Threading models of Class I (Rehm *et al.* 2002), Class II (Amara and Rehm 2003) and Class III PHA synthases (Jia *et al.* 2000). Catalytic triad residues (cysteine-Aspartate-Histidine) are circled.



Site-specific mutagenesis of the class I PHA synthase from *W. eutropha* provided evidence that the conserved residues **Cys-319, His-508** and **Asp-480** are directly involved in covalent catalysis (Gerngross *et al.* 1994; Jia *et al.* 2000). **Trp-425** has been postulated to play an important role in protein-protein interaction (dimerisation of the PhaC subunit) by generating a hydrophobic surface (Jia *et al.* 2001). Mutational analysis of residues **Cys-130, Cys-149, His-303, His-331** and **Asp-302** of PhaC from *A. vinosum* clearly indicated that the residues **Cys-149, His-331** and **Asp-302** are involved in covalent catalysis. Replacement of these residues almost abolish enzymatic activity (Jia *et al.* 2000). In class II synthases from *Pseudomonas aeroginosa* the replacement of the putative general base catalyst **His-480** strongly impaired enzymatic activity and replacement of conserved cysteine and aspartic acid abolished the enzyme activity completely. In contrast with class I and III PHA synthases, the replacement of the class II synthase catalytic **Cys-296** by serine resulted

in a still active enzyme (Amara and Rehm 2003). The replacement of **Trp-398** with phenylalanine or alanine caused inactivation of the enzyme, indicating the essential role of the amino acid, presumably in dimerization, as for class I synthases (Amara and Rehm 2003; Jia *et al.* 2001). Overall the class II enzymes from pseudomonads represent a rather distinct group with unique features not found in class I and class III enzymes.

Griebel *et al.* (1968) proposed a chain elongation mechanism that involved two thiol groups of the PHA synthases during the catalytic cycle, as was found in fatty acid synthases (Witkowski *et al.* 1997). The development of structural models for classes I-III PHA synthases based on identity with enzymes belonging to the α/β-hydrolase superfamily, and mutational analysis of various highly conserved residues in these PHA synthases, led to the proposal of a new catalytic mechanism for PHA synthases (Rehm 2003; Amara and Rehm 2003; Jia *et al*. 2000). In this new model, two thiol groups are proposed to play key roles in covalent catalysis. One thiol group serves as the loading site for 3-hydroxy-butyryl-CoA and the second thiol group serves as the priming and elongation site. The highly conserved cysteine residues have been demonstrated to be involved in covalent catalysis (Amara and Rehm 2003; Müh *et al.* 1999; Wodzinska *et al.* 1996). Some experimental evidences have shown that the conserved serine acts as the loading site (Hoppensack *et al.* 1999; Amara and Rehm 2003; Jia *et al*. 2000)

Although it was possible to isolate modified PHA synthases with enhanced activity and changed substrate specificity, the functional role of the affected amino acid residues contributing to the modified enzyme properties remains unclear. The overall biochemical and enzymological studies of wild-type PHA synthase, as well as modified PHA synthases, will further illuminate structure function relationships and the catalytic mechanism of the PHA synthases. Moreover, resolution of the three dimensional structure of the PHA synthase by X-ray crystal analysis will be a breakthrough in understanding of these interesting class of enzymes (Rehm 2003).

1.7 Phasins

Phasins represent a class of most probably noncatalytic proteins consisting of a hydrophobic domain, which is associated with the surface of the PHB granules, and of

a predominantly hydrophilic/amphiphilic domain exposed to the cytoplasm of the cell (Pötter and Steinbüchel 2005). These proteins are synthesized in very large quantities under the storage conditions, representing as much as 5% of the total protein (Weiczorek *et al.* 1995). The layer of phasins stabilizes PHA granules and prevents coalescence of separated granules. PhaP adheres very tightly to native as well as to artificial PHB granules. In the PHA_{SCL} accumulating bacteria *Rhodococcus ruber*, the anchoring region was located at the carboxy terminus, and it was demonstrated that phasin molecules truncated at the carboxy-terminal region lost their capability to bind to PHA granules (Pieper-Fürst *et al.* 1995). The size of the PHA inclusions and the PHA synthesis rate were found to be regulated by the addition of phasins in *in vitro* synthesis of PHA using purified PHA synthase and a suitable substrate (Jossek and Steinbüchel 1998). Addition of PhaP from *W. eutropha* increased the activity of class II PHA synthase from *Pseudomonas aeruginosa* by approximately 50% (Jurasek and Marchessault 2002). Several genetic and mutational studies with phasins of *W. eutropha* revealed the existence of four genes of phasin homologues-*pha*P1, *pha*P2, *pha*P3 and *pha*P4 (Pötter *et al.* 2004). These new unexpected findings will affect the current models of the structures of PHA granules and may also have severe impacts on the establishment of heterologous production systems for PHAs (Pötter and Steinbüchel 2005).

1.8 Transcriptional repressor (PhaR)

Further insights into the role of *pha*P1 were provided by the detection of the transcriptional repressor *pha*R in *W. eutropha* that regulates the expression of *pha*P1 (Pötter *et al.* 2002, York *et al.* 2002). *Pha*R interacts in this bacterium with upstream regulatory sequences of both *pha*P1 and *pha*R. Homologues to *pha*R were identified in various bacteria, for example, *S. melioti, Pa. denitrificans, A. vinosum*, and several other PHA_{SCL}-producing bacteria. A number of *W. eutropha* deletion strains were constructed to examine the role of *pha*R in PHB accumulation. *Pha*R is proposed to be a repressor protein of transcription that binds to the *pha*P1 upstream region in *W. eutropha*, thereby repressing expression of *pha*P1. Derepression of *pha*R requires biosynthesis and accumulation of PHB (Pötter *et al.* 2002).

1.9 PHA depolymerases

Since PHA is a storage compound for excess carbon, it is natural that microorganisms are equipped with a depolymerising system to recover the stored carbon. Several bacteria produce extracellular depolymerases, which are secreted into the environment to degrade PHA. Though the extracellular PHA depolymerases are well studied, intracellular depolymerases ($PhaZ_i$) have been far less investigated although they play an important role in PHA metabolism (Pötter and Steinbüchel 2005). Early studies on enzymatic depolymerization of PHA inclusions involved the utilization of soluble enzymes from *R. rubrum* to degrade native P(3HB) inclusions isolated from *B. megaterium* (Merrick and Doudoroff 1964). In *Z. ramigera* also the PHB depolymerase activity was found in the soluble enzyme fraction when assayed with protease treated P(3HB) inclusions. This led to the suggestion that PHA inclusions may be protected from attack by the depolymerase by proteins on the surface (Saito *et al.* 1992). The first nucleotide sequence of an intracellular depolymerase was published in 2001 and the enzyme was designated as PhaZ1 (Saegusa *et al.* 2002).

1.10 Structure and Dimensions of PHA granules

Several studies were conducted on the structure of PHA granules and granule-associated proteins in *Pseudomonas oleovorans* (De Smet *et al.* 1983; Foster *et al.* 1996; Stuart *et al.* 1996; Valentin *et al.* 1998), *Paracoccus denitrificans* (Maehara *et al.* 1999; Maehara *et al.* 2002) and with *W. eutropha* (Weiczorek *et al.* 1995; Pötter *et al.* 2002; Pötter *et al.* 2004; York *et al.* 2001; York *et al.* 2002; Dennis *et al.* 2003). Four types of granule-associated proteins are found within bacterial genera producing PHAs (i) PHA synthase, (ii) PHA depolymerases and 3HB-oligomer hydroxylase, (iii) phasins (PhaPs), which are thought to be the major structural proteins of the membrane surrounding the inclusion, and (iv) the regulator of phasin expresssion PhaR (Table 1.5).

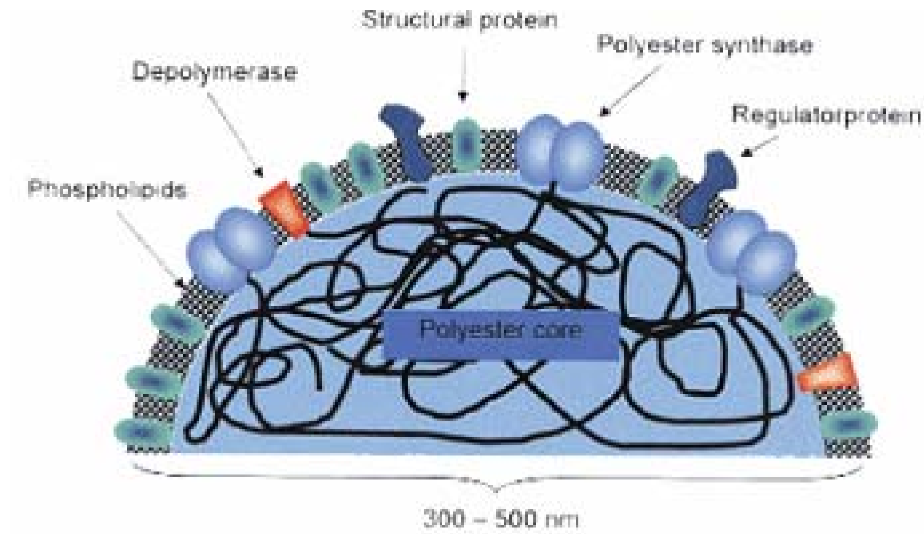
Early investigations revealed that the purified PHA granules contained proteins and lipids besides the PHB (Williamson and Wilkinson 1958; Griebel *et al.* 1968) Bacterial PHA granules are mostly between 200 and 500 nm in diameter (Anderson and Dawes 1990). Electron microscopic studies with *Bacillus cereus* and *B.*

megaterium revealed a dense membrane of a thickness of 15-20 nm at the surface of PHB granules (Lundgren *et al*.1964). It was also assumed that the PHA granules were covered with a layer of the PHB synthase protein (Ellar *et al*. 1968; Masamune *et al*. 1989). Chemical analyses have shown that inclusion bodies contain approximately 97.5% PHA, 2% protein, and 0.5% lipid (Griebel *et al.* 1968). The granule-associated proteins exhibited polymerase activity and also depolymerase activity (Griebel and Merrick 1971). De Koning and Maxwell (1993) proposed a model with a phospholipid monolayer at the surface of the PHA granules that contain proteins at their surface that are responsible for structure and function. AFM investigations at higher resolution clearly demonstrated that the rough inclusions show globular structures with 35 nm in diameter, and a central pore (Dennis *et al.* 2003). Mayer and Hoppert (1997) proposed a model of PHA granules that are formed with an amorphous polyester core by a lipid monolayer and PHA synthase covalently attached to the surface. Model of a PHA granule is presented in Fig. 1.5. An overview of various compounds associated to PHA granules are summarized in Table1.6.

Table 1.5 Granule-Associated Compounds of PHA Granules (Pötter and Steinbüchel 2005)

Representative species	Classes of PHA synthase	PHA synthase	PHA depolymerase or 3HB-oligomer hydroxylase	Regulator	Phasin
W. eutropha	I	PhaC	PhaZ1, PhaZ2, PhaZ3, PhaZ4, PhaZ5, PhaY	PhaR	PhaP1, PhaP2, PhaP3, PhaP4
P. oleovorans	II	PhaC1/PhaC2	PhaZ	PhaF/PhaI	unknown
A. vinosum	III	PhaE/PhaC	unknown	ORF4	unknown
B. megaterium	IV	PhaC/PhaR	unknown	unknown	PhaP

Fig. 1.5 Model of PHA granule (Rehm 2003)



1.11 Terminology of the genes and gene products

Genes encoding proteins involved in PHA biosynthesis are referred in alphabetical order as *pha*A (β-ketothiolase), *pha*B (acetoacetyl-CoA reductase), *pha*C (PHA synthase), *pha*G (3-hydroxy-acylcarrier protein Co-enzyme A transeferase), *pha*J (enoyl CoA hydratase) etc. The genes required for degradation of PHA are referred to in the reverse alphabetical order as *pha*Z (PHA depolymerase), *pha*Y*,* *pha*X*,* *pha*W etc. The genes for phasins whose expression is dependent upon PHA biosynthesis are termed as *pha*P. Other proteins that are granule-associated or aid in regulation or are of unknown function are referred to as *pha*F*,* *pha*I*,* *pha*Q*,* *pha*R etc. The origin of a gene or a protein is indicated by the first letter of the genus and the species and added as subscript *e.g*. PHA synthase of *W. eutropha* is written as *pha*C_{Re} (in case of gene) and PhaC_{Re} (in case of the gene product) (Rehm and Steinbüchel 1999).

Table 1.6 Overview of the Various Granule-Associated Proteins in Representative Species

Year	Strain	Granule-associated compounds	Reference
1964	*B. cereus* and *B. megaterium*	unknown membrane of 15-20 nm	Lundgren *et al.* (1964)
1968	*B. megaterium*	exclusive PHA synthase	Ellar *et al.* (1968)
1989	in general prokaryotes	exclusive PHA synthase	Masamune *et al.*(1989)
1991	*P. putida*	two PHA synthases and phospholipids	Huismann *et al.*(1991)
1993	*W. eutropha*	exclusive PHA synthase	Gerngross *et al.* (1993)
	in general prokaryotes	PHA synthase and phospholipids	De Koning and Maxwell (1993)
1994	*R. ruber*	PHA synthase, GA14 protein, phospholipid	Pieper-Fürst *et al.* (1994)
	in general prokaryotes	PHA synthase, phospholipids, and other proteins	Hocking and Marchessault (1994)
	P. oleovorans	two PHA synthases, PHA depolymerase, 18- and 43-kDa proteins and phospholipids	Foster *et al.* (1994)
1995	*W. eutropha*	PHA synthase, PhaP, unknown proteins	Wieczorek *et al.* (1995)
	P. oleovorans	43-, 55-, and 59-kDa proteins	Stuart *et al.* (1995)
1999	*P. oleovorans*	PhaF and PhaI	Prieto *et al.*(1999)
	B. megaterium	PhaC and PhaP	McCool and Cannon (1999)
	Pa. denitrificans	GA16	Maehara *et al.* (1999)
2001	*B. megaterium*	PhaR	McCool and Cannon (2001)
	W. eutropha	PhaZ1	Saegusa *et al*. (2001)
2002	*Pa. denitrificans*	PhaR	Maehara *et al.* (2002)
	W. eutropha	*D*-(-)-3-hydroxybutyrate-oligomer hydrolase	Saegusa *et al.* (2002)
	W. eutropha	PhaR	Pötter *et al.* (2002)
2003	*W. eutropha*	globular structures with central pore and phospholipids	Dennis et al. (2003)
2004	*B. megaterium*	PhaQ	Lee et al. (2004)
	W. eutropha	PhaP2, PhaP3, PhaP4	Pötter et al. (2004)

1.12 Molecular organization of PHA-biosynthetic genes

The first gene to be isolated was from *Zooglea ramigera* (Peoples *et al.* 1987), an interesting bacterium for biopolymer engineering since it produces both P(3HB) and extracellular polysaccharide (Easson *et al.* 1987). Since the original discovery of *phb* genes, many genes encoding enzymes from the PHA pathway have been cloned from different organisms that show different organizations of the genes. Based on the molecular data available till-date, seven types of arrangements have been observed for PHA synthesizing genes (Fig. 1.6) (Madison and Huisman 1999; McCool and Cannon 2001).

In *Ralstonia eutropha*, *Burkholderia cepasia*, *Acinetobacter* sp., *Alcaligenes latus* and *Pseudomonas acidophila* the three genes involved in PHA biosynthesis are arranged in tandem although not necessarily in same order (Peoples and Sinskey 1989a, b; Schembri *et al*. 1994; Umeda *et al*. 1998). As already reported, *W. eutropha* genes are arranged as *pha*CAB while in *Acinetobacter* sp. they are arranged as *pha*BCA. In *Z. ramigera***,** *Rhizobium melioti* and *Paracoccus denitrificans* the *pha*AB and *pha*C loci are separated (Peoples and Sinskey 1989c; Tombolini *et al*. 1995; Yabutani *et al*. 1995; Lee 1996; Ueda *et al*. 1996).

PHA polymerase in *Allochromatium vinosum*, *Thoicystis violacea* and *Synechocystis* is a two-subunit enzyme encoded by *phb*C and *phb*E genes. In these organisms *phb*AB *and phb*EC are in one locus but divergently oriented (Liebergesell and Steinbüchel 1992, 1993; Park *et al*. 1997; Oldenburg *et al*. 2000). So far in *Methylobacterium extorquens*, *Nocardia corallina*, *Rhizobium etli*, *Rhodococcus ruber* and *Rhodobacter sphaeroides* only PHB polymerase encoding gene has been identified (Pieper and Steinbüchel 1992; Hustede and Steinbüchel 1993; Cevallos *et al*. 1996; Hall *et al*. 1998). The PHA polymerase in *Aeromonas caviae* is flanked by a unique PHA biosynthesis enzyme encoded by *pha*J gene where in (R)-3-hydroxyacyl-CoA monomer units are supplied via the (R)-specific hydration of enoyl-CoA (Fukui and Doi 1997). In MCL-PHA producing *P. oleovorans and P. aeruginosa*, the *pha* loci contain two *pha*C genes (Huisman *et al*. 1991, Timm and Steinbüchel 1992) separated by the *pha*Z gene encoding for an intracellular depolymerase. Recently, Hang *et al*. (2002) have shown *Burkholderia caryophylli* to possess two synthases

genes interspersed with *pha*Z gene as in *Pseudomonas*. In *Streptomyces aureofaciens* NRRL 2209 PHA biosynthetic operon *pha*A_{Sa} was found to be absent, and the interesting feature of this operon is the presence of putative *pha*B_{Sa} gene within putative *pha*C_{Sa} gene (Ramachander2003).

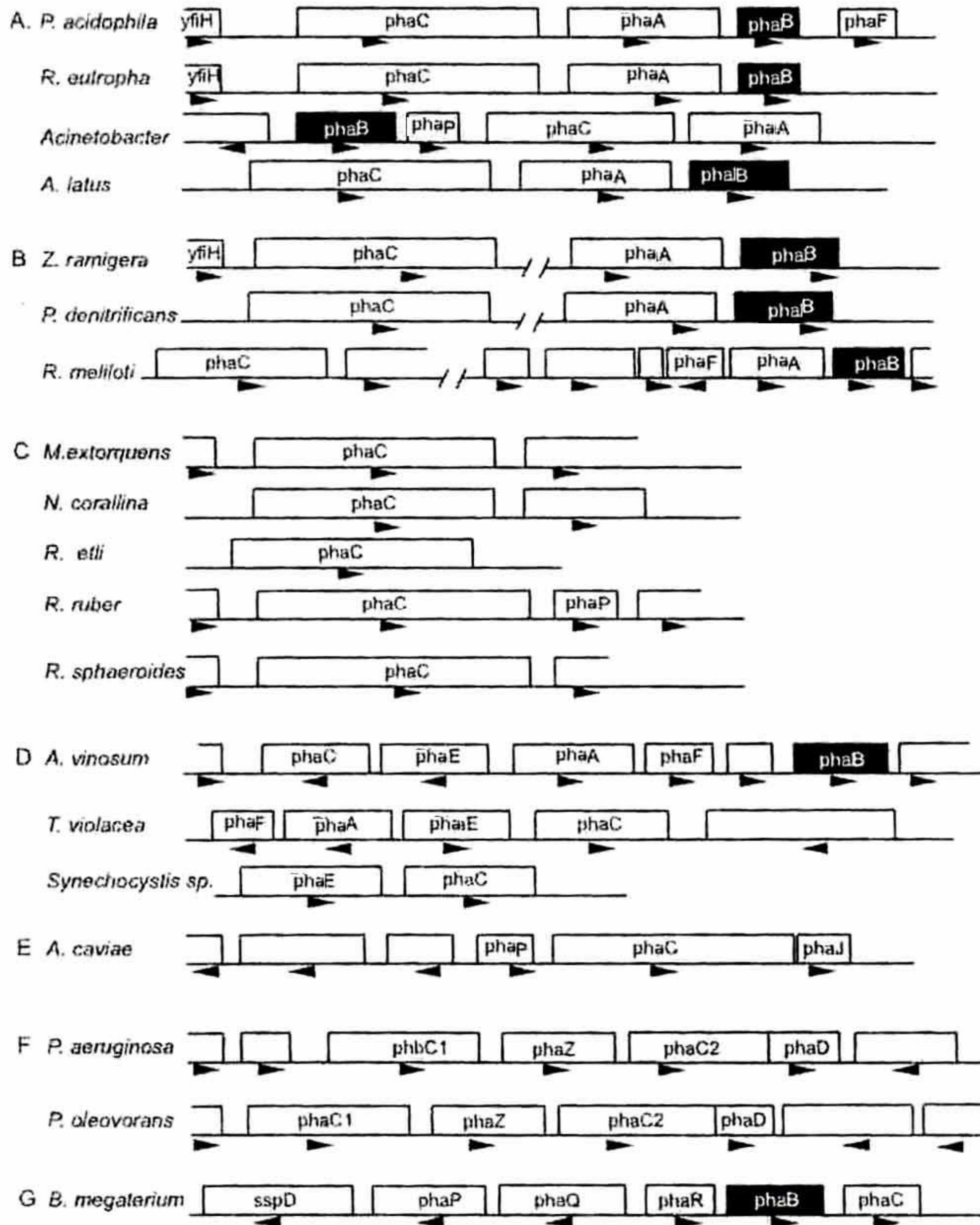


Figure 1.6: Organization of PHA biosynthesis and related genes

(modified from Madison and Huisman 1999)

In *Bacillus megaterium*, the *pha*C gene is similar to that of *Allochromatium vinosum* in size and sequence. However, unlike *pha*C_{Av}, the *pha*C_{Bm} requires *pha*R_{Bm} for PHA accumulation but not *pha*E (McCool and Cannon 1999, 2001). *pha*R_{Bm} is probably an orthologous replacement for *pha*E_{Bm}.

1.13 Metabolic pathways involved in the biosynthesis of PHA

Several studies have shown that different microorganisms are capable of producing PHA from various carbon sources ranging from inexpensive complex waste effluents like beet or cane molasses (Hangii 1990; Page 1992) to plant oils (Fukui and Doi 1998) and its fatty acids (Eggink *et al*. 1995; Tan *et al*. 1997), alkanes (Lageveen *et al*. 1988) as well as simple carbohydrates. Based on the monomer incorporated, various metabolic pathways have been shown to be involved (Steinbüchel 1991; Madison and Huisman 1999). Three major biosynthesis pathways have been elucidated as under which are illustrated in Fig. 1.8.

(i) Biosynthesis pathway (Pathway I) represented by *W. eutropha*

(ii) Biosynthesis (Pathways II and III) represented by Pseudomonads.

1.13.1 PHA biosynthesis pathway represented by *W. eutropha*

The enzymes involved in this biosynthesis pathway have been extensively studied (Anderson and Dawes 1990). In this bacterium, PHB is synthesized from acetyl-CoA by sequential action of three enzymes (Fig. 1.8 pathway I). Biosynthetic ketothiolase catalyses the formation of a carbon-carbon bond by a biological Claisen condensation of two acetyl-CoA moieties (Masamune *et al.* 1989). NADPH dependent acetoacetyl-CoA reductase catalyses the stereoselective reduction of acetoacetyl-CoA formed in the first reaction to R-(-)-3-hydroxybutyryl CoA. The third reaction of this pathway is catalyzed by the enzyme PHA synthase that catalyzes the polymerization of R-(-)-3-hydroxybutyryl-CoA to form PHB. The monomer for PhaC is provided by the action of β-ketothiolase and acetoacetyl-CoA-reductase. On the other hand, *R. rubrum* which shows similar PHA biosynthesis pathway as *W. eutropha*, the reductase gives rise to (S)-isomer of 3-HB-CoA. This is converted to (R)-type by two enoyl-CoA hydratases (Moskowitz and Merrick 1969). The (R)-3HB-CoA is then converted

to (R)-P(3HB) by PHA synthase. In *Aeromonas caviae* the (R)-specific enoyl-CoA hydratase (PhaJ) is involved in the production of P(3HB-co-3HHx) (Fukui and Doi 1997; Fukui *et al*. 1998).

Various carbon sources ranging from simple carbohydrates to plant oils act as substrates for the synthesis of PHA in these bacteria. However, it is observed that irrespective of the substrate provided the PHA monomer incorporated was always between C_3-C_5. *W. eutropha* accumulates copolymer of 3HB and 3HH when grown on substrates with odd number of carbon atoms (Akiyama *et al*. 1992). It is also capable of accumulating PHA from 4-HB, γ-butyrolactone and 1,4-butanediol which gives rise to P(3HB-co-4HB) copolymer (Doi *et al*. 1989).

1.13.2 PHA biosynthesis represented by Pseudomonads

This pathway is exhibited in Pseudomonads (*P. oleovorans)* belonging to rRNA homology group I. These synthesize PHA_{MCL} from various alkanes, alkanols or alkanoates (Lageveen *et al.* 1988), and generally do not synthesize PHA_{SCL}. In this group 3-hydroxyacyl CoA substrates of C_6-C_{14} are obtained from intermediates of fatty acid β-oxidation pathway (Fig 1.8 pathway II). Interestingly the monomer composition of the PHA is a reflection of the substrates provided (Lageveen *et al*. 1988). Even unsaturated monomers are incorporated when carbon sources are 1-alkenes (Lageveen *et al*. 1988). Specific enzymes like enoyl-CoA hydartases (PhaJ) (Fukui *et al*. 1999) and 3-ketoacyl-CoA-reductase (FabG) (Taguchi *et al*. 1999) are presumably involved in the conversion of fatty acid β-oxidation intermediates to suitable monomers to be polymerized by PHA synthase.

In contrast all other Pseudomonads belonging to rRNA homology group I except *P. oleovorans*, synthesize PHA containing MCL monomers from unrelated carbon sources, such as gluconate, glycerol, fructose, acetate or lactate (Anderson and Dawes 1990). Mainly PHA containing 3-hydroxydecanoate (3HD) as the predominant monomer is accumulated. The 3-hydroxyacyl monomers are derived from the *de novo* fatty acid biosynthesis pathway (Huijberts *et al*. 1994) (Fig. 1.8 pathway III). Usually (R)-3- hydroxyacyl-ACP is formed as a fatty acid biosynthesis intermediate. PhaG (3-hydroxyacyl-CoA-ACP transferase) converts it into (R)-3-hydroxyacyl-CoA. *P.*

aeruginosa, *P. aureofaciens*, *P. citronellis*, *P. mendocina* and *P. putida* are shown to possess this pathway. Even over expression of transacylating enzyme such as malonyl-CoA-ACP transacylase (FabD) also seems to generate monomers for PHA biosynthesis (Taguchi *et al*. 1999).

1.14 Regulation of PHA metabolism

The regulation of PHA synthesis is quite complex. It is exerted at different physiological and genetic levels (Kessler and Witholt 2001) such as:

(i) activation of *pha* gene expression due to specific environmental signals e.g. nutrient starvation

(ii) activation of the PHA biosynthetic enzymes by specific cell components or metabolic intermediates for PHA synthesis

(iii) inhibition of metabolic enzymes of competing pathways and therefore enrichment of required intermediates for PHA synthesis

(iv) a combination of all these

At the physiological level the PHA production is regulated by cofactor inhibition and availability of metabolites. At the genetic level, it is regulated through alternative sigma cofactors, two component regulatory systems, and autoinducing molecules. Further, the levels of PHA polymerase and phasins control the granule size and molecular weight of the PHA produced (Madison and Huisman 1999).

1.15 Potential PHA production systems

Potential PHA production systems include:

a. Production of PHAs by natural organisms,

b. Genetic engineering of PHA biosynthesis genes (in bacteria and higher organisms)

c. *In vitro* biosynthesis of PHAs.

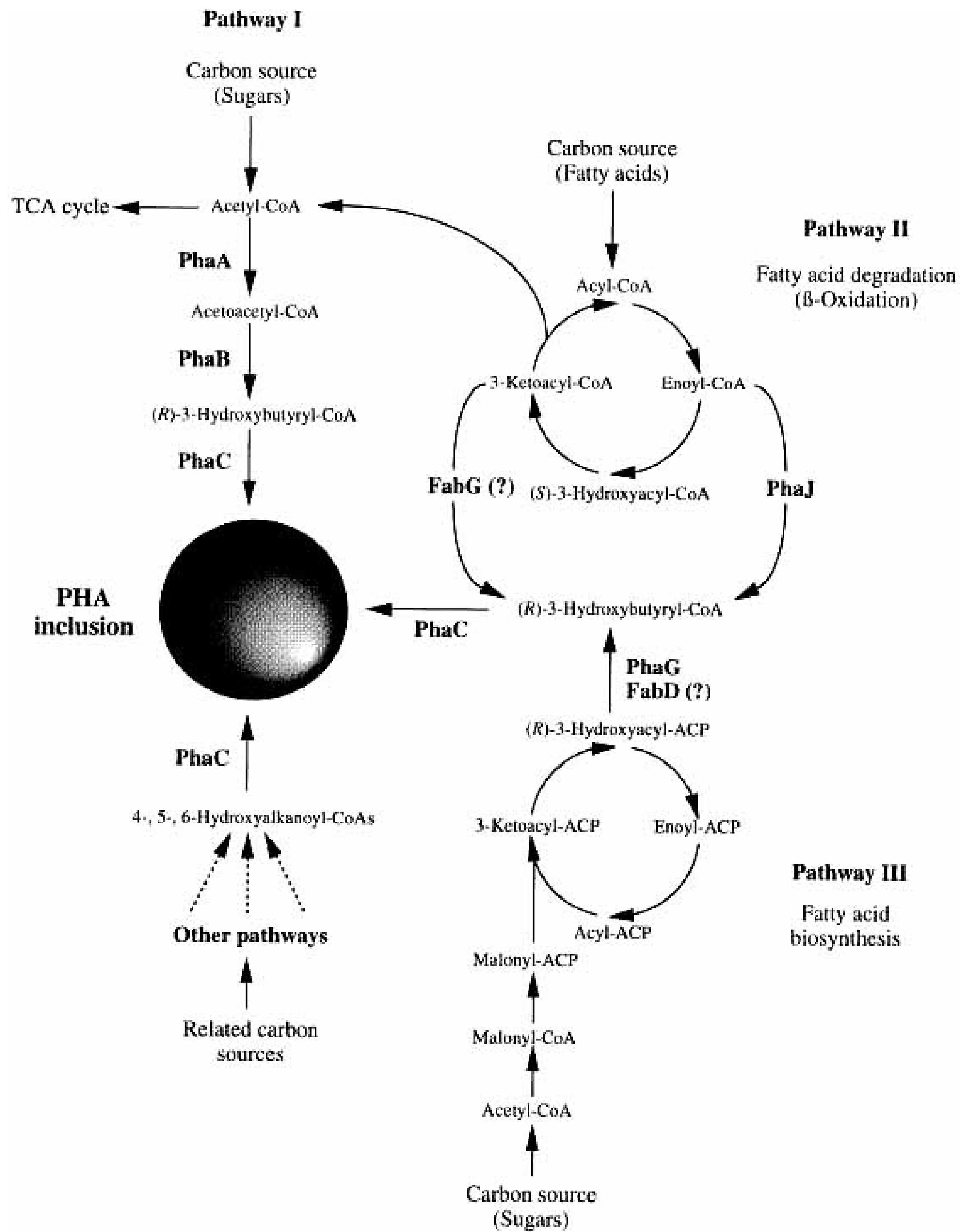


Figure 1.7 Biosynthesis pathways for PHA production
(Sudesh *et al*. 2000)

1.15.1 Production of PHAs by natural organisms

Wausteria eutropha has been most extensively used for PHB and PHBV production. The strains used extensively are the original PHB producer H16 (ATCC 17699) and its glucose utilizing mutant known as 11599 in the NCIMB collection. Other strains are ATCC 17697^{T}, *W. eutropha* SH-69, and a natural isolate, *Alcaligenes* sp. strain AK201. To achieve high productivity of a desired bioproduct, fed-batch cultures are usually grown with the controlled nutrient feeding, monitored dissolved oxygen (DO), pH, or carbon source as a feedback parameter (Kim *et al.* 1999, Ryu *et al.* 1997). Several strategies have been developed in academia for the efficient production of PHA using *Wausteria eutropha.* By controlling glucose concentration at 10 to 20 g/L during the fed-batch culture, the final cell mass, P(3HB) concentration, and P(3HB) content could be obtained in 50 hrs. resulting in the highest productivity of 2.42 g P(3HB)/L/h (Kim *et al.* 1994a). Using the same cultivation strategy, more than 110g/L of P(3HB-co-3HV) could be produced by feeding glucose and propionic acid (Kim *et al.* 1994b). Ethanol was used for production of P(3HB) by a mutant strain of *Wausteria eutropha,* but the highest concentration of P(3HB) obtained was 47 g/L (Alderete *et al.* 1993). P(3HB-co-3HV) could be produced by feeding ethanol with propanol or pentanol (Alderete *et al.* 1993; Park and Damodaran 1994). *Alcaligenes latus* is also being considered as a good candidate for the production of PHA owing to its fast growth and accumulation of PHA during its growth phase. Further it can utilize sucrose as a carbon source, which suggests that cheap beet or molasses can be used for the production of PHA. The use of methylotrophs was considered by ICI for the production of PHAs from methanol, which is one of the cheaper carbon sources. However, accumulation of less than satisfactory amounts made the recovery process difficult (Byrom 1987). Pseudomonads were also being considered for production of medium-chain-length (MCL) PHAs from MCL-alkanes, alkanols or alkanoates. In addition to these bacteria several bacteria including *Rhodospirilum rubrum, Halobacterium mediterranei, Rhizobium meliloti, Rhodococcus, Cornybacterium, Nocardia* strains are found to accumulate different types of PHAs utilizing a wide variety of carbon sources.

1.15.2 Genetic engineering of PHA biosynthesis genes

PHA production in natural producers is affected mainly due to the mobilizing enzymes in the form of depolymerases and esterases present *in vivo*. Moreover, many native organisms grow slowly. An alternative to increase the PHB content in the native organisms is to use a mutant devoid of PHA degradation pathways or heterologously express the genes in fast growing organisms lacking PHB biosynthesis and degrading genes.

For many purposes, ranging from academic importance to industrial production, PHA biosynthesis genes have been expressed in various heterologous systems. They have been expressed heterologously and the following facts have been unearthed:

1. Genes for PHA synthases or entire PHA biosynthesis pathways were identified during cloning by phenotypic complementation.
2. Expression in *E. coli* will make PHA synthase proteins more readily accessible for purification and therefore available in large quantities for further studies such as detailed biochemical analysis, generation of antibodies and *in vitro* PHA biosynthesis.
3. Heterologous expression allows one to provide the functionality of single enzymes or entire pathways.
4. Metabolic engineering may reveal novel pathways for synthesis and production of PHAs.
5. Other organisms may, for several reasons be much more suitable for the commercial production of PHA than the original host possessing the required gene(s). Therefore, organisms growing at faster rate grow to high cell densities and accumulate high amounts of PHB using cheaper carbon sources and are easy for downstream processing thus making them commercially important.

1.15.2.1 Expression in bacteria

A number of studies have successfully demonstrated heterologous expression of PHA synthases. Establishment of functionally active PHA biosynthesis pathways in *E. coli* requires not only a PHA synthase but also enzymes for conversion of

metabolites to (R)-isomers of hydroxyacyl-CoA thioesters that are used as substrates by respective PHA synthase. Native organisms defective in *pha*C gene such as *W. eutropha* $PHB^{-}4$ (Schlegel *et al*. 1970) and *P. putida* GPp104 (Huisman *et al*. 1991) were used for physiological studies and cloning experiments. These require only PHA synthase gene for phenotypic complementation so as to accumulate PHA and such strains are easily identified on agar plates by the enhanced opacity of the colonies (Schlegel *et al*. 1970), by staining the colonies with lipophilic dye Sudan black (Schlegel *et al*. 1970) or Nile blue A (Ostle and Holt 1982) or by enhanced fluorescent of colonies in the presence of Nile red (Spiekermann *et al*. 1999). A variety of heterologous systems have been used for high cell density cultivation and production of bulk amounts of PHAs, but recombinant *E. coli* and *W. eutropha* have been found to be of commercial importance (Lee and Choi 2000).

1.15.2.2 Expression in Yeast

In contrast to *E. coli* where the complete pathway had to be introduced for PHA formation to occur, P(3HB) was produced in yeast by expressing only part of the biosynthetic pathway. *Saccharomyces cerevisaie* does not require the expression of β-ketothiolase and acetoacetyl-CoA reductase genes for the PHA accumulation. These steps are catalyzed by the native Erg10 and Fox2 proteins, respectively which are involved in β-oxidation but functioning as β-ketothiolase and acetoacetyl-Co A reductase. However, P(3HB)was accumulated to only 0.5% of the cell dry weight (Leaf *et al*. 1996).

1.15.2.3 Expression in animal cells

Successful expression of PHA biosynthesis genes in animal cells has been achieved in the cells of insect *Spodoptera frugiperda* and *Trichopulsiani*. A baculovirus, *Autographa californica* nuclear polyhedrosis virus system was used to express $PhaC_{Re}$ in *Trichopulsiani* cells which accumulated PHA synthase in large amounts ranging to 50% of the total cell protein. This was used to purify large amounts of highly pure PHA synthase protein in one single liquid chromatography step for further biochemical characterization (Willams *et al*. 1996). In *Spodoptera*,

P(3HB) was synthesized when a dehydratase domain mutant rat fatty acid synthase cDNA and *pha*C_{Re} were expressed simultaneously thus, creating an alternative pathway for the biosynthesis of PHAs (Williams *et al*. 1996).

1.15.2.4 Expression in plants

In view of the flexibility of plants in expressing foreign proteins, it was of interest to explore the possibility of synthesizing PHAs in plants. The PHA biosynthesis genes from microorganisms have been successfully expressed in *Arabidopsis thaliana* (Poirier *et al*. 1992; Nawrath *et al*. 1994; Poirier *et al*. 1995), *Brassica napus* (Valentin *et al*. 1999), *Gossypium hirsutum* (John and Keller 1996), *Nicotiana tabacum* (Nakashita *et al*. 1999), *Solanum tuberosum* (Mittendorf *et al*. 1998) and *Zea mays* (Hahn *et al*. 1999). However, the PHAs accumulated in these transgenic plants contributed to only a small percent of the total cellular matter. In many cases stunted growth or impaired functions were observed (Steinbüchel and Hein 2001). To establish PHA biosynthesis in plants, *pha*C_{Re} and *pha*B_{Re} alone or together with *pha*A_{Re} have been used. In many cases *pha*A expression is not required as it is inherently present in plants and is available for PHB synthesis. Replacement of *pha*A_{Re} by *bkt*B_{Re} in plants enabled them to accumulate co-polymers (Slater *et al*. 1998; Valentin *et al*. 1999). PHB biosynthesis in transgenic plants has been established in various compartments of plant cells and tissues. Of all the transgenic plants *B. napus* stands out as a classic example wherein the PHA accumulation occurred in leucoplasts without affecting the overall growth of the plant (Valentin *et al*. 1999).

1.16 *In vitro* synthesis of PHB

An alternative approach to PHA synthesis is *in vitro* synthesis using isolated enzymes. This is of interest since specific functional groups could be incorporated into PHA. Though the studies in this direction were started by Gerngross and Martin in 1995, not much progress has been made. The main problems encountered are the stabilization of the polymerase enzyme, elimination of the lag phase and the high cost of the precursor, CoA thioester. Lenz *et al*. (1999) were successful in stabilizing the polymerase with the help of glycerol and eliminating the lag phase *in vitro*. The list of

the organisms where from PHA polymerases were purified and used for *in vitro* synthesis of PHA is listed in Table 1.7.

A system currently under development is the use of nano-technology to immobilize the PHA synthase on various supports including gold, glass and anodic alumina etc. such that *in vitro* synthesis of crystalline PHA is facilitated along with the reuse of the synthase (Paik *et al*. 2001). Once this system is developed the production cost of PHB may be reduced considerably.

1.17 Commercial status

Momentum is building up for biopolymers because of their complete biodegradability. Since 1960s when W. R Grace, an American company for the first time commercialized the bacterial PHA, many companies have come up with different forms of biopolymers (Table 1.8). In 1970s ICI Bioproducts, an UK based company started producing PHBV copolymer on a commercial scale from glucose/propionic acid utilizing *W. eutropha* (Steinbüchel 1991). This was marketed under the trade name BIOPOL and used for the manufacturing of biodegradable films and bottles. With the potential applications of these polymers being extended to various fields, the demand for the biopolymer is growing despite its high cost. This firm was taken over by Monsanto, USA and now the rights have been transferred to Metabolix, USA.

The potential for biomedical applications is very promising, since the added value to these special products is remarkably high (Lafferty *et al*. 1988; Hocking and Marchessault 1994; Duvernoy *et al*. 1995; Willams *et al*. 1999). Although research in this field is of unique complexity, it is both technologically and economically very compelling to succeed.

After extensive research in the fermentation and down stream processing of PHAs, the production costs have come down. Use of sucrose and other cheap carbon sources have further brought down the costs. In the present day market the cost per kg of PHBV has come down to $3.95 / kg at a production scale of 100,000 tons per year. This cost is supposed to come down eventually with the increase in the demand.

Table 1.7: ***In vitro* synthesis of PHA by various purified PHAs**

PHA synthase of *W. eutropha*	Gerngross and Martin (1995)
PHA synthase of *A. vinosum* with Propionyl CoA transferase of *Clostridium propionicum*	Jossek and Steinbüchel (1998)
PHA synthase of *A. vinosum* with Acetoacetyl-CoA reductase of *S. cerevisiae,* Propionyl-CoA transferase of *Clostridium propionicum*	Jossek and Steinbüchel (1998)
PHA synthase of *T. pfennigii*, butyrate kinase and Phosphotransbutyrylase of *C. acetobutylicum*	Liu and Steinbüchel (2000)
PHA synthase of *P. aeruginosa* and acyl-CoA synthetase of *Pseudomonas* sp.	Qi *et al.* (2000)

Table 1.8: List of the companies producing biopolymers from various sources.

Company	Commercial Name	Source
Metabolix, USA	BIOPOL (PHBV)	**Glucose and Propionic acid**
Novamont, Italy	Mater-Bi	Starch
Cargill Dow, USA	Natureworks (PLA)	Corn
Rhodenburg Biopolymers, Holland	Solanyl	Starch (Potato peels)
Procter and Gamble, USA	Nodax (PHBHx)	Low cost feeds
DuPont, USA	Sorona 3GT polyester	Corn Sugar

Despite the 75 years, on and off, of research of PHAs and 20 years of intense industrial interest, PHAs still appear to be far removed from large scale production. At present, two development programs on these biopolymers are receiving attention, namely (1) a joint program by the Proctor and Gamble Co. and Kaneka Corp. on a family of short and medium chain copolymers, especially on poly(3-hydroxybutyrate-co-3-hydrohexanoate), and (2) a program at Metabolix Inc. on PHAs for medical applications. The lack of commercialization of the initially promising bacterial poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymers has been generally attributed to the high investment for the fermentation and product recovery processes on a large scale and to the cost of the substrates. To reduce the latter limitation, alternative substrates are receiving attention, including starch and vegetable oils, but no breakthroughs in this area have been announced. Nevertheless, in the long run, it is possible that advances in our understanding and control of the genetic pathways involved in the biosynthesis of PHAs in microorganisms and plants could make the industrial scale production of these biopolymers competitive with oil-based synthetic polymers (Lenz and Marchessault 2005).

1.18 *Bacillus thuringiensis*

Bacillus thuringiensis is endospore forming Gram-positive soil bacterium that produces crystal proteins (*cry* proteins), which are toxic to many species of insects. *Bacillus thuringiensis* was first discovered in 1911 as a pathogen of flour moths from the province of Thuringia, Germany. *B. thuringiensis* (commonly known as 'Bt') is an insecticidal bacterium, marketed worldwide for control of many important plant pests - mainly caterpillars of the Lepidoptera (butterflies and moths) mosquito larvae and simuliid blackflies that vector river blindness in Africa. Since 1996, a wide range of crop plants have been genetically engineered to contain the delta-endotoxin gene from *Bacillus thuringiensis*. These "Bt crops" are now available commercially in USA. They include "Bt corn", "Bt potato", "Bt cotton" and "Bt soybean". Such plants have been genetically engineered to express part of the active Cry toxin in their tissues, so they kill insects that feed on the crops.

Bacillus species continue to be dominant bacterial workhorses in microbial fermentations owing to their faster growth rates. Some *Bacillus* strains have also been

developed and engineered as industrial producers of nucleotides, the vitamin riboflavin, the flavor agent ribose, and the supplement poly-γ-glutamic acid. Some strains of Bacillus are known to accumulate PHAs, however, they were not exploited for PHA production on commercial scale. Belma *et al.* (2000) identified 27 strains of *Bacillus* from soil that could accumulate PHB. They reported different quantities of PHB accumulation in *B. subtilis*, *B. firmus*, *B. sphaericus* and *B. pumilus* and *Bacillus thuringiensis*. Though the accumulation of PHAs was reported in *Bacillus thuringiensis* very little is known about the biosynthesis of the polymer in this bacteria.

Scope of the Present Thesis

Though the accumulation of PHAs has been first identified in *Bacillus megaterium* in 1926 by Lemoigne, the molecular characterization of the genes encoding for PHB synthesis have been studied very recently (McCool and Cannon 1999). Also the studies in this species have resulted in a new class of PHA synthases (Class IV) and recognition of new genes *pha*P, *pha*R, *pha*Q etc. There is not much study done on the microbes falling into this new class. The present study reports the isolation of *Bacillus thuringiensis* R1 that accumulates PHB utilizing a variety of carbon sources and isolation and characterization of an ~5 Kb gDNA fragment that is responsible for the accumulation of PHA.

The objectives of the present thesis are:

- Isolation, identification and characterization of PHA producing *Bacillus thuringiensis* R1
- Physical characterization of PHA from *Bacillus thuringiensis* R1
- PHA gene locus from *Bacillus thuringiensis* R1: Isolation, cloning, sequencing and expression in *E. coli*
- Sequence analysis of the ~5.0 kb genomic DNA fragment of *Bacillus thuringiensis* R1 harboring the PHB biosynthetic gene locus.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Ampicillin, Tris, IPTG, X-gal, SDS, bovine serum albumin, Nile blue-A, benzoic acid, EDTA, Ethidium bromide and poly-β-hydroxybutyrate were purchased from Sigma-Aldrich, USA. Agarose, restriction enzymes, T4 DNA ligase, RNase A, lysozyme, pronase, proteinase K were obtained from GIBCO-BRL (USA), Promega (USA) and Amersham (UK). pBKS+ vector was purchased from Stratagene (USA). Megaprime labelling kit and Hybond-N^+ membrane were obtained from Amersham (UK). Radiolabelled [α-^{32}P]-dATP was obtained from Bhabha Atomic Research Centre (BARC), India. X-ray films were obtained from Konica (Japan) or Kodak (USA). All other chemicals and solvents were of analytical grade and purchased from HIMEDIA, Qualigens Fine Chemicals and E-Merck Laboratories, India. Pipette tips and microcentrifuge tubes were purchased from Axygen (USA).

Table 2.1 Bacterial strains and plasmids used in the study

Strain or Plasmid	**Important features (reference or source)**
Bacillus thuringiensis R1	Organism isolated from soil from which PHA synthesizing genes were isolated
***E. coli* DH5α**	F^-; lac for blue/white screening (Gibco-BRL)
pBKS+	Cloning vector (Stratagene)
pGEMT	Cloning vector (Promega)
pSRAB	pSTV28 derivative that encodes *pha*A and *pha*B from *W. eutropha*
pSVA10	Plasmid harboring PHA biosynthetic genes from *Bacillus thuringiensis* R1

2.2 Bacterial culture conditions

E. coli and *Bacillus thuringiensis* R1 were grown at 37°C with shaking at 200 rpm in Luria Bertani (LB) broth and maintained on LB plates with 1.5% agar (Sambrook *et al.* 1989). For plasmid DNA preparation recombinant *E. coli* was grown in LB media supplemented with appropriate antibiotic. When required ampicillin (100 µg/ml) was added to the culture medium. For PHA accumulation *E. coli* and *Bacillus thuringiensis* R1 were grown in basal medium.

Nutrient agar Broth	**(g L^{-1})**
Beef extract	10
NaCl	5
Peptone	10
pH adjusted to 7.2	

Luria Bertani Broth (LB)	**(g L^{-1})**
Bacto-tryptone	10
Yeast extract	5
NaCl	10
pH adjusted to 7.0	

Basal Medium	**(g L^{-1})**
Yeast extract	5
Peptone	5
Na_2HPO_4	1
$MgSO_4$	0.2
Carbon Source	1.0%
pH adjusted to 7.2	

Agar-Agar was added at 1.5% concentration wherever required.

2.3 The Nucleic Acids

2.3.1 Genomic DNA isolation

Solutions

SET buffer: 75 mM NaCl, 25 mM EDTA (pH 8.0), 20mM Tris (pH 7.5)

Lysozyme: 50 mg mL^{-1}in water

Proteinase K: 20 mg mL^{-1}in water

SDS: 10% (may be preheated to 55°C)

TE buffer: 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0)

5M *NaCl*; *Chloroform*; *Isopropanol*; *70% Ethanol*

Genomic DNA of *Bacillus thuringiensis* R1 was extracted by using salting out method (Pospiech and Neumann 1995). *Bacillus thuringiensis* R1 was grown in LB medium, pelleted and suspended in 5 mL SET buffer. To the above solution 100 μL lysozyme (final conc. 1 mg mL^{-1}) was added and incubated at 37°C for 30-60 min. Further, 140 μL of proteinase K (final conc. 0.5 mg mL^{-1}) and 600 μL of 10% SDS solutions are added, mixed by inverting the tubes and incubated for 2 h at 55°C with occasional mixing. To the lysate 2 mL of 5 M NaCl (final conc. 1.25 M) was added and the mix was cooled to 37°C. To the mix 5 mL chloroform was added and mixed. The tubes were kept at room temperature (RT) for 30 min. and centrifuged at 4,500 x g at RT for 10 min. The supernatant was transferred to a fresh tube and 0.6 vol. isopropanol added to precipitate DNA, which was spooled out. The DNA was washed with 70% ethanol, air dried and dissolved in 1-2 mL of 10 mM Tris-1mM EDTA, pH 8.0 (TE) at 55°C.

The g-DNA obtained by this method was essentially free of RNA. Alternatively, to make the samples RNA free, 10 μL of RNase A (1 mg mL^{-1}) was added to the restriction enzyme (RE) digestion reaction mix or RNase A treatment was given separately and the g-DNA was purified by extraction with chloroform when needed for Polymerase Chain Reaction (PCR).

2.3.2 Plasmid DNA Isolation

Solutions

TEG Buffer (Sol. I): 25mM Tris-HCl (pH 8.0); 10mM EDTA (pH 8.0); 50mM Glucose.

Sol. II: 0.2 N NaOH, 1% SDS (freshly prepared)

Sol. III: 3.0 M Potassium acetate (pH 4.8)

Chloroform; *Absolute Ethanol; 3.0 M Sodium acetate; 70% Ethanol*; *Deionized water*

The alkaline lysis method of Sambrook *et al*. (1989) was improvised upon so that 12-24 samples are processed conveniently for plasmid DNA extraction within 3 h, with yields of 5-30 µg per 1.5 mL culture depending on the host strain and the plasmid vector. An important feature of this protocol was the use of PEG for purification, which resulted in precipitation of high quality super-coiled plasmid DNA free of contamination (PRISM™ 1995). This method is recommended for JM109, XL1 Blue, MV 1190 and highly recommended for DH5α and HB101. The bacterial cultures were grown overnight (O/N) at 37°C in LB broth, with appropriate antibiotic. About 1.5 to 3 mL culture was pelleted for 1 min at 4,000 rpm in a micro-centrifuge. The bacterial pellet was resuspended in 100 µL of TEG buffer by pipetting up and down, 200 µL of Sol. II was added, mixed by inversion till the solution becomes clear and incubated on ice for 5 min. The above solution was neutralized by adding 150 µL of Sol. III, mixed well and incubated on ice for 5 min. The cell debris was removed by centrifuging for 5 min. at 12,000 x g at room temperature. The supernatant was transferred to a clean tube; RNase A to a final concentration of 20 µg mL^{-1} (Sambrook *et al.* 1989) was added and incubated at 37°C for 20 min. To the above solution 400 µL of chloroform was added, mixed for 30 s and centrifuged for 5 min. at 12,000 x g. The upper aqueous layer was transferred to a clean tube, 0.1 volume sodium acetate and 1 mL of absolute ethanol was added with mixing and kept at -20°C for 1-2 hrs. The sample was centrifuged for 10 min., 12,000 x g at room temperature. The pellet was washed with 70% ethanol and dried under vacuum for 3 min. The dried pellet was dissolved in 40 µL of deionized water and to it 40 µL of PEG-NaCl (20% PEG; 2.5M NaCl) solution was added. The mixture was incubated on ice for 20 min. and the plasmid DNA pelleted out by centrifugation for 15 min, 4°C at 12,000 x g. The supernatant was

aspirated carefully, the pellet washed with 70% ethanol and air-dried. The dried pellet was resuspended in 20 µL of deionized water and stored at -70°C.

2.3.3 Extraction and purification of DNA from agarose gels

The restriction enzyme digested DNA was run on a 1.0% agarose gel in 1X TAE buffer (see Section 2.6.2). The gel was stained with ethidium bromide (0.5 µg mL^{-1}). Stained gel was viewed under a UV transilluminator and the fragment of interest excised from the gel and weighed. About 50-200 mg of gel slice was transferred to a 1.5 mL microcentrifuge tube and 0.5 mL GEX buffer (Auprep^{TM} GEL^{X} kit, Life Technologies, USA). The tube was incubated at 60°C for 5 to 10 min. with constant mixing every 2 min. until the gel gets completely dissolved. The gel mixture was cooled down to room temperature. A GEL^{X} column was placed on the collection tube and 0.7 mL dissolved gel mixture was loaded into the column. The assembly was centrifuged at 12,000 x g for 2 min. The flow-through was discarded and the same step was repeated for the rest of the mixture. The column was washed with 0.5 mL of WF buffer by centrifuging for 30-60 seconds. The flow-through was discarded and the column was washed with 0.7 mL of WS buffer. The flow-through was discarded and the column was centrifuged at 12,000 x g for 3 min. to remove residual ethanol. The column was placed onto a new 1.5 mL centrifuge tube. About 30-50 µL of elution buffer or sterile distilled water was added onto the center of the membrane. The column was allowed to stand for 1-2 min. and then centrifuged at 12,000 x g to elute the DNA. The eluted DNA was stored at -20°C.

2.4 Sub-Genomic library of *Bacillus thuringiensis* R1

The genomic DNA of *Bacillus thuringiensis* R1 was restriction digested to completion with *Sac* I-*Cla* I and fractionated on 1.2% low melting point (LMP) agarose gel in 1X TAE running buffer. DNA fragments 4-6 kb in size were isolated (Sambrook *et al.* 1989) and cloned into *Sac* I-*Cla* I digested pBKS+ vector. *E. coli* DH5α cells were transformed and the *lacZ* complementation was used to identify the colonies harboring the recombinant molecules.

2.5 Transformation of *E. coli* cells and selection of the transformants

Competent *E. coli* cells were made in 100 mM $CaCl_2$. LB medium (50 mL) was inoculated with 1% of the overnight grown culture and allowed to grow till 0.5 O.D. at 600 nm. The cells were harvested by centrifugation at 5,000 rpm at 4°C for 10 min., suspended in 100 mM ice-cold $CaCl_2$ and kept on ice for 30 min. Cells were centrifuged again and the pellet suspended in 1 mL of 100 mM ice-cold $CaCl_2$ and stored as aliquots of 200 μL at 4°C.

The competent *E. coli* cells, thus formed, were transformed according to Sambrook *et al*. (1989). DNA (~50 ng in a volume of 10 μL or less) was added to competent *E. coli* cells, mixed and kept on ice for 30 min. The cells were then incubated at 42°C for 2 min. To each tube 800 μL of LB broth was added and further incubated at 37°C for 1 h. About 100 μL of the transformed competent cells was spread onto LB plates containing appropriate antibiotic, IPTG and X-gal (Sambrook *et al.* 1989).

Solutions	*Stock*	*Final conc. in medium*
1) IPTG stock solution	200 mg mL^{-1} in sterile distilled water	40 μg mL^{-1}
2) X-gal Stock solution	20 mg mL^{-1} in dimethyl formamide	40 μg mL^{-1}

2.6 Southern Blotting

Solutions

1X TAE:	0.04 M Tris-Acetate (pH 8.0), 0.001 M EDTA (pH 8.0)
20X SSC:	3 M NaCl, 0.3 M Sodium citrate (pH 7.0)
Depurination solution:	0.25 N HCl
Denaturation solution:	1.5 M NaCl, 5 M NaOH
Neutalization solution:	0.5 M Tris-HCl (pH 7.4), 3 M NaCl
Gel loading dye (6X):	0.25% Bromophenol blue in 40% (w/v) sucrose in water

For Southern hybridization (Southern 1975) the DNA samples were electrophoresed on an agarose gel in 1X TAE buffer containing 0.5 μg mL^{-1} ethidium bromide. The gel was rinsed in deionized water (DW) and placed in depurination

solution for 15 min. The gel was rinsed in DW and immersed in denaturation solution for 30 min. with gentle shaking. The gel was again rinsed in DW and transferred to neutralization solution buffer for 45 min. The gel was next set up for capillary transfer of DNA to solid membrane support.

A tray was filled with the transfer buffer (20X SSC). A platform was made and covered with a wick, made from 2 sheets of Whatman 3MM filter paper saturated with transfer buffer and the gel was placed on it. It was surrounded with a saran wrap to prevent the transfer buffer being absorbed directly by the paper towels stacked above the membrane. A sheet of Hybond-N^+ membrane (Amersham, UK) of the exact gel size was wetted with deionized water followed by transfer buffer (20X SSC) and placed on top of the gel. A glass rod was rolled over the membrane to remove any trapped air bubbles between the membrane and the gel. One piece of Whatman 3MM paper wetted with 20X SSC was placed on the membrane followed by Whatman 3MM paper prewetted in 2X SSC. On this paper another dry Whatman 3MM paper was placed followed by a stack of absorbent paper towels. A glass plate was placed on the top of the paper towels. An ~0.5 kg weight was placed on the glass plate. Transfer of DNA was allowed to proceed for about 18 h. The membrane was marked for orientation, removed carefully and washed with 6X SSC. The membrane was air dried and baked for 2 h at 80°C to immobilize DNA onto the nylon membrane. Hybridization and autoradiography were carried out as described in the following section.

2.7 Colony blotting

A nylon membrane of appropriate size was selected and placed on the agar surface in a Petri dish bearing *E. coli* colonies. The membrane and the agar plate were marked using a sterile needle to mark for orientation of the colonies. The membrane was peeled off after 1 min. and placed with the colony side up on a pad of 3MM Whatman paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH). After 7 min. the membrane was removed and placed on a separate pad soaked in neutralizing buffer (1.5 M NaCl, 1.0 M Tris-HCl, pH 7.4). After 3 min. the blot was removed and kept on a filter paper soaked in 2X SSC. The blot was then transferred to a dry filter

paper and air dried with the colony side up. The membrane was baked at 80°C for 2 h to heat fix the DNA. Hybridization and autoradiography were carried out as described Section 2.9 below.

2.8 Random primer labelling

Random primer labelling of the probe was done using the Megaprime DNA labelling system supplied by Amersham, UK. Reaction (50 µL) was set up as follows:

25 ng of DNA (used as probe)	5.0 µL
Primer solution (Random hexanucleotides) (3.5 A_{260} units)	5.0 µL

Above mixture was heated in a boiling water bath for 10 min. and cooled to room temperature facilitating the primer annealing to the DNA.

10X reaction buffer (500 mM Tris-HCl, pH 8.0; 100 mM $MgCl_2$; 10 mM DTT; 0.5 mg mL^{-1} acetylated BSA)	5.0 µL
0.5 mM each of dCTP, dGTP, dTTP solutions (333 mM Tris-Cl pH 8.0; 33.3 mM $MgCl_2$; 10 mM β-Mercaptoethanol)	12.0 µL (4.0 µL each)
[α-^{32}P-dATP (Sp. activity 3000 Ci $mmol^{-1}$)	5.0 µL
Sterilie deionized water	16.0 µL
Exonuclease free Klenow fragment (2 U $µL^{-1}$)	2.0 µL
TOTAL VOLUME	50.0 µL

The reaction was carried out at 37°C for 45 min. The reaction was stopped by incubating the reaction mixture in a boiling water bath for 10 min. and snap chilled on ice.

2.9 Hybridization

Solutions

20X SSC:	See section 2.6
Hybridization buffer:	1% BSA; 1.0 mM EDTA, pH 8.0; 0.5 M Sodium phosphate, pH 8.0; 7% SDS
Low stringency wash buffer:	2 X SSC; 0.1% SDS
High stringency wash buffer:	0.2 X SSC; 1% SDS

The blots made in section 2.6 were prehybridized at 45°C in 30 mL of hybridization buffer for 6-8 h in a hybridization incubator (Robin Scientific, USA). The buffer was decanted and fresh buffer along with the denatured radiolabelled probe was added. Hybridization was carried out at 50°C for 14-18 h. The solution was decanted and the membrane washed with low stringency buffer at 55°C for 15 min. followed by a high stringency wash at 55°C for 15 min. The moist blot was wrapped in saran wrap and exposed to X-ray film at –70°C in a cassette with intensifying screen.

2.10 Sodiumdodecylsulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Laemmli (1970) SDS-PAGE system is the widely used electrophoretic system for protein separations. The resolution in a Laemmli gel is excellent because the treated peptides are stacked in a stacking gel before entering the separating gel. Following are the stock solutions required for the Laemmli gel electrophoresis.

Solutions

Monomer solution	30% acrylamide and 2.7% bis in water. Stored at 4°C in the dark.
Running Gel Buffer	3.0 M Tris-Cl (pH 8.8)
Stacking Gel Buffer	1.0 M Tris-Cl (pH 6.8)
10% SDS	10 g dissolved in 100 mL water
Initiator (APS)	10% Ammonium persulfate (make fresh)
Tank Buffer	0.025 M Tris; 0.192 M glycine; 0.1% SDS pH 8.3
Staining Solution	0.025% Coomassie Brilliant blue G in 40% methanol and 7% acetic acid
Destaining Solution	7% acetic acid; 5% methanol
Water Saturated n-Butanol	5 mL water in 50 mL n-butanol
2X loading Buffer	0.125 M Tris-Cl (pH 6.8); 4% SDS; 20% glycerol; 0.1% Bromophenol blue; 10% 2-mercaptoethanol

Table 2.2 Composition of 10% Sodiumdodecylsulfate - Polyacrylamide Gel

Stock solution	Seperating gel for 8 mL	Stacking gel for 5 mL
Buffer	1.0 mL	0.63 mL
Acrylamide	3.0 mL	0.83 mL
Distilled water	3.8 mL	3.40 mL
APS	100.0 µL	100.00 µL
SDS	75.0 µL	50.00 µL
TEMED	10.0 µL	5.00 µL

2.10.1 Preparation of the separating gel

A vertical slab gel (Hoefer Scientific, U.S.A.) was assembled as per the instructions provided by the manufacturer using 1.0 mm spacers. In a side-armed vacuum flask, 10% separating gel solution was made according to Table. 2.2, leaving out ammonium persulfate and TEMED. Flask was stoppered and vacuum applied for several minutes while stirring on a magnetic stirrer. TEMED and ammonium persulfate were added and mixed gently without generating bubbles. The solution was poured into the vertical gel slab with the help of a pipette leaving 1.5 cm from the top. The slab was overlaid with water-saturated n-butanol to remove trapped air bubbles and to layer evenly across the entire surface. When a sharp liquid-gel inter-surface was observed after the gel polymerization, the slab was tilted to pour off the overlay.

2.10.2 Preparation of the stacking gel

After the separating gel is casted and the overlay poured off, stacking gel was prepared according to Table. 2.2 leaving out ammonium persulfate and TEMED. As in the separating gel, this solution was degased. TEMED and ammonium persulfate were added, mixed and overlaid on the separating gel. A comb was inserted without creating air bubbles beneath the teeth of the comb. The gel was left for 4 h to polymerize.

2.10.3 Preparation of the sample

Equal parts of the sample and the loading buffer were mixed in a microfuge tube and kept in a boiling water bath for 90 seconds. The samples were centrifuged at 10,000 x g for 10 min., supernatant collected and used for electrophoresis.

2.10.4 Loading and running the gel

The comb from the gel was removed carefully. The wells were flushed with distilled water and drained off completely. Wells were filled with tank buffer and the samples were underlaid in each well using a syringe. Lower and upper buffer chambers were filled with tank buffer. Voltage was set between 70 – 80 volts. The run was stopped when the dye reached the bottom of the gel.

2.10.5 Staining and destaining the gel

The gel apparatus was disassembled and the gel was carefully transferred into the staining solution and allowed to stain with shaking for 4-6 h. The gel was removed and transferred into destaining solution until the bands were clearly visualized.

CHAPTER 3

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF PHA PRODUCING *BACILLUS THURINGIENSIS* R1

3.1 INTRODUCTION

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters and elastomers, which accumulate as cytoplasmic inclusions in certain bacteria during unbalanced growth conditions (Anderson and Dawes 1990; Doi 1990; Steinbüchel 1991) usually characterized by an excess carbon supply and the lack of one or more essential nutrients (Steinbüchel and Schlegel 1991). About 150 different hydroxyalkanoic acids have been identified as constituents of bacterial polyesters (Steinbüchel and Valentin 1995). Several researchers have explained that soil bacteria generally produce PHB. PHB production in these soil isolates increases if the convenient condition is made available. The controlled large-scale production of PHB by bacteria has become a subject of increasing interest with a hope of replacing the petrochemical based polymers with biodegradable polymers.

The first PHA, a homopolymer of poly-β-hydroxybutyrate (PHB) was reported by Lemoigne (1926, 1927) in *Bacillus megaterium.* Belma *et al.* (2000) identified 27 strains of *Bacillus* from soil that could accumulate PHB. They reported different quantities of PHB accumulation in *B. subtilis*, *B. firmus*, *B. sphaericus* and *B. pumilus* and *Bacillus thuringiensis*. Borah *et al.* (2002) reported the accumulation of PHB in *B. mycoides* RCJ B-017. Though the accumulation of PHAs was reported in *Bacillus thuringiensis* very little is known about the biosynthesis of the polymer in this bacteria. There are no studies available on the accumulation of the polymer as affected by media parameters. Also no molecular data is available about the genes involved in the process. Hence, *Bacillus thuringiensis* was chosen as an experimental system for the present study.

In 1872, Ferdin and Cohn, a student of Robert Koch, recognized and named the bacterium *Bacillus subtilis.* Bacilli are rod-shaped, Gram-positive, sporulating, aerobes or facultative anaerobes. The family's distinguishing feature is the production of endospores, one per cell, which are highly refractile resting structures formed within the bacterial cells. Spore formation, universally found in the genus, is thought to be a strategy for survival in the soil environment, wherein the bacteria predominate. These spores are resistant to heat, cold, radiation, desiccation, and disinfectants. There is great diversity in physiology among members of the genus, many species exhibit

wide range of physiologic abilities that allow them to live in every natural environment.

This chapter presents the isolation of a PHB producing *Bacillus thuringiensis* R1 strain from soil, identification and characterization of the isolate, effect of carbon sources on accumulation of PHA.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Isolation of PHA producing strain

Soil samples were collected from different locations on the National Chemical Laboratory, Pune campus. One gram of each sample was suspended in sterile deionised water and volume made up to 100 ml. The samples were shaken vigorously for 2 min. The supernatant was serially diluted from 10^1 to 10^8 times with 0.9% NaCl and 0.1 ml of the diluted samples was plated on nutrient agar medium plates. The plates were incubated at 37°C for 24-48 hrs.

3.2.2 Analytic method for isolation of PHA producing strain

Each bacterial colony obtained on the nutrient agar (section 2.2) medium plates was picked and grown in 5 ml of nutrient broth with shaking for 24 h at 37°C. These cultures were then used as the inoculum (1%v/v) for a 50 ml basal medium (section 2.2) supplemented with glycerol (1%v/v) as the carbon source. The cultures were grown for 24-48 hrs at 37°C with shaking at 150 rpm. After incubation, the cell cultures were centrifuged at 4000 x g for 10 min at 15°C. The cell pellet was washed with sterile deionised water, an aliquot stained with Nile blue-A and observed for orange fluorescence at an excitation wavelength of 460 nm (Ostle and Holt 1982). One such colony showing orange fluorescence was used for further studies.

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3.2.3 Identification of the isolate

The isolate was subjected to different morphological and biochemical tests for identification. The brief principles and the methodology of the various identification tests are given below.

- **Gram staining**: The gram stain is the most widely used staining procedure in bacteriology developed in 1884 by Hans Christian Gram. It is called a differential stain since it differentiates between Gram-positive and Gram-negative bacteria. Bacteria that stain purple with the gram staining procedure are termed Gram-positive; those that stain pink are said to be Gram-negative. This procedure is a differential staining method, meaning that bacteria will give different results depending on their cell wall chemistry. The Gram staining divides bacteria into two groups based on their reaction to the stain. Gram-positive bacteria will appear purple after the last step; Gram-negative bacteria will appear red. The size, shape and arrangement of the organisms can also be determined from a stained specimen.

  The four steps of the Gram staining can be summarized as follows:

  1. Primary stain - The smear is covered with crystal violet for one minute. All bacteria will take up this dye and appear purple. The excess dye is rinsed off with water.
  2. Mordant - Gram's iodine is added to interact for one minute with the crystal violet. Excess iodine is rinsed off with water.
  3. Decolorization - 95% ethanol is briefly (10-20 seconds) applied to the smear followed by a water rinse.
  4. Counterstain - Safranin is added for 20 seconds to dye any decolorized cells. It will not change the color of the cells that retain the crystal violet.

  The above procedure was followed to identify the bacteria isolated from soil.

- **Spore staining**: The Schaeffer-Fulton method is the most commonly used endospore staining technique, and it uses Malachite green as the primary stain. Once the endospore has absorbed the stain, it is resistant to decolorization, but the vegetative cell is easily decolorized with water (leaving the vegetative cells colorless). Finally, the vegetative cells are counterstained with Safranin to aid in their visualization. When viewed under a microscope, the endospores appear green, while the vegetative cells are red or pink. Using aseptic technique, a bacterial smear was prepared on a clean slide, air-dried and gently heat fixed. The

slide was covered with a piece of paper towel, and placed on a staining rack over the boiling water bath. The paper towel on the slide was flooded with Malachite green (primary stain) and the slide was steamed for five minutes. The slide was allowed to cool, and then rinsed with deionized water until the water runs clear. The slide was stained with Safranin (counterstain) for two minutes. Excess Safranin was rinsed off with deionized water, and the slide was blotted dry and observed under the microscope.

- **Indole test**: The principle involves the detection of the ability of an organism to breakdown tryptophan to indole. Some bacteria possess the enzyme tryptophanase, which converts the amino acid tryptophan to pyruvate and indole through a deamination reaction. The pyruvate, then, can be used in fermentation or respiration reactions. Production of indole is used as a test to differentiate tryptophanase positive and tryptophanase negative organisms. The chemicals/ reagents required are summarized below.

Tryptone Broth - Tryptone Water	
Tryptone	10.0 g
NaCl	5.0 g
Distilled Water	1 L
Kovacs reagent (prepared in fume hood)	
p-dimethylaminobenzaldehyde	50 g
amyl or butyl alcohol	750 mL
HCl (conc.)	250 mL

The ingredients were dissolved in water, cooled to room temperature and pH adjusted to 7.5 and autoclaved. The culture was inoculated in tryptone water and incubated at 37°C for 48 hours. One mL of Kovacs reagent was added observed

for colour change immediately. Bright pink colour in the top layer indicates the presence of indole.

- **Methyl Red (MR) test**: This test is based on the ability of an organism to produce and maintain stable acid end products from glucose fermentation. The chemicals/ reagents required are summarized below.

Buffered Glucose Broth	
Proteose Peptone	5.0 g
D-glucose	5.0 g
Na_2HPO_4	5.0 g Distilled water to 1 L

The ingredients were added to water, heated gently with stirring to dissolve completely, and autoclaved. The culture was inoculated in buffered glucose broth and incubated at 37°C for 48 hours. A few drops of methyl red solution were added to the culture and observed for colour change. Red colour indicates the MR positive culture while MR negative culture gives a yellow colour.

- **Voges Proskauer (VP) test**: This test is done to detect the production of acetylmethylcarbinol (acetoin), a natural product formed from pyruvic acid in the course of glucose fermentation. The requirements are given below.

Buffered Glucose Broth	
Proteose Peptone	5.0 g
D-glucose	5.0 g
Na_2HPO_4	5.0 g Distilled water to 1 L

The ingredients were added to water, heated gently with stirring to dissolve completely and sterilized. The culture was inoculated in buffered glucose broth and incubated at 37°C for 3 days. Approximately 3 ml of alphanaphthol, was added

followed by 1 ml of 40% KOH, mixed well and allowed to stand for 30 minutes. Pink colour indicates VP(+) culture while no change in colour indicates VP(-) culture.

- **Citrate Utilization (CU)**: This test is done to determine if an organism is capable of utilizing citrate as sole carbon source for metabolism with resulting alkalinity. The culture was streaked on a Simmon's citrate agar plate and incubated at 37°C for 48 hours.

Simmons Citrate Agar	
$MgSO_4.7H_2O$	0.2 g
$(NH4)H_2PO_4$	1.0 g
K_2HPO_4	1.0 g
Sodium Citrate	2.0 g
NaCl	5.0 g
Bromothymol blue	0.08 g
Agar	15.0 g
Distilled water to	1 L

The ingredients were dissolved and then autoclaved. The growth of the culture indicates that the culture is CU(+). Growth on the medium is accompanied by a rise in pH to change the medium from its initial green colour to deep blue. This test aids in the differentiation of genera and species.

- **Casein hydrolysis**: The enzyme caseinase is excreted out of the cells (an exoenzyme) into the surrounding media, catalyzing the breakdown of milk protein, called casein, into peptides and amino acids. This reaction causes the milk agar, normally the opacity of real milk, to clear around the growth area. Skim milk agar plate was prepared and the culture streaked on the plate. A zone of clearance around the growth area identifies the presence of the enzyme caseinase.
- **Urease hydrolysis:** Urease is an enzyme that breaks the carbon-nitrogen bond of amides to form carbon dioxide, ammonia, and water. Urease can be **detected by**

plating bacteria onto an amide containing medium, specifically urea. When urea is broken down, ammonia is released and the pH of the medium increases (becomes more basic). This pH change was detected by a pH indicator that turns pink in a basic environment. A pink medium indicates a positive test for urease.

- **Nitrate Reduction:** To determine the ability of an organism to reduce nitrate to nitrites or free nitrogen gas. The culture was inoculated in a nitrate broth and incubated for 5 days at 37°C. To each nitrate broth culture 1 ml of sulphanilic acid and 1 ml of alphanaphthalamine was added. The production of a red colour occurs in the presence of nitrite and indicates the ability of the organism to reduce nitrate to nitrite.
- **Catalase test**: Metabolic reactions that occur in the presence of water and oxygen often result in the formation of hydrogen peroxide (H_2O_2). This compound is toxic to cells. Therefore, most organisms that can grow in the presence of oxygen possess catalase, an enzyme that converts hydrogen peroxide to water and oxygen. A colony was touched to a capillary tube and dipped in 3% H_2O_2. Production of bubbles indicates that the culture is catalase positive.
- **Cytochrome oxidase test**: This test is done to determine the presence of oxidase enzymes. Cytochrome oxidase C is an enzyme that reduces (adds electrons to) oxygen. This enzyme, therefore, is an oxygen reductase. Cytochrome oxidase is the last step in the electron transfer system in most aerobic organisms. It transfers electrons from the electron transport chain to oxygen, forming water in the process. Not all aerobic organism, though, possess cytochrome oxidase. Some species possess alternative electron transport molecules. Because this enzyme is present in some, but not all bacteria, microbiologists have developed a rapid means of testing for the presence of oxidase. The reagent (impregnated into strips of filter paper) contains tetramethyl-p-phenylenediamine which serves as an alternate substrate for the cytochrome oxidase reaction. In the reduced state the reagent is colourless but when oxidised it becomes purple. A piece of the oxidase test paper was held with forceps and touched onto an area of heavy growth. Colour change to purple immediately (with in 10 seconds) indicates that the culture is cytochrome oxidase positive.

- **Carbohydrate fermentation tests (acid production from carbohydrates)**: This test is used to determine the ability of an organism to ferment various simple carbohydrates (sugars). The indicator, phenol red / Andrade's indicator will turn yellow below pH 6.8 and a darker pinkish-red above pH 7.4. If the organism metabolizes the carbohydrate, subsequent acid production will result in lowered pH. If the organism does not ferment the carbohydrate, the pH may remain neutral. If the organism does not ferment the carbohydrate and also utilizes the peptone, accumulation of the ammonia as a degradation product will raise the pH.

Requirements :-

1. Sterile tubes of sugar fermentation medium with inverted Durham's tubes
   - A. Glucose broth.
   - B. Sucrose broth.
   - C. Mannitol broth.
   - D. Xylose broth.
   - E. Arabinose broth.
   - F. Meso-inositol broth.
   - G. Raffinose broth.
   - H. Rhamnose broth.
   - I. Salicin broth.
   - J. Galactose broth.

Composition of peptone water base medium:

Peptone- 1.0 gm.
NaCl - 0.5gm.
Distilled water-100ml.

pH - 7.5; Indicator added as per choice. Specific sugar such as Sucrose, Glucose, or lactose is added.

Composition of indicator used.

a) Andrade's indicator (1%)
   Acid Fuchsin - 0.5 gm
   1 N Sodiumhydroxide -16ml.
   Distilled water-100ml.

b) Phenol red indicator-0.2% aqueous phenol red solution.

A loopful suspension of the culture is inoculated into the sugar medium and observed for colour change.

3.2.4 Culture conditions for the Bacillus thuringiensis R1 isolate

The *Bacillus thuringiensis* R1 cells were grown for 70 hrs in a basal medium containing glycerol (1%v/v). Samples were harvested at different time intervals; the O.D. at 600 nm, pH, dry weight and PHB accumulation was recorded. To study the effect of various carbon sources on the growth of *B. thuringiensis* R1 and PHB accumulation, cells were cultivated for 36 h (stationary phase) in the basal medium supplemented with glycerol, glucose, molasses, table sugar, fructose, maltose, lactose, liquid glucose, acetic acid, lactic acid, ethanol and β-hydroxybutyrate. Each carbon source was used at a final concentration of 1% (w/v) or (v/v) in the medium except for β-hydroxybutyrate that was used to a final concentration of 0.50% (w/v) in the medium. For biphasic growth studies the *B. thuringiensis* R1 cells were grown in the basal medium for 36 hrs, harvested and reinoculated in the basal medium supplemented individually with glycerol (1%v/v), molasses (1%w/v), table sugar (1%w/v) and β-hydroxybutyrate (0.50%w/v). Different combinations of glycerol and propionic acid were also used in the medium to explore the possibility of production of the copolymers. The dry cell mass was used for GC analysis to identify and quantify the accumulated PHA.

3.2.5 Extraction of the polymer from the cells

Modified method of Hahn *et al*. (1993) was used to recover the polymer from the *B. thuringiensis* R1 cells. Briefly, the cells were harvested by centrifugation at 2000 × *g*, washed twice with deionized water and freeze-dried under vacuum. The lyophilized cell pellet was shaken for 90 minutes at 37°C with chloroform and 30% sodium hypochlorite (1:1). The dispersion was centrifuged at 4000 × *g*, at room temperature for 10 min. Lower chloroform phase that contained the solublised polymer was recovered and the polymer precipitated by the addition of 4 volumes of methanol.

3.2.6 Gas chromatography analysis

Acidic propanolysis of dried cell pellet was carried out as described previously (Riis and Mai 1988). About 100mg of the dry cell pellet was taken in a tightly sealable vial. Two mL of 1,2-Dichloroethane (DCE), 2 mL of acidified propanol (1 vol. concentrated HCl + 4 vol. n-propanol) and 200 μL of the internal standard (2.0 g benzoic acid in 50 mL n-propanol) were added to the pellet and incubated at 85°C for 4 h. The mixture was shaken from time to time. After cooling to room temperature, a mixture of 2 mL DCE and 2 mL acid propanol was added. The sample was centrifuged at 10,000 x g for 5 min. the clear supernatant was collected and an aliquot injected into the gas chromatograph. GC analysis was performed using a Shimadzu GC 17-A gas chromatograph. A 0.32 mm diameter BP1 capillary column (J&W Scientific Co., USA) of 25 m length was used. The analysis started at 80°C for 5 min followed by a 7°C/min. rise in temperature to the final temperature of 200°C. Nitrogen (5 ml/min) was used as the carrier gas. The quantitative evaluation was effected by the peak areas of the hydroxybutyl and the benzoyl esters formed.

3.3 RESULTS

3.3.1 Screening, isolation and characterization of a PHA producing microorganism

One particular bacterial isolate from the soil samples when stained with Nile blue A and viewed under an excitation wavelength of 460 nm, showed characteristic orange fluorescence, indicating the possible presence of PHA(s) in the cells. Further the production of PHA was confirmed by extraction of the polymer from the cells. Based on biochemical characteristics, the isolate was characterized and identified as the endospore forming, Gram-positive *Bacillus thuringiensis,* and designated as the strain R1. *Bacillus thuringiensis* belongs to Superkingdom-Bacteria; Phylum-Firmicutes; Class-Bacilli; Order-Bacillales; Family-Bacillaceae; Genus-*Bacillus*; Species group- *Bacillus cereus* group; Species- *Bacillus thuringiensis.* Morphological and physiological characteristics of the strain *Bacillus thuringiensis* R1 are summarized in Table 1.

3.3.2 Growth and accumulation of PHB by Bacillus thuringiensis R1 in Basal medium with glycerol

The *B. thuringiensis* R1 cells grown for 24 h in the glycerol (1%v/v) containing basal medium were harvested. GC analysis of the dried and esterified cells revealed the accumulation of poly-β-hydroxybutyrate (PHB). The cells were next cultured in presence of glycerol (1%v/v) for 70 h to study growth and PHB accumulation patterns. The pH of the medium dropped from 7.2 to 5.5 during this period. The *B. thuringiensis* R1 cells entered stationary phase of growth after 36 h of incubation (Fig. 3.1). Maximum dry cell biomass (3.90g/L of the medium) and PHB accumulation (34.18% of the dry cell mass) coincided with the advent of the stationary phase of growth.

3.3.3 Effect of different carbon sources on growth and accumulation of PHB in Bacillus thuringiensis R1

The *B. thuringiensis* cell dry mass formation and PHB accumulation, varied with the carbon source (Fig.3.2). Maximum dry cell biomass (3.90g/L of the medium) and PHB accumulation (34.18% of the dry cell mass) was observed with glycerol as the carbon source. It was 3.11g/L in presence molasses, 2.67g/L in presence of lactose and 2.01g/L in presence of table sugar. Least cell growth was observed in presence of ethanol and lactic acid. Dry cell mass in presence of the monomer β–hydroxybutyric acid (0.5%w/v) was 1.5g/L.

PHB accumulation in presence of table sugar, molasses and fructose was 28.23%, 23.06% and 19.76% respectively. PHB accumulation in presence of lactose was 12.72% and 4.61% in presence of lactic acid. Only 9.23% PHB accumulated in presence of glucose in the medium. Acetic acid and ethanol did not support any PHB accumulation. Marginal accumulation of PHB (2.10% on cell dry mass basis) was observed with the incorporation of maltose in the basal medium. PHB accumulation was 52.50% of dry cell mass in presence of the β–hydroxybutyric acid monomer in the medium.

Table 3.1. Morphological and physiological characteristics of the *B. thuringiensis* R1

Characteristic	Response
Gram staining	Positive
Spore staining	Positive
Cell shape	Rods
Density	Opaque
Elevation	Convex
Margin	Wavy
Motility	Negative
Fluorescence and Pigments	Nil
Indole test	Negative
Methyl red test	Positive
Voges Proskauer test	Negative
Citrate utilization	Positive
Casein hydrolysis	Positive
Urea hydrolysis	Negative
Nitrate reduction	Positive
Catalase test	Positive
Cytochrome oxidase test	Positive
Acid production from	
Glucose	Positive
Sucrose	Positive
Mannitol	Negative
Arabinose	Negative
Xylose	Negative
Meso-inositol	Negative
Raffinose	Negative
Rhamnose	Negative
Salicin	Negative
Galactose	Negative

Fig. 3.1 Growth of *Bacillus thuringiensis* R1 in Basal medium with glycerol (1%v/v)

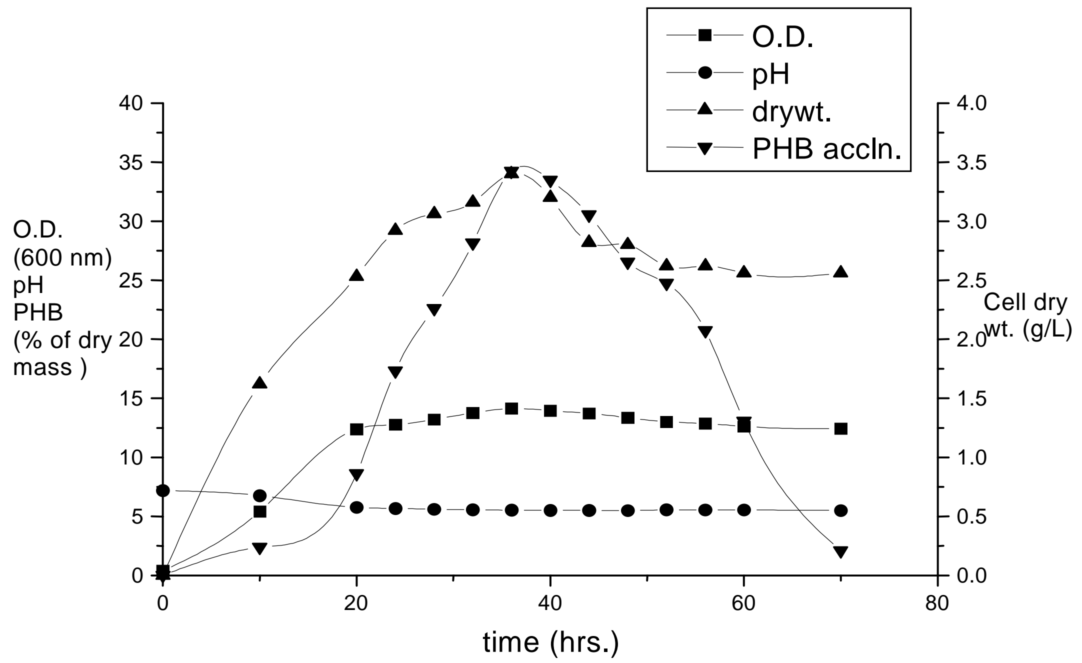
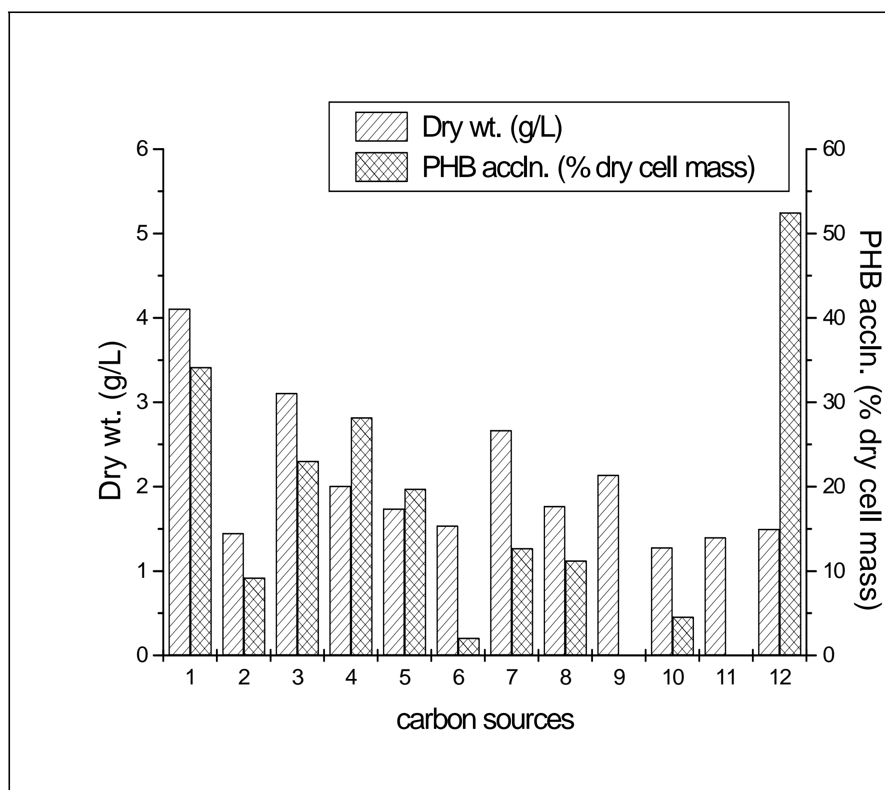


Fig. 3.2 Effect of different carbon sources on growth and accumulation of polyhydroxybutyrate in *Bacillus thuringiensis* R1 at 36 hrs. 1 Glycerol, 2 Glucose, 3 Molasses, 4 Table sugar, 5 Fructose, 6 Maltose, 7 Lactose, 8 Liquid glucose, 9 Acetic acid, 10 Lactic acid, 11 Ethanol and 12 β–hydroxybutyric acid.



3.3.4 Biphasic growth studies in Bacillus thuringiensis R1

Under biphasic growth conditions, the *B. thuringiensis* R1 cell dry weight in presence of glycerol (1%v/v) increased from 4.11g/L to 6.11g/L of the medium and PHB accumulation from 34.23% to 64.10% of dry cell mass. When table sugar was used in the biphasic study there was an increase in dry weight from 2.01g/L to 2.70g/L and PHB content from 28.23% to 31.36% of dry cell mass. There was no increase of PHB content in the biphasic growth of *B. thuringiensis* R1 when either the monomer or molasses were used as the carbon source (Table 3.2).

Table 3.2. Biphasic growth studies in *Bacillus thuringiensis* R1

Carbon source	Dry wt. (g/L) after		PHB accumulation (% dry cell mass) after	
	1st 36 h of Phase I	2nd 36 h of Phase II	1st 36 h of Phase I	2nd 36 h of Phase II
Glycerol	4.11	6.11	34.23	64.10
Table sugar	2.01	2.70	28.23	31.36
Molasses	3.15	3.18	23.06	23.06
β-hydroxybutyrate	1.50	1.58	52.50	52.50

3.3.5 Gas chromatography analysis

GC analysis of the dried and esterified *B. thuringiensis* R1 cells grown for 36 hrs in presence of different carbon sources, revealed two peaks. One corresponding to the propyl ester of 3-hydroxybutyrate (3-HB) and the other to the propyl ester of the internal standard benzoic acid (Fig. 3.3a). The cells grown in presence of a combination of glycerol (1%v/v) and propionic acid (0.25%v/v) revealed an additional peak corresponding to the propyl ester of 3-hydroxyvalerate (3-HV) (Fig. 3.3b). However, the accumulation of the copolymer was low (6.46% PHB with a 0.02 mol fraction of 3-HV).

Fig. 3.3a. Gas chromatograms of the propyl ester of the authentic Polyhydroxybutyrate sample (A) and the *Bacillus thuringiensis* R1 cells grown in medium containing glycerol (B). Propyl ester of PHB and benzoic acid are represented by the peaks 1 and 2 respectively.

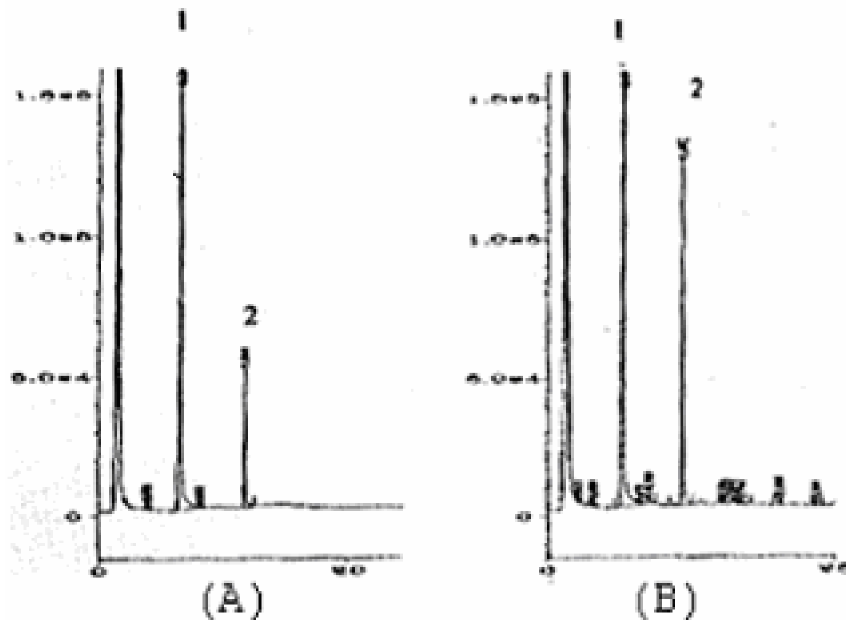
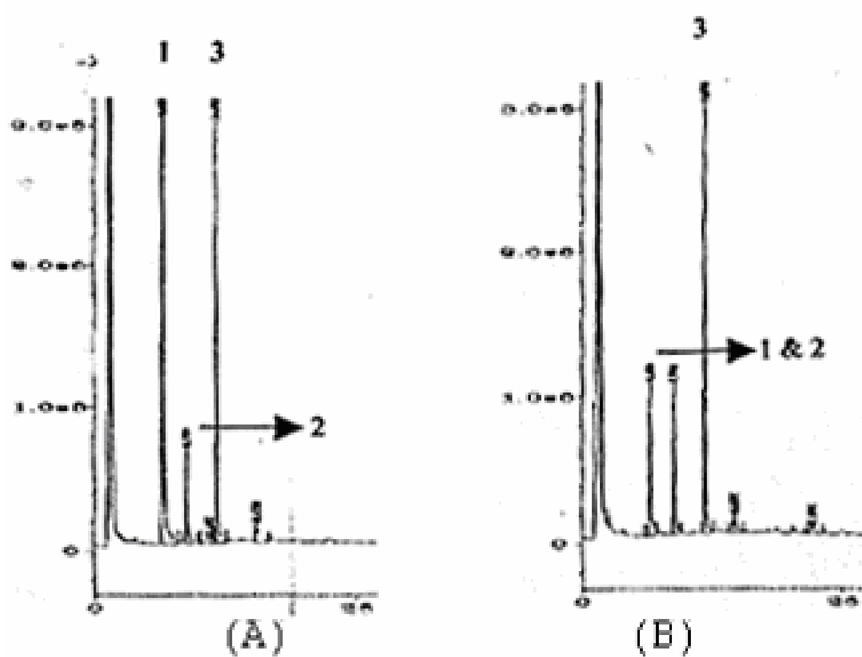


Fig. 3.3b. Gas chromatograms of (A) authentic polyhydroxybutyrate-valerate copolymer and (B) dried *Bacillus thuringiensis* R1 cells grown in medium containing propionic acid (0.25%v/v) and glycerol (1.0%v/v). The peaks 1, 2 and 3 represent propyl ester of 3-hydroxybutyrate, 3-hydroxyvalerate and benzoic acid respectively.



3.4 DISCUSSION

The soil isolate was identified as Gram-positive *Bacillus thuringiensis* R1 based on the morphological characteristics and biochemical tests. Gram-positive and Gram-negative bacteria stain differently because of fundamental differences in the structure of their cell walls. The Gram-positive cell wall appears thick and consists of numerous interconnecting layers of peptidoglycan. Also interwoven in the cell wall of Gram-positive bacteria are teichoic acids. Generally, 60% -90% of the gram-positive cell wall is peptidoglycan. The Gram-negative cell wall, on the other hand, contains a much thinner, single layer of peptidoglycan only two or three layers thick. This is surrounded by an outer membrane composed of phospholipids, lipopolysaccharide, and lipoprotein. Only 10% - 20% of the Gram-negative cell wall is peptidoglycan. With the current theory behind Gram staining, it is thought that in Gram-positive bacteria, the crystal violet and iodine combine to form a larger molecule that precipitates out within the cell. The alcohol/acetone mixture then causes dehydration of the multilayered peptidoglycan in the Gram-positive cell wall, thus decreasing the space between the molecules and causing the cell wall to trap the crystal violet-iodine complex within the cell (Kaiser 2002). Spore formation, universally found in the genus, is thought to be a strategy for survival in the soil environment, wherein the bacteria predominate. Aerial distribution of the dormant spores probably explains the occurrence of *Bacillus* species in most habitats examined. An endospore is not a reproductive structure but rather a resistant, dormant survival form of the organism that are quite resistant to high temperatures (including boiling), most disinfectants, low energy radiation, drying, etc. The completed endospore consists of multiple layers of resistant coats (including a cortex, a spore coat, and sometimes an exosporium) surrounding a nucleoid, some ribosomes, RNA molecules, and enzymes that allow the organism to survive in the condition of starvation (Kaiser 2002). The biochemical characterization of the isolate confirmed it to be a strain of *Bacillus thuringiensis*.

Belma *et al*. (2000) have reported PHB accumulation in *B. thuringiensis* cells. However, no systematic study has been carried out to study the accumulation of PHAs by these cells as influenced by media and culture conditions. The GC analysis revealed that *B. thuringiensis* R1 cells accumulate PHA(s) with a single carbon

source. Maximum cell mass formation and the accumulation of the polymer coincided with the beginning of the stationary phase of the cell growth cycle. Thereafter, the amount of the accumulated PHB declined possibly due to PHB metabolism (Jaeger *et al.* 1995). GC analysis of the *Bacillus thuringiensis* R1 cells revealed that the cells supported synthesis of the PHB homopolymer when supplied with a single carbon source. However, glycerol supported maximum cell growth as well as PHB accumulation (except for when the hydroxybutyrate monomer was used). The utilization of glycerol for growth and PHB formation ensues conversion of glycerol to acetyl CoA *via* either glycolysis or the methylglyoxal pathways (Ramachander *et al.* 2002). Some of the Bacillus species have been reported to accumulate 6-36% PHB of the cell dry mass (Belma *et al.* 2000). PHB accumulation of 64.10% in the biphasic growth cycle of *B. thuringiensis* R1 cells is the highest recorded for this bacterium thus far. This indicates that while the first phase is used for the development of the biomass, the second phase in presence of glycerol is preferentially utilized for PHB production by the cells. Glycerol, which is the by product of the soap industry can be utilized as the carbon source for efficient PHB production by the *Bacillus thuringiensis* R1 cells. Table sugar and molasses being cheap sources from the massive sugar industry in India may also be used for cost effective production of PHB on a large scale. This is the first report of utilization of glycerol, table sugar or molasses for the production of PHB by *Bacillus thuringiensis* cells. Borah *et al.* (2002) reported the use of sucrose as the cheaper source for the production of PHB by *Bacillus mycoides* RLJ B-017. Similarly, *Bacillus* Jma5 (Wu *et al.* 2001) (25-35%) and *Bacillus megaterium* (Gouda *et al*. 2001) (40.80%) accumulate PHB during fermentation with a molasses. *Bacillus megaterium* has also been reported to accumulate PHB in presence of glucose (39.90%)(Gouda *et al*. 2001). Belma *et al.* (2000) have reported PHB accumulation of 8.00% and 7.54% in *Bacillus thuringiensis* strains D1 and D2 respectively in glucose containing medium. In the present study glucose did support high accumulation of PHB (9.23%). This may be possibly the result of medium acidosis since the cell mass formation was also low. It is also documented that glucose in the medium represses the expression of phosphotransbutyrylase (Ptb) gene that is needed for PHB production (Vazquez *et al.* 2003). Glucose is degraded through the EMP pathway to yield pyruvate and acetate as

the main products. Acetate is partially converted into PHB, which is consumed during sporulation in *Bacillus* sp. (Benoit *et al.* 1990). Although acetate is a precursor in acetyl-coA synthesis (Anderson and Dawes 1990) it did not support PHB accumulation by the *B. thuringiensis* strain R1. Though there was increased accumulation of PHB (52.50%) with the use of β-hydroxybutyrate (sodium salt) as the carbon source in the media, it is not feasible to use the monomer as a carbon source owing to its high cost. Growth of the *B. thuringiensis* culture in two phases resulted in the increase in the PHB production when glycerol or table sugar was used as the carbon source. This indicates that the fully grown culture of *B. thuringiensis* could utilize the carbon source supplied in the second phase, essentially for the accumulation of PHB. This biphasic growth for PHB accumulation may be used as a strategy for increased PHB production on a commercial scale. *Bacillus thuringiensis* R1 cells grown in the presence of glycerol and propionic acid were found to accumulate P(HB-co-HV) copolymer, however, the accumulation of PHA was considerably low which could be the inhibitory effect of propionic acid as a toxic substrate on growth and accumulation of PHB (Ramsay *et al*. 1990).

3.5 CONCLUSIONS

- A Gram-positive soil microorganism, *Bacillus thuringiensis* R1 capable of accumulating polyhydroxyalkanoates has been isolated.
- Maximum growth and PHB accumulation was obtained at 36 hrs when *Bacillus thuringiensis* R1 was grown in shake flasks.
- Maximum accumulation of PHB was found in the medium containing glycerol as the sole carbon source followed by molasses and sucrose.
- Biphasic growth cycle of *B. thuringiensis* R1 cells resulted in PHB accumulation of 64.10%.
- *Bacillus thuringiensis* R1 is found to accumulate PHB when grown on a single carbon source.
- PHB-V was accumulated when glycerol and propionic acid were used in the medium as the carbon sources.

CHAPTER 4

PHYSICAL CHARACTERIZATION OF PHA FROM *BACILLUS THURINGIENSIS* R1

4.1 INTRODUCTION

PHB and its co-polymers have been industrially produced since 1982 as substitutes for petroleum based plastics. Global environment concerns and solid waste management problems have generated much interest in the development of biodegradable plastics that retain the desired physical and chemical properties of the conventional synthetic plastics. Non-degradability and persistence in the environment of the fossil fuel based plastics has prompted the scientific community to look for degradable alternatives with comparable properties. The discovery of naturally occurring biodegradable polymers like polyhydroxyalkanoates (PHAs), polylactides, polyglycolic acids, polysaccharides and their blends has opened avenues for their utilization (Leaversuch 1987; Dawes 1990; Chum 1991; Steinbüchel 1991; Graham 1993; Chang 1994).

A better understanding of the physical and thermal properties of biopolymers will help us design these with improved properties for commercial purpose. Poly(3-hydroxybutyrate)P(3HB), the most widely spread PHA belonging to the short-chain-length (scl) family, is highly crystalline, hard and brittle (Holmes 1988; Doi 1990; Hocking and Marchessault 1994). Medium-chain-length (mcl) PHAs on the other hand have a low degree of crystallinity and are soft and sticky (Gross *et al.* 1989; Marchessault *et al.* 1990; Preusting *et al.* 1990). These properties limit the applications of mcl-PHAs as thermoplastic materials (Hammond and Liggat 1995; Amass *et al.* 1998). In addition to this medium chain length PHAs have been found unsuitable for applications due to their low melting and decomposition temperature and low glass transition temperatures (Gross *et al.* 1989; He *et al.* 1999). It has been shown that co-polymers of various PHAs possess superior thermal and mechanical properties than the homopolymers (Steinbüchel 1991). Analytical techniques such as GC, NMR, FTIR and improving upon the protocols from time to time has helped to identify, characterize and quantitate PHAs in relatively quick time (Riis and Mai 1988; Rozsa *et al.* 1996; Hong *et al.* 1999; Misra *et al.* 2000). Though, many organisms produce PHB, it's properties differ depending upon the organism producing it (Van der Walle *et al.* 2001). This necessitates physical characterization of the polymer from *Bacillus thuringiensis* R1.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Organisms and growth

Bacillus thuringiensis R1 grown and maintained as enumerated in Chapter 2.

4.2.2 Recovery of the Biopolymer from the cells

This is enumerated in section 3.2.5 of chapter 3.

4.2.3 Scanning electron microscopy

PHA sample purified from *B. thuringiensis* R1 cells was sonicated in water at 20 KHz for 3 cycles of 5 min. each. A drop of the suspension was dried on a brass stub under an IR lamp and later coated with gold using Polaron sputter unit. SEM photographs were taken with a LEICA Stereoscan 440 Scanning electron microscope equipped with a Phoenix EDAX attachment.

4.2.4 ^{1}H NMR spectroscopy

The ^{1}H NMR of the extracted polymer was carried out using Bruker Ac200 at 24°C (Rozsa *et al.* 1996). The polymer was solubilized in $CDCl_3$.The samples were analysed in 5mm sample tubes in chloroform-d. The spectra were referenced to internal tetramethylsilane.

4.2.5 FTIR analysis

The polymer sample (2 mg) was thoroughly mixed with KBr (Spectroscopic grade). KBr treated pellet (15 mg) was dried at 100°C for 4 h (Misra *et al.* 2000). FTIR spectrum was taken using a Perkin Elmer (USA) model 1720 Fourier Transform IR spectrometer.

4.2.6 Determination of molecular weight and molecular weight distribution by GPC

The molecular weight and molecular weight distribution of the polymer was determined by gel-permeation chromatography (GPC) using a Waters 515 pump with four Stryagel HR columns. Monodisperse polystyrene and chloroform were used as

the molecular weight standard and the mobile phase respectively. For each analysis 250 µl of 0.1% (w/v) polymer solution in $CHCl_3$ was injected.

4.2.7 Differential Scanning Calorimetry

To determine the morphological state of the polymer, the melt temperature and the enthalpy of fusion were measured using a Perkin Elmer Differential Scanning Calorimeter (DSC-7). Samples (10-15 mg) were encapsulated in aluminium pans and heated from 0 to 200°C at a rate of 20°C/min. The melting temperature (T_m) was taken at the peak of the melting endotherm.

4.2.8 Thermogravimetric analysis of PHA

Thermogravimetric analysis of the polymer sample was done using Rheometric TG analyser (Rheometric Scientific, USA). The analysis was carried out under nitrogen flow rate of 15 ml/min. with scanning rate of 10°C/min. The temperature was increased until weight loss was 20%. After cooling rapidly to room temperature (100°C/min), the residue was recovered. This residue was completely soluble in chloroform.

4.2.9 Purification of inclusion bodies from Bacillus thuringiensis R1

Purification of the inclusion bodies from the cell lysate of *Bacillus thuringiensis* R1 was done with modifications of the extraction procedure described by McCool *et al.* (1996). Briefly, cells were pelleted and resuspended in a minimum volume of 10mM Tris-HCl pH 8.0, 1 mM EDTA, 20 mM $MgSO_4$, 0.25 M sucrose at 4°C. Lysozyme was added to a final concentration of 1 mg/ml followed by incubation at 37 ^{o}C for 1 hour. To ensure proper lysis, cells were treated with NaOCl (30%). Aliquots of 1.8 ml of cell lysate were loaded on sucrose step gradient in centrifuge tubes (Beckman) and consisted of 1.5 ml each of the following sucrose concentrations 2.00 M, 1.66 M, 1.33 M, 1.00 M and 1.0 ml of 0.66 M in TE (10mM Tris-HCl pH 8, 1 mM EDTA). The tubes were centrifuged at 30,000 rpm for 2 hours. The inclusion bodies, which banded about mid tube were collected, washed in 20 volumes of TE and stored at 4°C.

4.2.10 Separation of polypeptides associated with PHA inclusion bodies

Inclusion bodies were purified as described above followed by suspension in 1x TE buffer with 2% SDS. An equal volume of sample buffer was added prior to boiling for 5 min., and samples were centrifuged for 3 min. to pellet PHA, the supernatant was loaded on an SDS 10% polyacrylamide gel (as described in Section 2.9) and run at 20 mA for 2 h. The gel was stained by Coomassie blue staining procedure.

4.3 RESULTS AND DISCUSSION

PHB granules isolated from the *B. thuringiensis* cells observed under scanning electron microscope showed stable spherical configuration with an average diameter of 5 microns (Fig. 4.1).

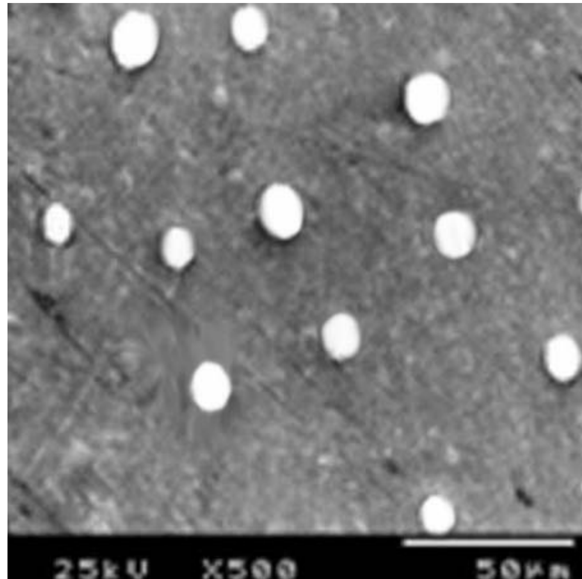


Fig. 4.1 Scanning electron microscopy (SEM) of the gold coated PHB granules obtained from *Bacillus thuringiensis* R1. PHB granules of uniform spherical shape with a stable configuration are seen. The size of the granule is 5 µm. Bar represents 50 µm.

The NMR spectra identified the polymer as an isotactic homopolymer (Fig. 4.2). The spectrum revealed the presence of three groups of signals characteristic of PHB homopolymer. The doublet at 1.3 ppm was attributed to the methyl group coupled to one proton; the doublet of the quadruplet at 2.57 ppm to the methylene group adjacent to an asymmetric carbon atom bearing a single proton and the multiplet at 5.28 ppm to the methyne group. Chloroform-d gave a chemical shift signal at 7.25 ppm. The NMR signal multiplicity by a proton as a quadruplet or octet in case of protons of CH_2 group was obtained due to proton coupling in isotactic form unlike in syndiotactic where duplet signal is obtained due to coupling (Fig.3). Similar spectral signatures have been reported for PHB isolated from *Bacillus cereus* (Labuzek *et al.* 1994) and *Bacillus circulans* (Rozsa *et al.* 1996).

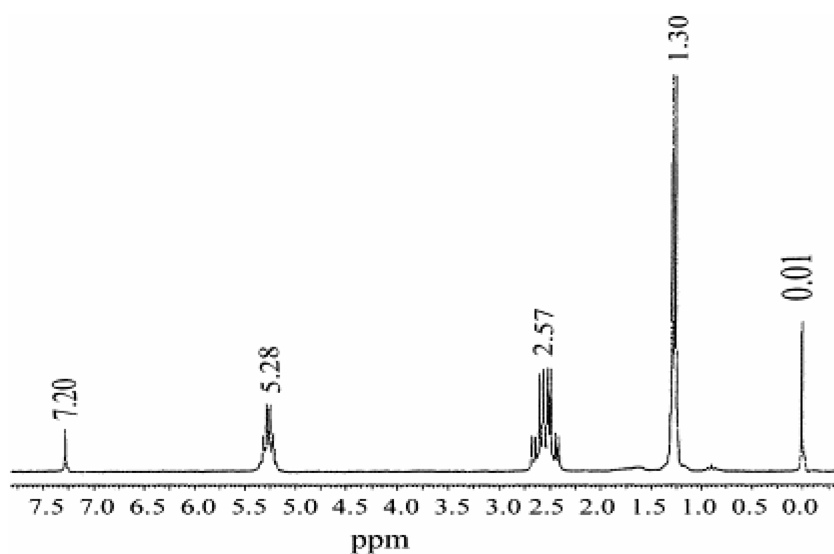


Fig. 4.2 ^{1}H NMR spectra of the PHB recovered from *Bacillus thuringiensis* R1 showing a doublet, doublet of quadruplet and a multiplet at 1.3, 2.57 and 5.28 ppm which are due to proton coupling. The signal at 7.25 ppm is characteristic of chloroform-d.

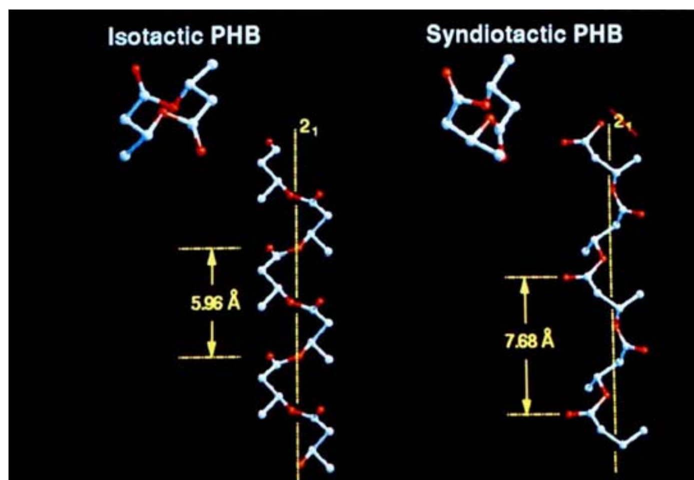


Fig. 4.3 Arrangement of molecules and pendant groups in an isotactic and syndiotactic PHB are shown. In isotactic form the pendant group is always away from the main chain and are arranged towards one side of the plain unlike in syndiotactic wherein the pendant groups are alternately arranged.

Hong *et al.* (1999) communicated a rapid method to identify and differentiate cells producing scl- or mcl- PHA using FTIR. FTIR analysis of PHA extracted from *Bacillus thuringiensis* R1 cells revealed two absorption bands at 1280 cm^{-1} and 1735 cm^{-1} corresponding to C=O and C-O stretching groups respectively (Fig. 4.4). Rozsa *et al.* (1996) have shown that the FTIR absorption band at about 1730 cm^{-1} is a characteristic of carbonyl group and a band at about 1280-1053 cm^{-1} characterizes the valence vibration of the carboxyl group. These are characteristics of the polyhydroxybutyrate.

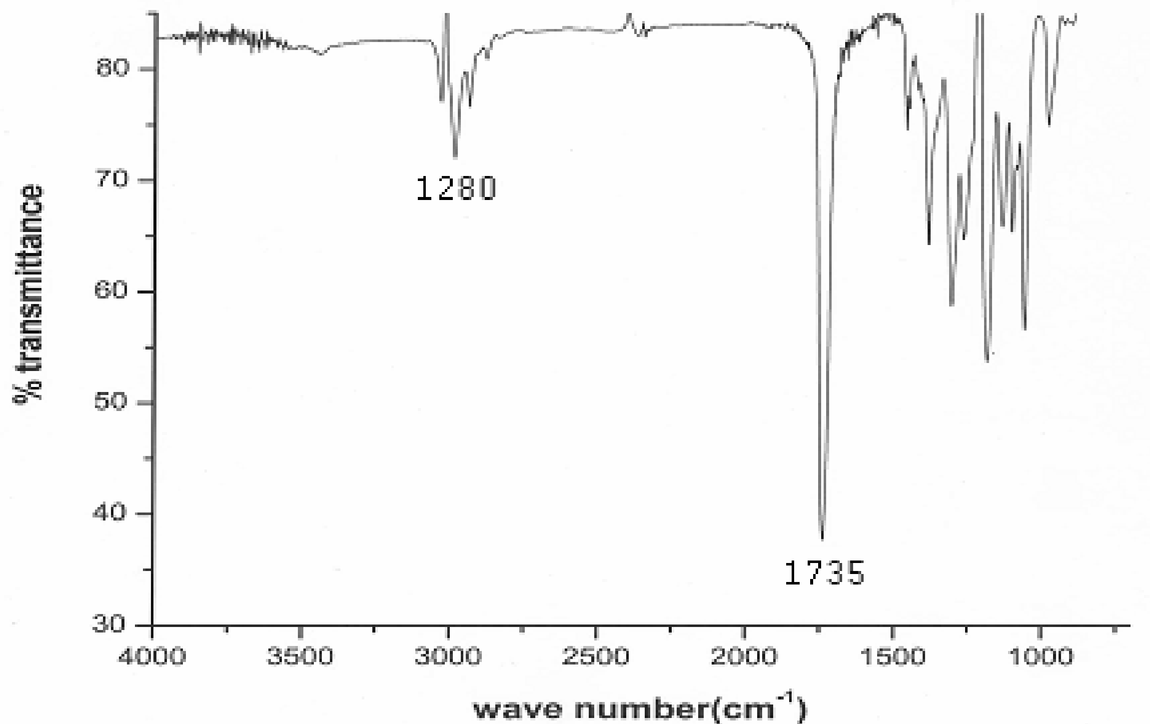


Fig. 4.4 FTIR spectra of polymer extracted from *Bacillus thuringiensis* R1. The absorption bands at 1280 and 1735 cm^{-1} correspond to C=O and C-O of PHB, respectively.

The thermodynamic properties of a polymer are dependent on its number-average molecular mass and the bulk properties connected with large deformations are largely determined by weight-average molecular mass (Van Krevelen 1972). Molecular weight of PHB is dependent on the physiological background or on the abundance of PHA synthase present within the cell (Rehm and Steinbüchel 1999; Madison and Huisman 1999). The GPC analysis revealed that the polydispersity index (Q) (defined as Mn/Mw), number average molecular weight (Mn), weight average molecular weight (Mw) were 1.77, 5.8526 x 10^4 and 1.0385 x 10^5 respectively (Fig. 4.5).

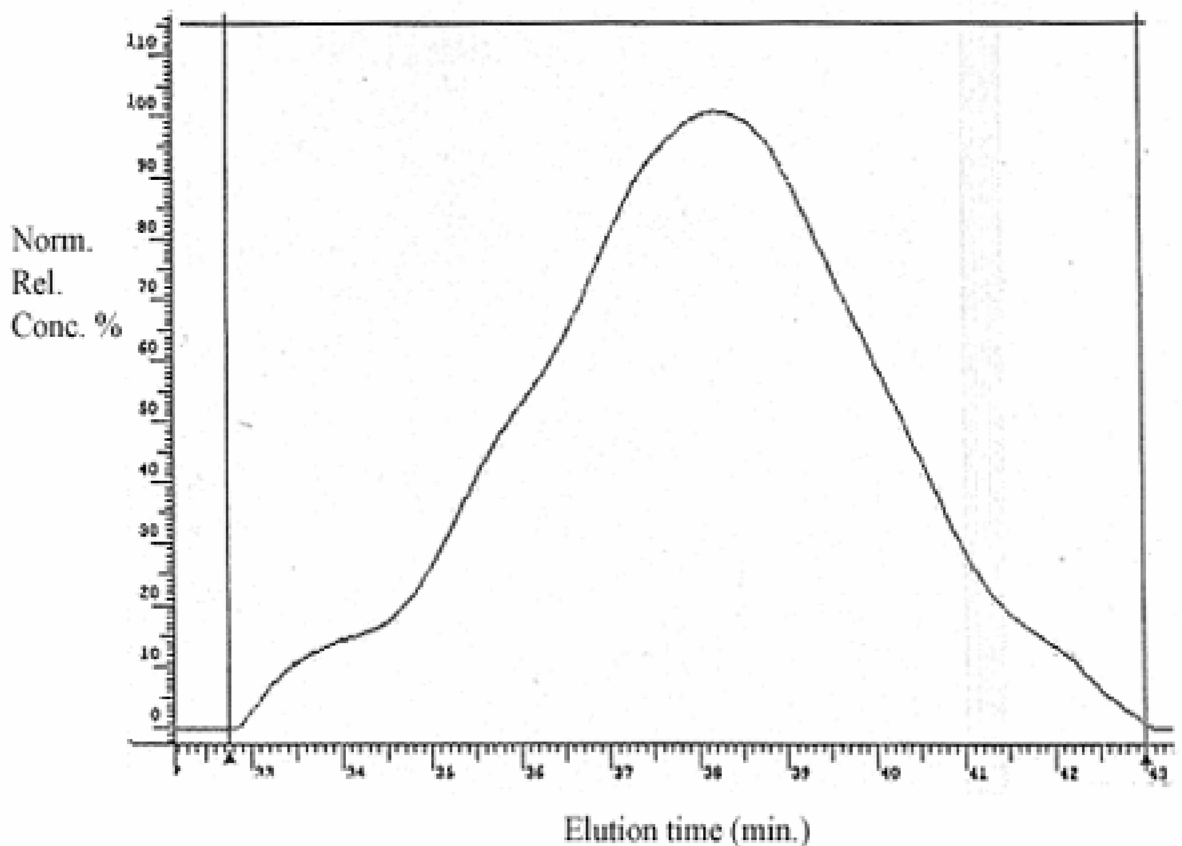


Fig. 4.5 Gel permeation chromatography of the purified PHB sample from *Bacillus thuringiensis* R1

The melting temperature and the enthalpy of fusion of the polymer were 165.6°C and 84.1 J/g respectively. The polymer degraded rapidly between 225°C and 270°C with a peak at 261°C (Fig. 4.6). The high enthalpy of fusion (84.1 J/g) suggests high crystalline nature of the recovered PHB that was calculated to be of 60 - 65 % assuming the enthalpy of fusion of 100% crystalline sample to be 146 J/g. The melting temperature of PHB (165.6°C) in the present study was slightly lower than reported for PHB from *B. cereus* (170°C) (Labuzek *et al.* 1994) and *B. circulans* (173°C) (Rozsa *et al.* 1996). The difference between the melting temperature and the decomposition temperature (261°C) was high enough to facilitate processing of the polymer.

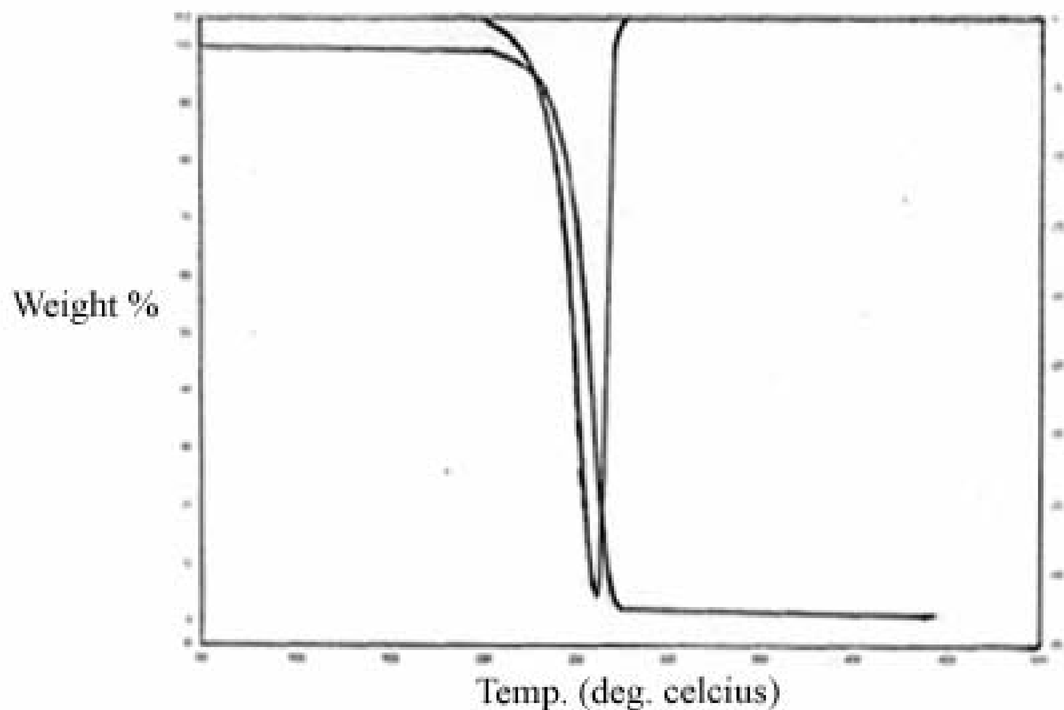


Fig. 4.6 Differential Scanning Colorimetry of the polymer purified from *Bacillus thuringiensis* R1.

PHA inclusion bodies from *Bacillus megaterium* are known to contain 97.7% PHA, 1.87% protein and 0.46% lipid with protein and lipid forming an outer layer (Griebel *et al.* 1968). PhaC and PhaP are found to be the two most abundant proteins that copurify with inclusion bodies and essential for PHB accumulation in *Bacillus megaterium* (McCool and Cannon 2001). . The SDS gel when stained with Coomassie blue showed two bands (~45 kDa and ~40 kDa) that may correspond to the proteins associated with inclusion bodies (Fig. 4.7).

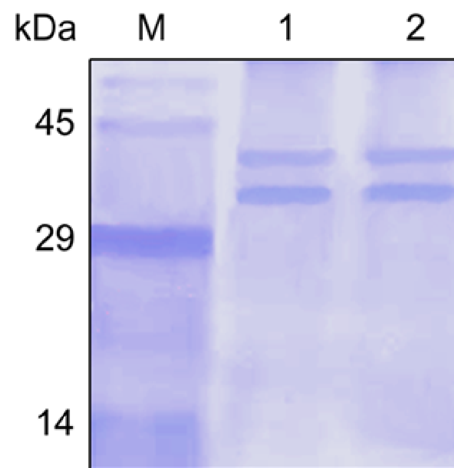


Fig. 4.7 Inclusion body associated proteins purified from *Bacillus thuringiensis* R1 cell lysate.

4.4 CONCLUSIONS

- SEM of the recovered polymer revealed a uniform spherical shape with a stable configuration with a granule size of 5 microns.
- NMR and FTIR studies have shown the accumulated polymer to be isotactic homopolymer of PHB when *Bacillus thuringiensis* R1 was grown on a single carbon source.
- Molecular weight of the PHB synthesized and accumulated by *Bacillus thuringiensis* R1 was found to be around 1.0385×10^5 by GPC.
- DSC and TGA results show a shift in the thermal degradation temperature pattern of the polymer, which is found to be at 261°C.
- Inclusion bodies associated proteins of ~45 kDa and ~40 kDa were purified from *Bacillus thuringiensis* R1 cell lysate.

CHAPTER 5

PHA GENE LOCUS FROM *BACILLUS THURINGIENSIS* R1: ISOLATION, CLONING, SEQUENCING AND EXPRESSION IN *E. COLI*

5.1 INTRODUCTION

The class of PHA synthesized by a bacterium is dictated by the organism's metabolic background and its PHA-biosynthesis genetic composition and function. The PHA biosynthesis genetic system (*pha* gene locus) is divided into four classes on the basis of the organization of the gene locus and the structure – function properties of the PHA synthase (polymerase) enzymes. The class I *pha* gene locus, as exemplified by that of *Waustersia eutropha*, consists of *pha*C (coding for PHA synthase), *pha*A (β-ketothiolase), and *pha*B (acetoacetyl-CoA reductase) genes. The class II *pha* operon, found in Pseudomonads, comprises of two PHA synthase genes (*pha*C1 and *pha*C2) flanking a PHA depolymerase gene (*pha*Z). The class III *pha* operon is distinguished by the presence of *pha*C and *pha*E genes coding for the two hetero-subunits of its PHA synthase. Adjacent to the two synthase genes are the *pha*A and *pha*B genes that are transcribed in the opposite direction. The class IV *pha* locus found in *Bacillus* species constitute the *pha*R and *pha*C genes coding for the two hetero-subunits of the PHA synthase and a *pha*B between the two genes (McCool and Cannon 1999; McCool and Cannon 2001; Satoh *et al.* 2002).

5.1.1. Molecular biology of PHA synthesizing genes

Till 1980's research was limited to the identification of microorganisms synthesizing PHAs, characterization of the PHAs and their copolymers. With advancements in the molecular biology tools, characterization of the genes involved in PHA biosynthesis gained momentum. Though, the number of microorganisms shown to accumulate PHAs is increasing every year, the molecular data regarding the PHA biosynthesis genes remains rather limited.

Different strategies have been employed to identify PHA biosynthesis genes (Table 5.1). Of these only a few allow successful identification of the PHA biosynthesis genes encoding enzymes with novel and / or unusual features (Rehm and Steinbüchel 2001).

Timm *et al.* (1994) found that the class I *pha*C gene of *W. eutropha* was not useful as a general hybridization probe to identify PHA synthase genes. Based on conserved regions of the PHA synthases of *W. eutropha* and *Pseudomonas oleovorans*, these authors designed 30-mer oligonucleotides for use as a universal

probe for *pha*C, these researchers detected and subsequently cloned the class II *pha* gene loci of *Pseudomonas citronellolis, Pseudomonas* sp. DSM 1650, *Pseudomonas mendocina,* and both the class I and class II *pha* loci of *Pseudomonas* sp. GP4BH1. The probe was not tested on classes III and IV *pha* genes and most likely would not detect these genes by virtue of the basis of their design.

Table 5.1 Strategies for screening and identification of PHA biosynthesis genes (Source: Rehm and Steinbüchel 2001)

Strategy	Screening technology
A	Enzymatic analysis
B	Homologous gene probes obtained by transposon insertions
C	Heterologous gene probes from well characterized genes
D	Consensus oligonucleotides derived from multiple alignments
E	Oligo nucleotides derived from N-terminal or internal amino acid sequences of PHA synthases
F	Opaque and fluorescent colonies in PHA negative host after heterologous expression
G	Growth after detoxification of media due to removal of fatty acids
H	Genome sequence analysis and functional assignments of PHA biosynthesis genes

Polymerase chain reaction (PCR) provides a means for rapid detection of specific genes in organisms. Lopez *et al.* (1997) reported the use of PCR to identify *pha*C positive organisms in river water environmental samples using nondegenerate primers derived from *W. eutropha.* However, they obtained an essentially nonspecific PCR product mixture which necessitated further confirmation of the *pha*C gene by Southern blot hybridization. Solaiman *et al.* (2000) subsequently developed the first PCR procedure that specifically detects class II *pha* genes, wherein the primers were designed on basis of the highly conserved nucleic acid sequences of the class II *pha* loci of *Pseudomonas*. Zhang *et al.* (2001) reported PCR cloning of the class II *pha* genes on basis of degenerate primers derived from the consensus sequences of ORF1,

*pha*Z and *pha*D genes of *Pseudomonas*. Sheu *et al.* (2000) designed a broad specificity PCR procedure using degenerate semi-nested primer pairs and isolated 38 PHA-positive strains by colony PCR and semi-nested PCR. PCR detection of the *pha* genes in *Bacillus* was reported by Shamala *et al.* (2003) using non-degenerate primer pairs designed solely on the basis of the class IV *pha*C of *Bacillus megaterium* by employing a primer-design program. However, because of the simplistic approach by which these primers were designed, a global detection of class IV *pha* genes could not be expected (Solaiman and Ashby 2005). This scenario necessitated homologous probes to screen the PHA gene locus from the *Bacillus thuringiensis* R1 genomic library.

The scope of the present chapter was to obtain a homologous probe to locate the *pha*C gene and subsequently use it to isolate the *pha* gene locus. In the present study different primers were used to amplify the *pha*C gene from *Bacillus thuringiensis* R1. The putative *pha*C gene was sequenced and upon sequence conformity it was used as a probe to screen the subgenomic library of *Bacillus thuringiensis* R1 for the *pha* operon.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Organisms and growth

Bacillus thuringiensis R1 and *E. coli* were grown and maintained as detailed in Chapter 2.

5.2.2 DNA extraction and manipulation

Genomic DNA, plasmid DNA extraction, Southern hybridization were done as described in Chapter 2.

5.2.3 Polymerase Chain Reaction (PCR)

Two sets of primers were used to amplify *pha*C gene from *B. thuringiensis* RI. The first set of primers (all 26-mers) were based on the multiple sequence alignment results of 19 *pha*C genes (Sheu *et al.* 2000). The primer set I sequences are as below:

Forward primer I *pha*C F1 (26 mer):

5'(d)ATCAACAA(GGG/A) T(TT/A)CTAC(AA/G)TC(CC/T)T(CC/G)GACCT3'

Forward primer II *pha*C F2 (26 mer):

5' (d)GT(CCC/GG)TTC(GGG/AA)T (GGG/CC) (AAA/GG) T(CC/G)(TT/A) (CCC/GG)CTGGCGCAACCC 3'

Reverse primer *pha*C R4 (26 mer):

5'(d)AGGTAGTTGT(TT/C)GAC(CCC/GG)(AAA/CC)(AAA/CC)(GGG/A) TAG(TTT/G)TCCA3'

Two reactions were set up using the two different forward primers.

The second set of primers used earlier to screen the sub-genomic library of *Bacillus* sp. INT005 (Satoh *et al.* 2002) for *pha*C gene was a kind gift from Dr. Yasuharu Satoh (Division of Molecular Chemistry, Graduate school of Engineering, Hokkaido university, Sapporo 060-8628, Japan).

The primer set II sequences are as below:

Forward primer P5 (30 mer):

5' (d)AAGGATCCACTACATTCGCAACAGAATGGG 3'

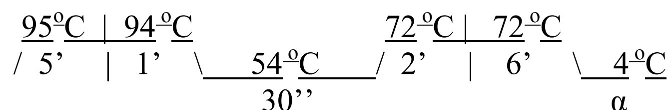
Reverse primer P6 (30 mer):

5' (d)AACTGCAGTTACTTAGAGCGCTCGTCAAGC 3'

The PCR reaction mixture consisted of:

SMQ	:	5.2 µL
Templete DNA	:	3.0 µL (10 ng)
DNTPs	:	2.0 µL (266 µM)
10X Taq Pol. Buffer	:	1.5 µL
15 mM $MgCl_2$	:	1.0 µL
Forward primer	:	1.0 µL (10 picomoles)
Reverse primer	:	1.0 µL (10 picomoles)
Taq Polymerase	:	0.3 µL (1U)
Total volume	:	15.0 µL

The PCR reaction was set up as below.



5.2.4 Cloning the PCR product

The PCR amplicons were gel purified (Section 2.3.3) and cloned into pGEMT-Easy Vector (Promega, USA).

5.2.5 Restriction mapping

Plasmid DNA from recombinant *E. coli* was digested with different restriction enzymes. Single and double digestions were done as recommended by the manufacturer to generate the restriction enzyme map of the insert DNA.

5.2.6 DNA Sequencing and sequence analysis

Beckman Coulter CEQ™ 8000 Genetic Analysis System was used to perform DNA sequencing reactions using CEQ™ DTCS Quick Start Kit Dye Terminator Cycle sequencing kit provided by the manufacturer. Sequencing was bi-directional and accomplished by primer walking. Database searches and sequence analysis were performed using BLAST (National Center for Biotechnology Information, National Institute of Health, Bethesda, MD, USA).

5.2.7 Southern hybridization

The genomic DNA from *Bacillus thuringiensis* R1 was restriction digested with *Sac* I, *Cla* I, *BamH* I, *Xba* I, *Hind* III, *EcoR* I, and their combinations. Southern blotting, random primer labeling and Southern hybridization were done as described in sections 2.6, 2.7 and 2.8 respectively.

5.2.8 Sub-genomic library construction

Sub-genomic library construction and transformation were done as described in Section 2.4 and 2.5 of Chapter 2. *Bacillus thuringiensis* R1 genomic DNA (100 µg) was digested to completion with the restriction enzymes *Sac* I/*Cla* I (double digestion). The restriction fragments were size separated by agarose gel electrophoresis. The fragments in the size range of 4-6 kb were purified by gel extraction and cloned into the *Sac* I/*Cla* I digested pBluescript KS+ plasmid vector.

5.2.9 Screening for PHA synthesizing genes

Recombinants from the sub-genomic library were screened by amplifying the partial *pha*C gene sequence with primer set II (P5 forward and P6 reverse). Colony hybridization was done as described in section 2.9 of Chapter 2. The recombinant clones were streaked on basal media agar plates supplemented with glycerol (1% v/v) and Nile red (0.5 µg/mL media) (Spiekermann *et. al* 1999). In addition to the above tests the PHB synthesis was checked by extraction of the polymer from recombinant *E. coli* as described in Section 3.2.5.

5.3 RESULTS AND DISCUSSION

5.3.1 PCR amplification of the putative PHA synthase gene

Using the PCR set I primers *pha*C F1 and *pha*C R4 no amplification was observed, whereas an amplicon of ~600 bp was observed when the combination of *pha*C F2 and *pha*C R4 primers were used (Fig. 5.1). The amplicon was cloned into pGEMT vector (Promega, USA), designated as pRDC1 and the insert sequenced.

The sequence of the pRDC1 insert and its homology results are presented in Figures 5.2, 5.3a and 5.3b respectively. A search with the BLAST program with the deduced amino acid sequence of the 657 bp insert from pRDC1 revealed 100% amino acid sequence similarity, and 99% nucleotide sequence similarity with the DNA-directed RNA polymerase, beta' subunit of *Bacillus* sps.

Fig. 5.1 PCR amplification of *Bacillus thuringiensis* R1 genomic DNA. Lanes: 1 &2 - *pha*C F2 & *pha*C R4 amplified PCR fragment; 3 & 4 - *pha*C F1 & *pha*C R4 amplification; 5 - λ *Eco* RI /*Hind* III marker.

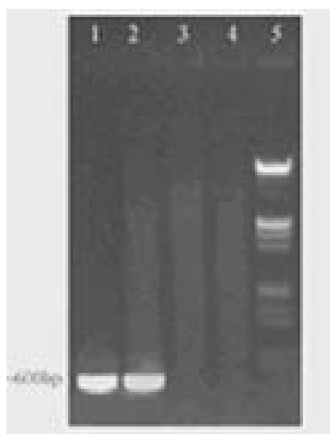


Fig. 5.2 Nucleotide sequence of pRDC1 insert

```
  1 CGATTTCATT GCTGGCGCAA CCCCACCTGT ATGGAACGT CGCATCGTTA
 51 ACTGTGTACC TGGCTCACCG ATAGATTGAG CTGCGATAAT ACCTACCGCT
101 TCCCCTACTT CTACGTCTGT TCCAGTTGCT AAGTTACGAC CGTAACACTT
151 CTTACATACA CCATGGCGAG TGTTACACGT AAACGCTGAA CGAATGTTTA
201 CAGTTTCAAC ACCCGAGTTT TCAACAATAT GAGCGATATC TTCAGTAATT
251 AATTGATTTT CAGCAACTAA TACTTCACCT GTTTCAGGAT GTTTTACAGT
301 TTTTCTTGCA AAACGTCCAA CAAGACGATC ATATAATGAC TCAATAACTT
351 CATTACCCTC TTTAATCGCA CCAATTAATA AACCGCGATC TGTTCCACAA
401 TCATCTTCAC GAACGATTAC ATCTTGTGCA ACGTCAACAA GACGACGTGT
451 TAAGTAACCA GAATCGGCAG TTTTCAGTGC TGTATCGGCA AGACCTTTAC
501 GCGCACCATG CGTAGAGATG AAGTATTCAA GTACTGTTAA ACCTTCACGG
551 AAACTTGATT TGATTGGAAG TTCGATGATA CGACCAGATG GATTGGCCAT
601 CAGACCACGC ATACCAGCAA GCTGAGTAAA GTTCGATGCG TTACCACGGG
651 CACCGGA
```

Fig. 5.3a Nucleic acid sequence similarity of pRDC1 with partial sequence of DNA-directed RNA polymerase, beta' subunit from *Bacillus sps.* (represented as Bac).

```
pRDC1  17   GCAACCCCACCTGTATGGAACGT-CGCATCGTTAACTGTGTACCTGGCTCACCGATAGAT
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Bac 114795 GCAACCCCACCTGTATGGAACGTACGCATCGTTAACTGTGTACCTGGCTCACCGATAGAT

pRDC1 76    TGAGCTGCGATAATACCTACCGCTTCCCCTACTTCTACGTCTGTTCCAGTTGCTAAGTTA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114735 TGAGCTGCGATAATACCTACCGCTTCCCCTACTTCTACGTCTGTTCCAGTTGCTAAGTTA

pRDC1 136    CGACCGTAACACTTCTTACATACACCATGGCGAGTGTTACACGTAAACGCTGAACGAATG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114675  CGACCGTAACACTTCTTACATACACCATGGCGAGTGTTACACGTAAACGCTGAACGAATG

pRDC1  196 TTTACAGTTTCAACACCCGAGTTTTCAACAATATGAGCGATATCTTCAGTAATTAATTGA
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114615 TTTACAGTTTCAACACCCGAGTTTTCAACAATATGAGCGATATCTTCAGTAATTAATTGA

pRDC1  256 TTTTCAGCAACTAATACTTCACCTGTTTCAGGATGTTTTACAGTTTTTCTTGCAAAACGT
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114555 TTTTCAGCAACTAATACTTCACCTGTTTCAGGATGTTTTACAGTTTTTCTTGCAAAACGT

pRDC1  316 CCAACAAGACGATCATATAATGACTCAATAACTTCATTACCCTCTTTAATCGCACCAATT
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114495 CCAACAAGACGATCATATAATGACTCAATAACTTCATTACCCTCTTTAATCGCACCAATT

pRDC1  376 AATAAACCGCGATCTGTTCCACAATCATCTTCACGAACGATTACATCTTGTGCAACGTCA
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114435 AATAAACCGCGATCTGTTCCACAATCATCTTCACGAACGATTACATCTTGTGCAACGTCA

pRDC1  436 ACAAGACGACGTGTTAAGTAACCAGAATCGGCAGTTTTCAGTGCTGTATCGGCAAGACCT
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114375 ACAAGACGACGTGTTAAGTAACCAGAATCGGCAGTTTTCAGTGCTGTATCGGCAAGACCT
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pRDC1  496 TTACGCGCACCATGCGTAGAGATGAAGTATTCAAGTACTGTTAAACCTTCACGGAAACTT
           ||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Bac 114315 TTACGCGCACCATGCGTAGAGATGAAGTACTCAAGTACTGTTAAACCTTCACGGAAACTT

pRDC1  556 GATTTGATTGGAAGTTCGATGATACGACCAGATGGATTGGCCATCAGACCACGCATACCA
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114255 GATTTGATTGGAAGTTCGATGATACGACCAGATGGATTGGCCATCAGACCACGCATACCA

pRDC1  616 GCAAGCTGAGTAAAGTTCGATGCGTTACCACGGGCACCGGA  656
           |||||||||||||||||||||||||||||||||||||||||
Bac 114195 GCAAGCTGAGTAAAGTTCGATGCGTTACCACGGGCACCGGA  114155
```

Fig. 5.3b Amino acid sequence similarity of pRDC1 with partial sequence of DNA-directed RNA polymerase, beta' subunit from *Bacillus sps.* (represented as Bac).

```
pRDC1 657 SGARGNASNFTQLAGMRGLMANPSGRIIELPIKSSFREGLTVLEYFISTHGARKGLADTA
Bac   732 SGARGNASNFTQLAGMRGLMANPSGRIIELPIKSSFREGLTVLEYFISTHGARKGLADTA

pRDC1 477 LKTADSGYLTRRLVDVAQDVIVREDDCGTDRGLLIGAIKEGNEVIESLYDRLVGRFARKT
Bac   792 LKTADSGYLTRRLVDVAQDVIVREDDCGTDRGLLIGAIKEGNEVIESLYDRLVGRFARKT

pRDC1 297 VKHPETGEVLVAENQLITEDIAHIVENSGVETVNIRSAFTCNTRHGVCKKCYGRNLATGT
Bac   852 VKHPETGEVLVAENQLITEDIAHIVENSGVETVNIRSAFTCNTRHGVCKKCYGRNLATGT

pRDC1 117 DVEVGEAVGIIAAQSIGEPGTQLTMRTFHTGGVA 16
Bac   912 DVEVGEAVGIIAAQSIGEPGTQLTMRTFHTGGVA 945
```

Sheu *et al.* (2000) designed primers *pha*C F1, *pha*C F2 & *pha*C R4 based on the multiple sequence alignment of 13 *pha*C genes from 13 Gram-negative bacteria. They used these primers to screen 38 PHA producing environmental isolates (30 Gram-positive, 5 Gram-negative and 3 Gram variable strains) and reported that the individual PCR products are suitable for use as specific probes for genomic library screening. Interestingly, one of the bacterial test samples used by these authors was *Bacillus megaterium*, however, the amplicon obtained was not sequenced to confirm its classification (Solaiman and Ashby 2005). The present study proves that *pha*C F1, *pha*C F2 and *pha*C R4 primers cannot be used to amplify the class IV *pha*C gene, since the nucleic acid sequence is not homologous to those of class I and class II genes (Solaiman and Ashby 2005). Hence, the primers designed by Sheu *et al.* (2000) cannot be used as universal degenerate primers to amplify the *pha*C gene from *Bacillus* sp. Shamala *et al.* (2003) designed non-degenerate primers based on sequence analysis of class IV synthases from *Bacillus* sps., however, their standard species (*B. circulans* and *B. brevis*) did not produce the expected amplicons (Solaiman and Ashby 2005).

With the primer set II an ~1 Kb DNA fragment was amplified (Fig. 5.4). The amplicon was cloned into pGEMT vector and the clone designated as pRDC2. The clone pRDC2 was digested with different restriction enzymes (Fig. 5.5a & Fig. 5.5b) and a restriction map generated for the insert DNA (Fig. 5.6). The nucleotide sequence of pRDC2 is presented in Fig. 5.7. The homology search of the 1083 nucleotide sequence showed 99% homology to the nucleotide and amino acid sequences with poly(R)-hydroxyalkanoic acid synthase (*pha*C) gene of different *Bacillus sps.* (Fig. 5.8a and 5.8b). However, the sequence showed only 82% similarity to the nucleotide sequence of *Bacillus megaterium*. The putative lipase box **(G-X-C-X-G-G)** of the PHA synthase is represented in the figure. Because of high sequence similarity with other *pha*C gene sequences from *Bacillus* sps., the insert from pRDC2 (putative *pha*C gene) could be used as probe to locate the *pha* operon and screen the *Bacillus thuringiensis* R1 genomic library.

Fig 5.4 PCR amplification of with set II *Bacillus thuringiensis* R1 genomic DNA. Lane1: λ/*Hind* III marker, Lanes 2 & 3: P5P6 amplified PCR fragment.

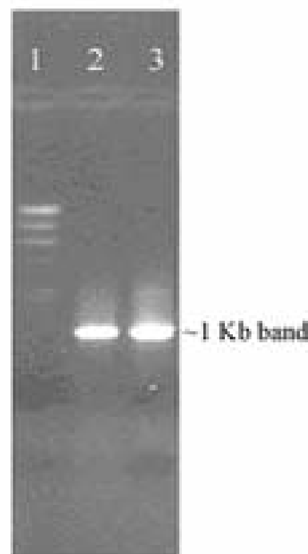
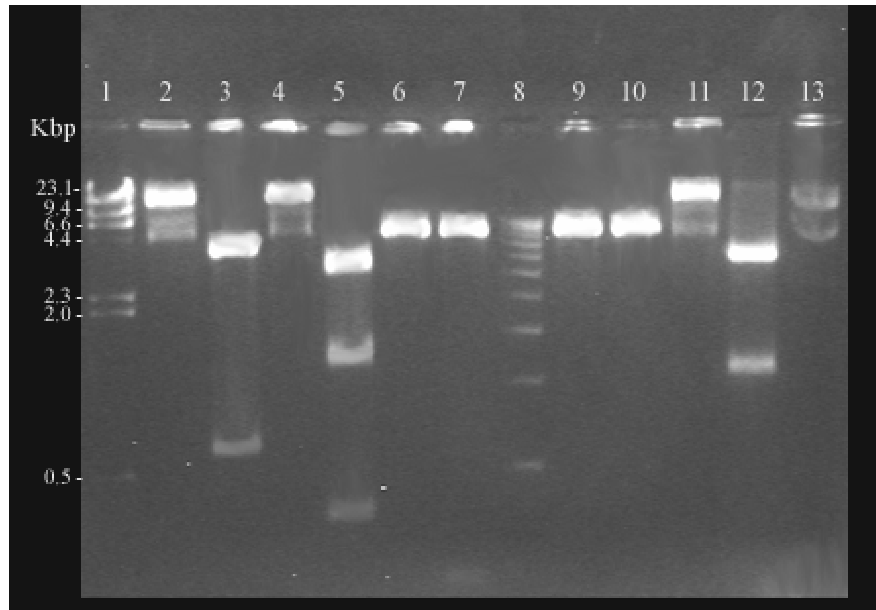
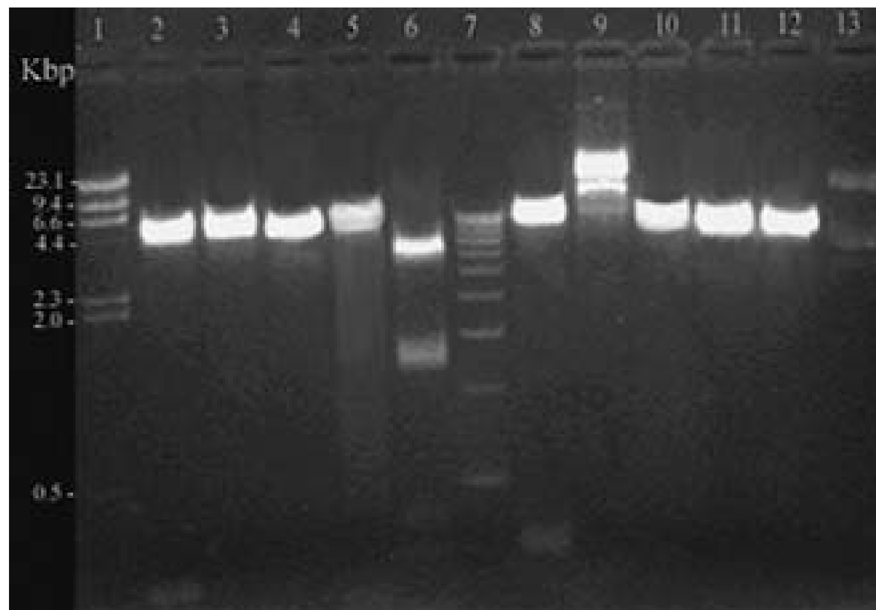


Fig. 5.5a Agarose gel electrophoresis of restriction digested pRDC2 plasmid



Lanes: 1. : λ/Hind III marker; 2: pRDC2/*Cla* I; 3: pRDC2/*Hinc* II; 4: pRDC2/*Sac* I; 5: pRDC2/*Pvu* II; 6: pRDC2/*Nco* I; 7: pRDC2/*Nde* I; 8: 500 bp ladder; 9: pRDC2/*BamH* I; 10: pRDC2/*Hind* III; 11: pRDC2/*EcoR* V; 12: pRDC2/*EcoR* I; 13: uncut pRDC2

Fig. 5.5b Agarose gel electrophoresis of restriction digested pRDC2 plasmid



Lanes: 1. : λ/Hind III marker; 2: pRDC2/*Nco* I/*Hind* III; 3: pRDC2/*Nco* I/*BamH* I; 4: pRDC2/Nco I/*Sma* I; 5: pRDC2/*Ava* I/*Nde* I; 6: pRDC2/*Pvu* II/*Nde* I; 7: 500 bp ladder; 8: pRDC2/*Acc* I; 9: pRDC2/*Ava* I; 10: pRDC2/*Apa* I; 11: pRDC2/*Acc* I; 12: pRDC2/*Pst* I; 13: uncut pRDC2

Fig. 5.6 Restriction map of pRDC2 insert DNA (not to scale)

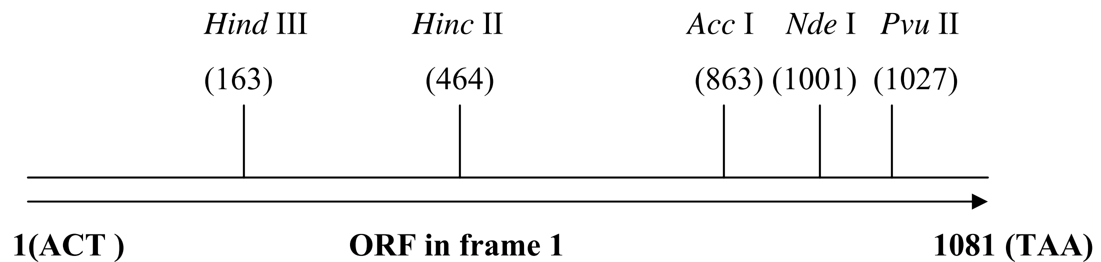


Fig. 5.7 Nucleotide sequence of pRDC2 insert DNA.

```
             P5 forward
   ------------------------------->
  1 ACTACATTCGCAACAGAATGGGAAAAGCAATTAGAGCTATACCCAGAAGA

 51 GTATCGAAAAGCATACCGCCGAGTGAAAAGGGCGAGTGAAATTTTATTAC

101 GTGAACCAGAGCCGCAAGTCGGATTAACGCCGAAAGAGGTTATTTGGACG

151 AAGAATAAGACGAAGCTTTATCGCTACATTCCAAAACAAGAAAAAACACA
                Hind III
201 AAGAGTTCCAATTCTATTAATATATGCTCTTATTAATAAACCATATATTA

251 TGGACTTAACTCCTGGAAATAGTTTAGTGGAATATCTAGTGGACCGTGGT

301 TTTGATGTGTATATGCTTGATTGGGGCACATTTGGTTTAGAAGATAGTCA

351 TTTGAAATTTGATGATTTCGTGTTTGATTATATTGCAAAAGCAGTGAAAA

401 AAGTAATGCGAACTGCAAAATCGGACGAGATTTCTTTACTTGGTTATTGC

451 ATGGGTGGAACGTTAACTTCTATTTATGCGGCACTTCATCCGCACATGCC
               Hinc II
501 AATTCGCAACTTAATCTTTATGACAAGTCCTTTTGATTTCTCTGAAACAG

551 GATTATATGGTCCTTTACTAGATGAAAAATATTTTAACTTAGATAAAGCA

601 GTTGATACATTTGGCAATATTCCGCCAGAAATGATTGATTTCGGAAACAA
```

```
651  AATGTTAAAACCAATTACAAACTTTGTGGGACCATACGTTGCTTTAGTAG

701  ATCGTTCAGAGAATGAGCGTTTCGTTGAAAGCTGGAGGTTAGTTCAAAAG

751  TGGGTTGGCGATGGCATTCCGTTCCCAGGTGAATCATACAGACAGTGGAT

801  TCGTGATTTTTATCAAAATAATAAACTCGTTAAGGGTGAACTCGTTATTC

851  GCGGACAAAAGGTAGACCCTGGAAATATTAAGGCGAATGTCTTAAATATT
                                      Acc I

901  TCCGGGAAACGTGATCATATCGCTCTGCCATGCCAAGTAGAAGCATTGCT

951  AGATCATATTTCTAGCACAGATAAACAATATGTATGTTTACCAACAGGGC

1001 ATATGTCTATCGTTTACGGCGGAACAGCTGTAAAACAAACGTATCCGACG
     Nde I                     Pvu II
1051 ATTGGAGATTGGCTTGACGAGCGTTCAAAGTAA 1083

                 <-------------------------
                      P6 reverse
```

Fig. 5.8a Nucleic acid sequence similarity of pRDC2 insert DNA with the *pha*C gene from *Bacillus sps.* (represented as Bac).

```
pRDC2 1     ACTACATTCGCAACAGAATGGGAAAAGCAATTAGAGCTATACCCAGAAGAGTATCGAAAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 113451  ACTACATTCGCAACAGAATGGGAAAAGCAATTAGAGCTATACCCAGAAGAGTATCGAAAA

pRDC2 61    GCATACCGCCGAGTGAAAAGGGCGAGTGAAATTTTATTACGTGAACCAGAGCCGCAAGTC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 113511  GCATACCGCCGAGTGAAAAGGGCGAGTGAAATTTTATTACGTGAACCAGAGCCGCAAGTC

pRDC2 121   GGATTAACGCCGAAAGAGGTTATTTGGACGAAGAATAAGACGAAGCTTTATCGCTACATT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 113571  GGATTAACGCCGAAAGAGGTTATTTGGACGAAGAATAAGACGAAGCTTTATCGCTACATT

pRDC2 181   CCAAAACAAGAAAAAACACAAAGAGTTCCAATTCTATTAATATATGCTCTTATTAATAAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 113631  CCAAAACAAGAAAAAACACAAAGAGTTCCAATTCTATTAATATATGCTCTTATTAATAAA

pRDC2 241   CCATATATTATGGACTTAACTCCTGGAAATAGTTTAGTGGAATATCTAGTGGACCGTGGT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 113691  CCATATATTATGGACTTAACTCCTGGAAATAGTTTAGTGGAATATCTAGTGGACCGTGGT

pRDC2 301   TTTGATGTGTATATGCTTGATTGGGGCACATTTGGTTTAGAAGATAGTCATTTGAAATTT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 113751  TTTGATGTGTATATGCTTGATTGGGGCACATTTGGTTTAGAAGATAGTCATTTGAAATTT

pRDC2 361   GATGATTTCGTGTTTGATTATATTGCAAAAGCAGTGAAAAAAGTAATGCGAACTGCAAAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 113811  GATGATTTCGTGTTTGATTATATTGCAAAAGCAGTGAAAAAAGTAATGCGAACTGCAAAA

pRDC2 421   TCGGACGAGATTTCTTTACTTGGTTATTGCATGGGTGGAACGTTAACTTCTATTTATGCG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 113871  TCGGACGAGATTTCTTTACTTGGTTATTGCATGGGTGGAACGTTAACTTCTATTTATGCG

pRDC2 481   GCACTTCATCCGCACATGCCAATTCGCAACTTAATCTTTATGACAAGTCCTTTTGATTTC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 113931  GCACTTCATCCGCACATGCCAATTCGCAACTTAATCTTTATGACAAGTCCTTTTGATTTC
```

```
pRDC2 541   TCTGAAACAGGATTATATGGTCCTTTACTAGATGAAAAATATTTTAACTTAGATAAAGCA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 113991 TCTGAAACAGGATTATATGGTCCTTTACTAGATGAAAAATATTTTAACTTAGATAAAGCA

pRDC2 601   GTTGATACATTTGGCAATATTCCGCCAGAAATGATTGATTTCGGAAACAAAATGTTAAAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114051 GTTGATACATTTGGCAATATTCCGCCAGAAATGATTGATTTCGGAAACAAAATGTTAAAA

pRDC2 661   CCAATTACAAACTTTGTGGGACCATACGTTGCTTTAGTAGATCGTTCAGAGAATGAGCGT
            ||||||||||||||||| || |||||||||||||||||||||||||||||||||||||||
Bac 114111 CCAATTACAAACTTTGTTGGTCCATACGTTGCTTTAGTAGATCGTTCAGAGAATGAGCGT

pRDC2 721   TTCGTTGAAAGCTGGAGGTTAGTTCAAAAGTGGGTTGGCGATGGCATTCCGTTCCCAGGT
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Bac 114171 TTCGTTGAAAGCTGGAGGTTAGTTCAAAAGTGGGTTGGCGATGGCATTCCGTTCCCGGGT

pRDC2 781   GAATCATACAGACAGTGGATTCGTGATTTTTATCAAAATAATAAACTCGTTAAGGGTGAA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114231 GAATCATACAGACAGTGGATTCGTGATTTTTATCAAAATAATAAACTCGTTAAGGGTGAA

pRDC2 841   CTCGTTATTCGCGGACAAAAGGTAGACCCTGGAAATATTAAGGCGAATGTCTTAAATATT
            |||||||||||||||||||||||||||| || ||||||||||||||||||||||||||||
Bac 114291 CTCGTTATTCGCGGACAAAAGGTAGACCTTGCAAATATTAAGGCGAATGTCTTAAATATT

PRDC2 901   TCCGGGAAACGTGATCATATCGCTCTGCCATGCCAAGTAGAAGCATTGCTAGATCATATT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114351 TCCGGGAAACGTGATCATATCGCTCTGCCATGCCAAGTAGAAGCATTGCTAGATCATATT

pRDC2 961   TCTAGCACAGATAAACAATATGTATGTTTACCAACAGGGCATATGTCTATCGTTTACGGC
            |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||
Bac 114411 TCTAGCACAGATAAACAATATGTATGTTTACCGACAGGGCATATGTCTATCGTTTACGGC

pRDC2 1021 GGAACAGCTGTAAAACAAACGTATCCGACGATTGGAGATTGGCTTGACGAGCGTTCAAAG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bac 114471 GGAACAGCTGTAAAACAAACGTATCCGACGATTGGAGATTGGCTTGACGAGCGTTCAAAG

pRDC2  1081   TAA  1083
               |||
Bac  114531   TAA  114533
```

Fig. 5.8b Amino acid sequence similarity of pRDC2 with poly(R)-hydroxyalkanoic acid synthase from *Bacillus sps.* (represented as Bac).

```
PRDC2    1 TTFATEWEKQLELYPEEYRKAYRRVKRASEILLREPEPQVGLTPKEVIWTKNKTKLYRYI
Bac      2 TTFATEWEKQLELYPEEYRKAYRRVKRASEILLREPEPQVGLTPKEVIWTKNKTKLYRYI

PRDC2  181 PKQEKTQRVPILLIYALINKPYIMDLTPGNSLVEYLVDRGFDVYMLDWGTFGLEDSHLKF
Bac     62 PKQEKTQRVPILLIYALINKPYIMDLTPGNSLVEYLVDRGFDVYMLDWGTFGLEDSHLKF
                                 Putative lipase box
PRDC2  361 DDFVFDYIAKAVKKVMRTAKSDEISLLGYCMGGTLTSIYAALHPHMPIRNLIFMTSPFDF
Bac    122 DDFVFDYIAKAVKKVMRTAKSDEISLLGYCMGGTLTSIYAALHPHMPIRNLIFMTSPFDF

PRDC2  541 SETGLYGPLLDEKYFNLDKAVDTFGNIPPEMIDFGNKMLKPITNFVGPYVALVDRSENER
Bac    182 SETGLYGPLLDEKYFNLDKAVDTFGNIPPEMIDFGNKMLKPITNFVGPYVALVDRSENER

PRDC2  721 FVESWRLVQKWVGDGIPFPGESYRQWIRDFYQNNKLVKGELVIRGQKVDPGNIKANVLNI
Bac    242 FVESWRLVQKWVGDGIPFPGESYRQWIRDFYQNNKLVKGELVIRGQKVDLANIKANVLNI

PRDC2  901 SGKRDHIALPCQVEALLDHISSTDKQYVCLPTGHMSIVYGGTAVKQTYPTIGDWLDERSK
Bac    302 SGKRDHIALPCQVEALLDHISSTDKQYVCLPTGHMSIVYGGTAVKQTYPTIGDWLDERSK
```

5.3.2 Construction of Bacillus thuringiensis R1 sub-genomic library

Bacillus thuringiensis R1 genomic DNA was digested with restriction enzymes *Sac* I*, Cla* I*, BamH* I*, Xba* I, *Hind* III*, EcoR* I*,* and their combinations (Fig. 5.9) and hybridized with ^{32}P labelled ~1 kb insert from pRDC2. The Southern hybridization showed positive signals in all the lanes (Fig. 5.10). The probe hybridized to restriction fragments of different sizes (Table 5.2). The pRDC2 plasmid with the putative *pha*C gene served as the positive control. To construct a sub-genomic library of *Bacillus thuringiensis* R1, genomic DNA was digested with *Cla* I and *Sac* I (Fig. 5.11). Fragments ranging from 4-6 kbp were shotgun cloned into *Cla* I and *Sac* I digested pBluescript KS+.

5.3.3 Screening the sub-genomic library of Bacillus thuringiensis R1 for PHB operon

The recombinant clones were PCR screened for the *pha*C gene operon using set II P5 and P6 primers. With one of the clones an approximately 1 kb fragment (Fig. 5.12) was amplified, which upon sequencing was homologous to the pRDC2 insert. The recombinants were further screened by colony hybridization using putative *pha*C gene probe from pRDC2. The probe hybridized to one recombinant colony of the *Bacillus thuringiensis* R1 sub-genomic library (Fig. 5.13). The same colony also showed weak fluorescence when plated on Basal medium with Nile red and exposed to ultraviolet light. The positive clone was checked by growing the recombinant *E. coli* cells in Basal media and PHB extraction by cell lysis. However, the recombinant clone accumulated only minimal amount of PHB (3.0% of dry cell mass) as compared to *Bacillus thuringiensis* R1 (34.0% of dry cell mass) in basal media with glycerol as the carbon source. The PHA+ clone was designated as pSVA10. The clone harbors an ~5.0 Kb genomic DNA fragment from *Bacillus thuringiensis* R1.

5.3.4 Restriction mapping of pSVA10

Plasmid DNA from pSVA10 was digested with *Apa* I, *Kpn* I, *Hinc* II, *Nde* I, *Xho* I, *Acc* I, *Xba* I, *Hind* III, *Sac* I, *Cla* I, *EcoR* I, *Pst* I, *Sma* I, *BstB* I, *BstY* I, *Xcm* I, *Nsi* I, *Sty* I, *EcoR* V, *Sca* I, *Mlu* I, *Bgl* II. The representative photographs are shown in Figures 5.14a, b, c, d. The results of the digestions are summarized in Table 5.3.

Fig. 5.9 Agarose gel electrophoresis of restriction digested genomic DNA of *Bacillus thuringiensis* R1 (Bt)

Lanes:1. pRDC2 (plasmid uncut); 2. Bt/*Cla* I; 3. *Bt/Sac* I; 4. Bt/*Cla* I/*Sac* I; 5. λ/*Hind* III marker; 6. Bt/*BamH* I; 7. Bt/ *Xba* I; 8. Bt/*BamH* I/*Xba* I; 9. λ /*Hind* III marker; 10. Bt/*EcoR* I; 11. Bt/ *Hind* III; 12.Bt/*EcoR* I/*Hind* III; 13. Undigested Bt genomic DNA

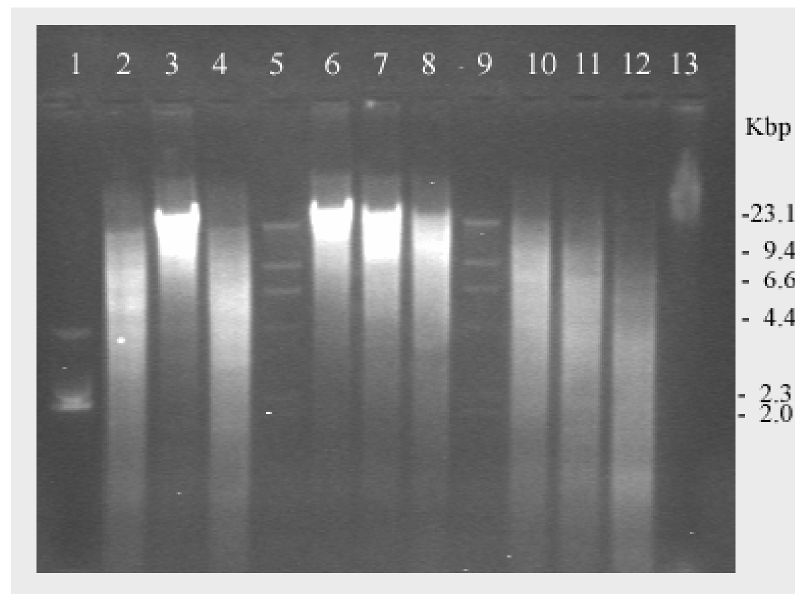


Fig. 5.10 Southern hybridization of the gel shown in Fig 5.9 with the insert from pRDC2

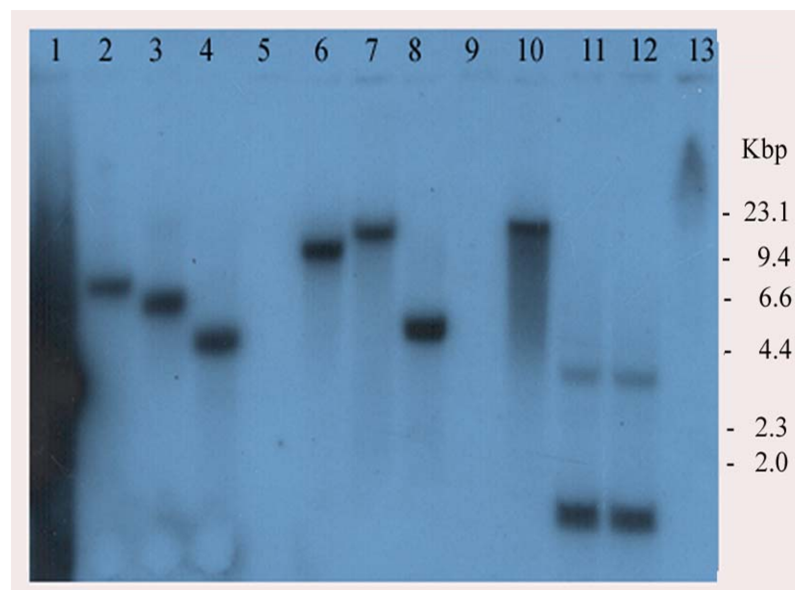


Table 5.2 Summary of Southern blot analysis of the *Bacillus thuringiensis* R1 digests using putative *pha*C gene as the probe.

Lane	Restriction enzymes used	Hybridization signals (approx. size)
2.	*Cla* I	~9.0 kb
3.	*Sac* I	~7.0 kb
4.	*Cla* I & *Sac* I	~5.0 kb
6.	*BamH* I	~10.0 kb
7.	*Xba* I	>10.0 kb
8.	*BamH* I & *Xba* I	~6.0 kb
10.	*EcoR* I	>10.0 kb
11.	*Hind* III	~ 1.4 kb
12.	*EcoR* I & *Hind* III	~ 1.4 kb

Fig. 5.11 Agarose gel electrophoresis of restriction digested genomic DNA of *Bacillus thuringiensis* R1

Lanes: 1, 2, 4 and 5 - Bt/*Cla* I/*Sac* I; 3- λ /*Hind* III marker

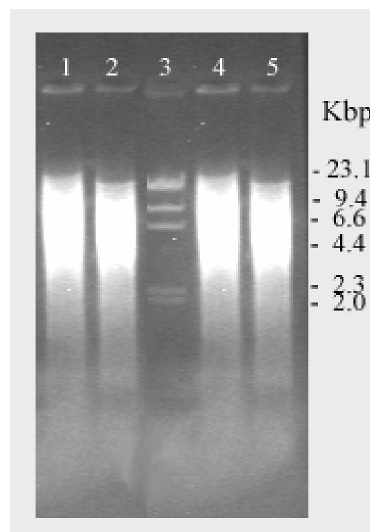
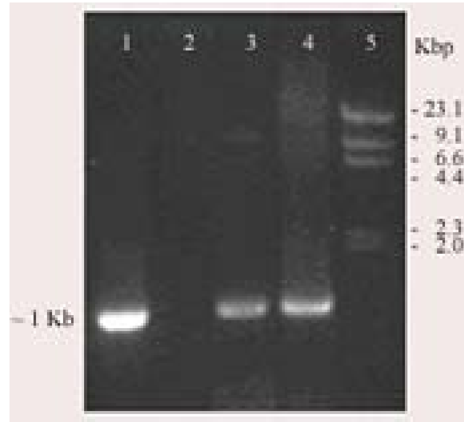


Fig. 5.12 PCR amplification using with P5 P6 set II primers



Lanes: 1: Bt; 2: pBSK+; 3: pRDC2; 4: pSVA10 as template DNA;
5: λ/*Hind* III marker

Fig. 5.13 Colony hybridization representative autoradiogram for screening of subgenomic library of *Bacillus thuringiensis* R1. The 1 kb fragment from pRDC2 was used as the probe for hybridization.

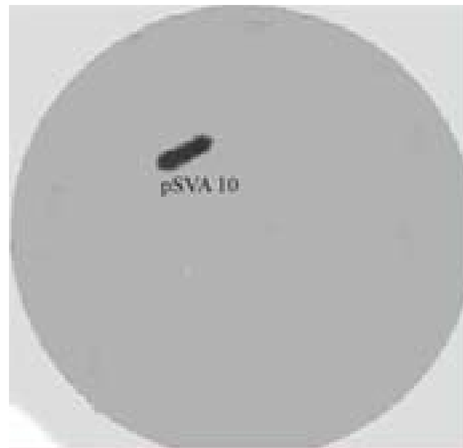
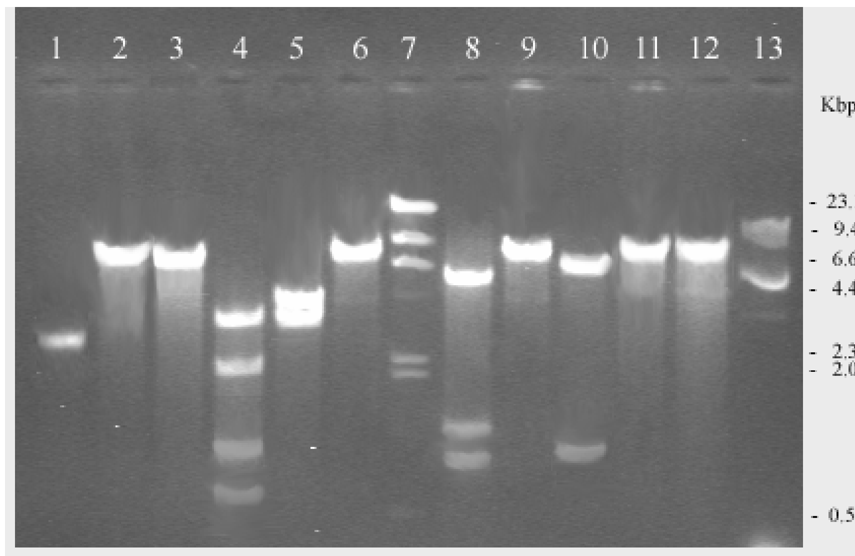
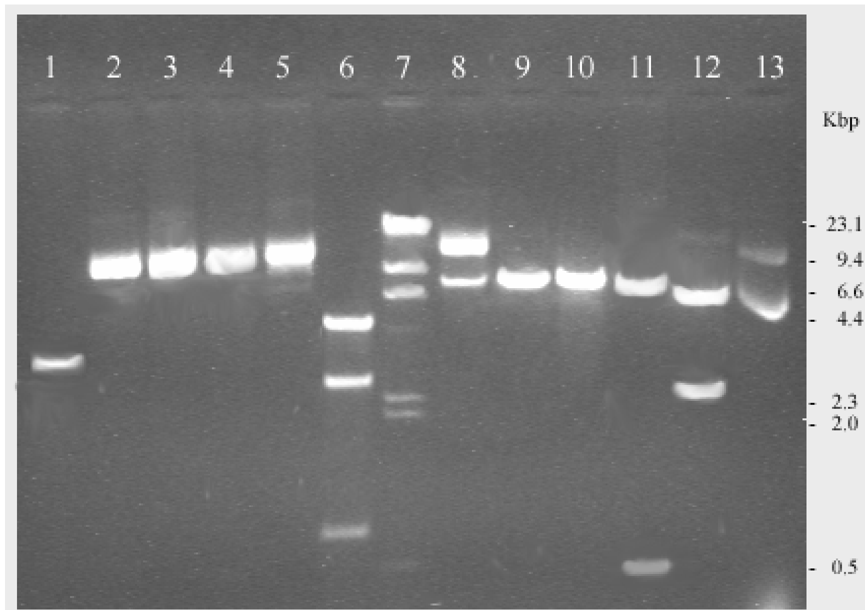


Fig. 5.14a Restriction digestions of pSVA10



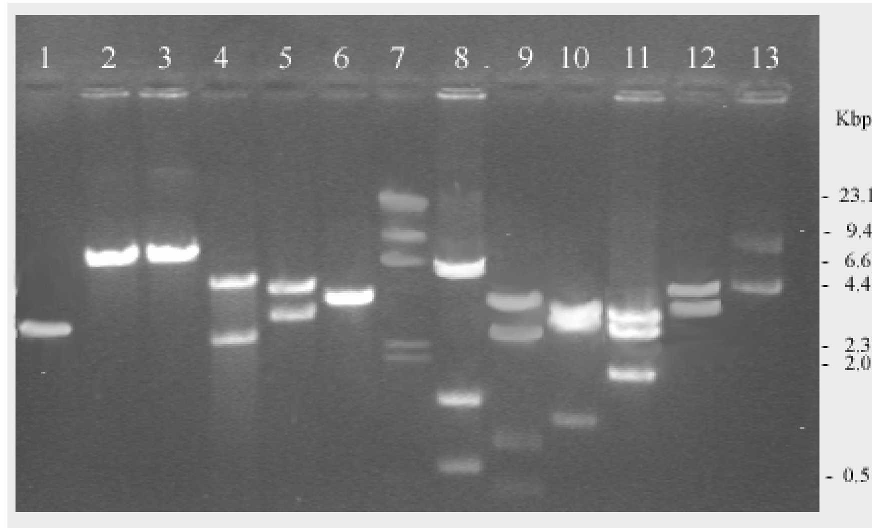
Lanes: 1: pBS linear; 2: pSVA10/*Apa* I; 3: pSVA10/*Kpn* I; 4: pSVA10/*Hinc* II; 5: pSVA10/*Nde* I; 6: pSVA10/*Xho* I; 7: λ/*Hind* III marker; 8: pSVA10/*Acc* I; 9: pSVA10/*Xba* I; 10: pSVA10/*Hind* III; 11: pSVA10/*Sac* I; 12: pSVA10/*Cla* I; 13: uncut pSVA10.

Fig. 5.14b Restriction digestions of pSVA10



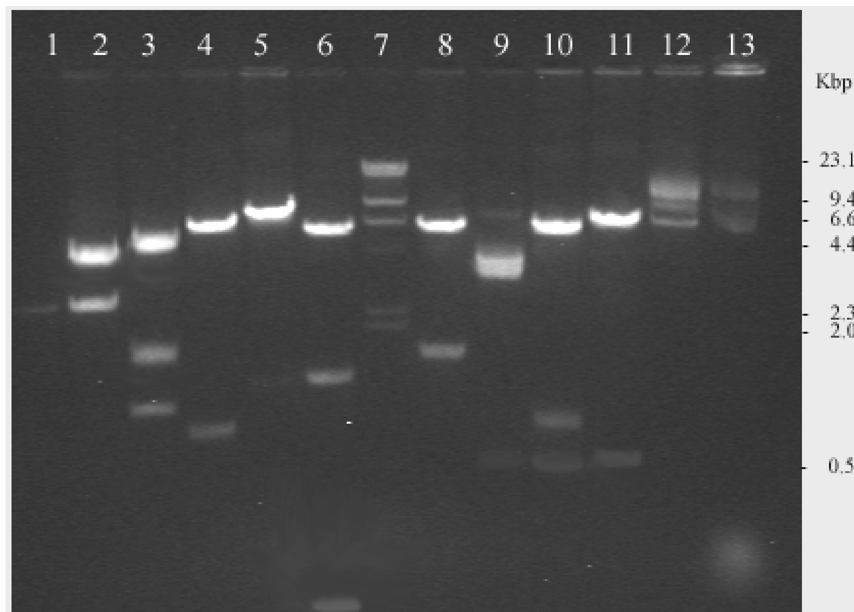
Lanes: 1: pBS linear; 2: pSVA10/*EcoR* I; 3: pSVA10/*Pst* I; 4: pSVA10/*Sma* I; 5: pSVA10/*BstB* I; 6: pSVA10/*BstY* I; 7: λ/*Hind* III marker; 8: pSVA10/*Xcm* I; 9: pSVA10/*Nsi* I; 10: pSVA10/*Sty* I; 11: pSVA10/*EcoR* V; 12: pSVA10/*Sca* I; 13: uncut pSVA10.

Fig. 5.14c Restriction digestions of pSVA10



Lanes: 1: pBS linear; 2: pSVA10/*Mlu* I; 3: pSVA10/*Bgl* II; 4: pSVA10/*Cla* I/*Mlu* I; 5: pSVA10/*Cla* I/*Bgl* II; 6: pSVA10/*Cla* I/*Sty* I; 7: λ/*Hind* III marker; 8: pSVA10/*Xho* I/*Hind* III; 9: pSVA10/*Hind* III/*Nde* I; 10: pSVA10/*Xho* I/*Nde* I; 11: pSVA10/*Xho* I/*Sca* I; 12: pSVA10/*Xho* I/*Xba* I; 13: uncut pSVA10.

Fig. 5.14d Restriction digestions of pSVA10

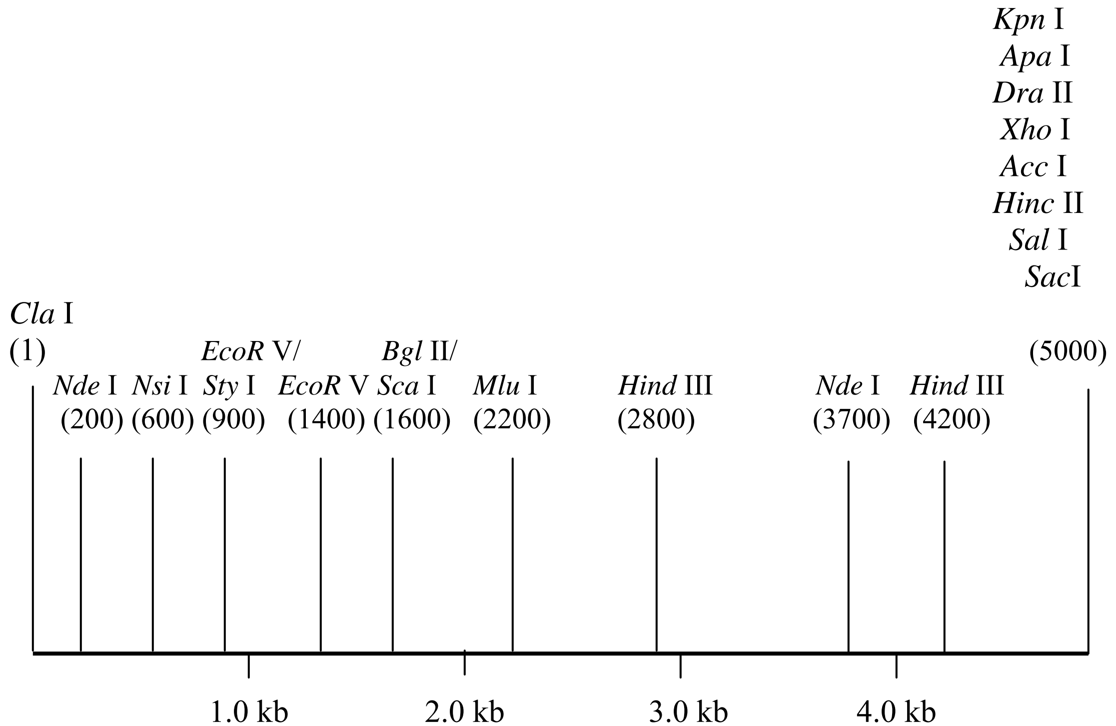


Lanes: 1. pBS linear; 2: pSVA10/*Cla* I/*Sac* I; 3: pSVA10/*Sca* I/*Sac* I; 4: pSVA10/*Sac* I/*Sty* I; 5: pSVA10/*Sac* I/*Xcm* I; 6: pSVA10/*Sac* I/*Xba* I; 7: λ/*Hind* III marker; 8: pSVA10/*Sac* I/*Bgl* II; 9: pSVA10/*Cla* I/*EcoR* V; 10: pSVA10/*Sac* I/*EcoR* V; 11: pSVA10/*Sac* I/*Nsi* I; 12: pSVA10/*Xcm* I; 13: uncut pSVA10

Table 5.3 Summary of restriction digestions of pSVA10

S.No.	Restriction enzymes	Approx. fragment sizes in basepairs
1.	*Apa* I	8000
2.	*Kpn* I	8000
3.	*Hinc* II	3500 2200 1500 800
4.	*Nde* I	4500 3500
5.	*Xho* I	8000
6.	*Acc* I	5000 1800 1200
7.	*Xba* I	8000
8.	*Hind* III	6600 1400
9.	*Sac* I	8000
10.	*Cla* I	8000
11.	*BstY* I	4500 2700 900
12.	*Nsi* I	8000
13.	*Sty* I	8000
14.	*EcoR* V	7500 500
15.	*Sca* I	5400 2600
16.	*Mlu* I	8000
17.	*Bgl* II	8000
18.	*Cla* I + *Sac* I	5000 3000
19.	*Cla* I + *Mlu* I	5200 2800
20.	*Cla* I + *Bgl* II	4500 3500
21.	*Cla* I + *Sty* I	4000 4000
22.	*Xho* I + *Hind* III	5800 1400 800
23.	*Hind* III + *Nde* I	4000 2600 900 500
24.	*Xho* I + *Nde* I	3500 3200 1300
25.	*Xho* I + *Sca* I	3400 2600 2 000
26.	*Xho* I + *Xba* I	4300 3700
27.	*Sac* I + *Sca* I	5400 1600 1000
28.	*Sac* I + *Sty* I	7100 900
29.	*Sac* I + *Xba* I	6550 1300 150
30.	*Sac* I + *Bgl* II	6400 1600
31.	*Cla* I + *EcoR* V	3900 3600 500
32.	*Sac* I + *EcoR* V	6600 900 500
33.	*Sac* I + *Nsi* I	7400 600

Fig. 5.15 Restriction map of pSVA10 (not to scale)



Among the restriction enzymes used *Apa* I, *Kpn* I, *Xho* I, *EcoR* I, *Pst* I, *Sma* I and *BstB* I do not cut the insert. The enzymes *Sca* I, *Mlu* I, *Bgl* II, *Sty* I, *Nsi* I cut the insert only once while *EcoR* V and *Hind* III have cut the insert twice. The predicted restriction map is presented in Figure 5.15.

5.3.5 Sequence of ~5.0 kb insert from pSVA10

The complete sequence of the insert in the pSVA10 was found to be 4787 bp (Fig. 5.16). This was deposited with NCBI GenBank and is available under the accession number **DQ000291**.

Fig. 5.16 4,787 bp sequence of the cloned *Bacillus thuringiensis* R1 g-DNA fragment (GenBank Acc. No. DQ000291)

```
  1 GAGCTCCCAC ATTTTTCTTA CTTGATGGAA GTAAATAGAT TTTTTGAGTT
 51 TTCCTCGAAC TTTTTTGCGA GTTCCAACTG CTTTAACTTG AACTCTTCCA
101 AAAAACCTTC TAACTGTTTT TGAGCCTCTT CACGTTGTAA TTGTTGTTGT
```

```
 151 TCAATAAATT GTTTTGTTGT TTCTTCAAAT TGGCCGCTTG TTTGAGTAAG
 201 AATAGACAAA GATGTTTTCG TAGGAGAAAC AGTAAGCTGC TGCATATGAG
 251 CAGAAAGTTC CTTCCATTTC TCTTGCCACT CATTAATTTG ATCATTTAAG
 301 GAGTTTCCAG TTAATTGCTT CACGTAATCT GTATATTGAT TATTGAACTG
 351 AGCAGTGAAT TGTTGCAGTT CTTTTTCTAG TTCATCTACT CCTGATGTTA
 401 ATTTATGCAA AGCATCTTGT TGTTGTTTTA ATGTTTCTAA AGTAAGTTGC
 451 TCTAATTGTT TCCCAGCTGA AGAGAAAAGG GAAAGTGATT GAGACCAGTT
 501 TTTCCAAAAT GCATCGACTA ATTCGTATGG TTTAGTTTCC ATTGTATGAC
 551 CCCCAAATGT TTTTTTACAT ATTGTGAACG CAGATTACTC TGCTGTATCA
 601 CCAGGTGAAG ATGAAGAATC TTTTTCATCC TTTTCTGGAG AAGTAGAAAA
 651 TGGAAAGAAT GCATTGGTGT ATAACGTGAA AAACGTCCGC AACATATTTT
 701 GATAATGTTC AAAAGAAGTC GCACATGTTG TTAAATATTG CTCTCCATAT
 751 TCAGTTAAAG AATAAATTCT TTTGGCTGGC CCTCCGTCGC TTGTATCCCA
 801 AGTAGAAGAA ATAAGGTTTT CTTTTTCTAA TTTGCGTAGT GTTCTGTACA
 851 CATTACCTTG GTCAACAGAA GAGAAGCCGA TATCCATTAG CATTTGAATA
 901 AGTTTGTAAC CATGAAGACT CCAGTCTTTA AGACAGAGAA GTAAGAAGGG
 951 AACTAAGAAG TTTTTTGGCA TGGAGTTTGG TTGTTTTGCT TGGTTGTTCT
1001 CCAAACTTTC GCGTTGTTCT GGTTCATTAT GTAGCATGAT TGTGCATCAC
1051 CTCATGAATC AGTAAGATGG AATTTTTTGC TTATAAGTGC AATTTACACC
1101 TATCTGTTTT TAAAGTCAAT ACATTTGTTA GAAAATAGAA AAAAATAAAA
1151 TATAGACTTG AAGTTTTATT CAGAATATTA GAAAATAATG AATAGGTTGA
1201 ATTGTTTCAA AAACGAGAAA GGGAGTGTAG GTAAAGTGAT TGATCAAAAA
1251 TTCGATCCAC TACAAGCATG GAAAAATGCT TATGAACAAA CCGAAACATT
1301 TTGGGGAAAA GCGCTCAATG AAACAATTAA AACAGAAGAA TATTCTGCTT
1351 GGATGGGCAG CGTTCTAGAT TTGAATTTGT TTTATCAAAA AGCATTAAAT
1401 GATACGACAA AAAATTATTT AGAGCAGGTG AATGTGCCTA CGAAAGAGGA
1451 TATCGCTAGA GTGGCTACGC TTGTTATTAA CTTAGAAAAT AAAGTGGATA
1501 ACATTGAGGA GTTTCTAGAA GAGAAGGTAG AGTCAGTAGG ACAAGCTCCT
1551 ACATTAAAGC GTGATGTTAC GAAAGTAAAA CAAGATCTTC GCACATTAGA
1601 AACGAAGGTT GATCAAATTT TAGAATTGCT AGAAAAGCAA AATGCAGTAC
1651 TAGCGAAACT ACAAGAACCT GTAAAGGAAG AAGTAAAACC TGCGAATAAG
1701 CCAGAAAATA AAAAGTGATA ATAGCGCTTG TTTATAACAG ATAAAAGAGA
1751 GAGGGAGAAT CTCTTTTCTA TGTATATACA GCGAAACATA ATTATATAGG
1801 GACTCCATAG TATGAGTACC AAAATGATTC ATAATAGTCG TTCACAAATT
1851 CATTTAAGGG GGAAAAGAAA TGGTTCAATT AAATGGAAAA GTAGCAATCG
1901 TAACAGGTGG GGCAAAAGGG ATTGGTAAAG CGATTACAGT AGCGCTAGCA
1951 CAAGAGGGAG CAAAAGTTGT TATTAACTAT AACAGCAGCA AAGAAGCAGC
2001 TGAAAACTTA GTAAATGAAC TAGGAAAAGA AGGACATGAC GTTTATGCAG
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2051 TTCAAGCGGA TGTTTCTAAA GTAGAAGATG CAAACCGACT TGTAGAAGAA
2101 GCAGTGAATC ATTTTGGTAA AGTTGATATT CTTGTTAATA ATGCTGGTAT
2151 TACAAGAGAT CGTACATTTA AAAAGTTAAA TCGTGAAGAT TGGGAACGCG
2201 TAATTGACGT GAACTTAAGC AGTGTGTTTA ATACGACAAG CGCGGTACTT
2251 CCATACATAA CGGAAGCAGA AGAAGGTAGA ATTATTAGCA TTTCTTCTAT
2301 TATTGGTCAA GCGGGTGGAT TTGGACAAAC AAACTACTCA GCAGCAAAAG
2351 CAGGTATGCT AGGATTTACA AAATCATTAG CATTAGAGCT TGCAAAAACA
2401 AATGTAACTG TAAACGCTAT TTGCCCAGGA TTTATTGATA CTGAAATGGT
2451 AGCAGAAGTA CCAGAAGAAG TACGTCAAAA AATCGTTGCA AAAATCCCGA
2501 AAAAACGTTT TGGTCAAGCA GATGAAATTG CAAAAGGTGT AGTATACCTA
2551 TGCCGTGACG GTGCTTATAT TACAGGTCAG CAATTAAACA TTAACGGCGG
2601 ATTATATATG TAATGAAGTA AGAAAAAAGT GCATATCCAT AGCAGGAATG
2651 CACTTCTTTT TTTAGAAGGA AATTGACCAA AAAGGAGATA GAAAAATGAC
2701 TACATTCGCA ACAGAATGGG AAAAGCAATT AGAGCTATAC CCAGAAGAGT
2751 ATCGAAAAGC ATACCGCCGA GTGAAAAGGG CGAGTGAAAT TTTATTACGT
2801 GAACCAGAGC CGCAAGTCGG ATTAACGCCG AAAGAGGTTA TTTGGACGAA
2851 GAATAAGACG AAGCTTTATC GCTACATTCC AAAACAAGAA AAAACACAAA
2901 GAGTTCCAAT TCTATTAATA TATGCTCTTA TTAATAAACC ATATATTATG
2951 GACTTAACTC CTGGAAATAG TTTAGTGGAA TATCTAGTGG ACCGTGGTTT
3001 TGATGTGTAT ATGCTTGATT GGGGCACATT TGGTTTAGAA GATAGTCATT
3051 TGAAATTTGA TGATTTCGTG TTTGATTATA TTGCAAAAGC AGTGAAAAAA
3101 GTAATGCGAA CTGCAAAATC GGACGAGATT TCTTTACTTG GTTATTGCAT
3151 GGGTGGAACG TTAACTTCTA TTTATGCGGC ACTTCATCCG CACATGCCAA
3201 TTCGCAACTT AATCTTTATG ACAAGTCCTT TTGATTTCTC TGAAACAGGA
3251 TTATATGGTC CTTTACTAGA TGAAAAATAT TTTAACTTAG ATAAAGCAGT
3301 TGATACATTT GGTAATATCC CGCCAGAAAT GATTGATTTC GGAAACAAAA
3351 TGTTAAAACC AATTACAAAC TTTGTGGGAC CATACGTTGC TTTAGTAGAT
3401 CGTTCAGAGA ATGAGCGTTT CGTTGAAAGC TGGAGGTTAG TTCAAAAGTG
3451 GGTTGGTGAT GGCATTCCGT TCCCAGGTGA ATCATACAGA CAGTGGATTC
3501 GTGATTTTTA TCAAAATAAT AAACTCGTTA AGGGTGAACT CGTTATTCGT
3551 GGACAAAAGG TAGACCTTGC AAATATTAAG GCGAATGTCT TAAATATTTC
3601 CGGGAAACGT GATCATATCG CTCTGCCATG CCAAGTAGAA GCATTACTAG
3651 ATCATATTTC TAGCACAGAT AAACAATATG TATGTTTACC AACGGGGCAT
3701 ATGTCTATCG TTTACGGTGG AACAGCTGTA AAACAAACGT ATCCGACGAT
3751 TGGAGATTGG CTTGACGAGC GTTCAAAGTA AAAATGAAAA ATCCAACTAG
3801 CGTTGTGTTA GTTGGATTTT TTTATGGAAA ATAACAAAAC ATAGAAAGGA
3851 GAAAATTATA TTGTAGGAGT ATAATTATGA ACCTTATAAC TGGTAAGTTG
3901 TTTTGGAATA GAGGAGTTTC TGTACCTTGT TATCCACCGT TAGAAAATGA

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3951 TATGATATGT GATGTGCTTG TAGTCGGAAG TGGCGAAGCA GGTGCCCATA
4001 TAGCGTACTT TTTAGCGAAA ATTGGCATGA GTGTGATGCT TATTGAAAAA
4051 AGAGAAATTG CATGTGGTAG TACATTTGCA AATGTAGGTT TATTACAGTT
4101 TTTTCATGAT AAATCGTTAA CCTCACTTAT TCATACATTT GGTGAAGAAA
4151 AAGGGGTACG AGCATATAAG CTTTGTTACG AAGCGTTACG AACAATGGAG
4201 AAAGTTGTAT CGACTCTCGA TATTGAACCA CAGTTTATTC CACGAAATAG
4251 TTTATATTAT GCAAGTAAAA GTGAAGATGT CTCATCTTTA CAAGAGGAGT
4301 ACAATACGTT ACAGAAATAT GGATTTCCTG TTGAGTATTT TACAGAAGTT
4351 GATATTAAGG AACGTTATTC CTTTACCAAA CAAGCGGCAT TATATACACA
4401 TGGTGATGCT GAGGTAAATC CATATTTATT AGCGCATAGT CTTTTGCATA
4451 AAGCAAATCA AATGGGTGCT ATTATACATG AACATACAGA GGCCCTACAT
4501 ATAAAAAAAC GTCAAAATGA TTTAATTTGT TATACGAAAA CCGGAAATCA
4551 AATTGTAGCA AAGAATATTA TTATGGCGAC GGGCTATGAA GCGCTTTTTG
4601 GGATGAAAGA AAAAAATACA CCAGTAGAGA CATCTTATGC AGTTGTGACA
4651 AATGAAATAG ATCAGTTTGA GGGCTGGCAT GAGCGATCAT TGATTTGGGA
4701 AACAGCACGT CCCTACTTAT ATTTTCGGAC GTATAAAAAC CGCATTATGG
4751 TAGGCGGATT AGATGAAGCG ATGCAAATTC AATCGAT
```

5.4 CONCLUSIONS

- The set I primers (*pha*C F1, *pha*C F2 & *pha*C R4) could not amplify the *pha*C gene. However, *pha*C F2 & *pha*C R4 amplified a different gene i.e., DNA-directed RNA polymerase, beta' subunit of the *Bacillus* sps. genome.
- An ~1 kb gDNA fragment amplified with set II primers (P5 and P6) coded for the *Bacillus thuringiensis* putative *pha*C gene which could be used as probe to locate the PHA gene locus.
- The putative *pha*C gene is designated as pRDC2 has the putative lipase box **(G-X-C-X-G-G)** of the PHA synthase.
- An ~5 kb *Sac* I – *Cla* I insert form *Bacillus thuringiensis* R1 confers the ability of PHA accumulation to recombinant *E. coli* designated as pSVA10. PHB accumulation, however, was in negligible amounts.
- A restriction map was generated for pSVA10.
- The complete sequence of the insert in the pSVA10 was found to be 4787 bp and the sequence was deposited with NCBI GenBank and is available under the accession number **DQ000291.**

CHAPTER 6

SEQUENCE ANALYSIS OF THE ~5.0 KB GENOMIC DNA FRAGMENT OF *BACILLUS THURINGIENSIS* R1 HARBORING THE PHB BIOSYNTHETIC GENE LOCUS

6.1 INTRODUCTION

Intensive research in physiology, biochemistry and molecular genetics of polyhydroxyalkanoates (PHAs) metabolism has over the years expanded our knowledge of the biosynthesis of these biopolymers in bacteria. Many new enzymes and pathways involved in the metabolic process have been identified. In addition, many genes encoding the enzymes of the pathways have been cloned and characterized (Steinbüchel and Hein 2001).

Identification and characterization of PHA synthesizing genes from different organisms will help in metabolic engineering (Hein *et al.* 1998) gene shuffling (Stemmer 1994) and directed evolution (Moore *et al.* 1997) to engineer new or improved PHA synthase(s) for the synthesis of novel biodegradable polymers. Insight gained at molecular level will provide necessary tools for developing the biopolymer engineering approach to produce these materials in recombinant organisms and it would aid in their *in vitro* production as well.

Genes related to PHA biosythesis are often clustered in the bacterial genomes (Rehm and Steinbüchel 1999; Rehm and Steinbüchel 2001). In *W. eutropha* belonging to the class I PHA synthases the genes *pha*C, *pha*A and *pha*B constitute the *pha*CAB operon (Slater *et al*. 1988; Schubert *et al*. 1988; Peoples and Sinskey 1989a, b). All pseudomonads belonging to class II PHA synthases possess two different *pha*C genes separated by *pha*Z encoding the PHA depolymerase. In bacteria possessing class III PHA synthases, *pha*C and *pha*E are linked and most probably constitute a single operon (Rehm 2003). The class IV synthase genes found in bacteria belonging to the genus *Bacillus* comprise *pha*R and *pha*C coding for the two hetero-subunits of the active PHA synthase and are separated by *pha*B (McCool and Cannon 2001; Satoh *et al.* 2002).

The scope of the present chapter is to analyze the PHA gene locus and identify different genes involved in PHA biosynthesis in the recombinant *E. coli* harboring the PHA synthesizing genes from *B. thuringiensis* R1. This chapter describes the sequence analysis of the 4787 bp PHA gene locus in pSVA10 and complementation studies to increase the PHB production in pSVA10.

6.2 EXPERIMENTAL PROCEDURES

6.2.1 Sequence analysis

Open Reading Frame (ORF) and restriction analysis of the 4787 bp insert (Accession no. **DQ000291**) in pSVA10 was done using pDRAW32 version 1.1.61**.** Sequence comparisons and alignments were performed with the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information). The Promoter analysis and RBS (Ribosome Binding site) prediction was done by SoftBerry BPROM (http://www.softberry.com/) and Web GeneMark version 2.5 (http://opal.biology.gatech.edu/GeneMark/) (Borodovsky and McIninch 1993). Protein parameters were determined by ExPasy tools (http://au.expasy.org/tools/). Codon usage was determined by http://bioweb.pasteur.fr/cgi-bin/seqanal/codonw.pl software.

6.2.2 Complementation studies

To increase the PHB production in pSVA10, plasmid pSRAB that included *pha*A and *pha*B (Satoh *et al.* 2002) was introduced into pSVA10 in order to synthesize monomers. The plasmid pSRAB is pSTV28 derivative that was constructed by ligating 2.3 Kb *Pst* I fragment that encoded *pha*A and *pha*B from *W. eutropha*. PSTV28/29 DNA are plasmid vectors that are reconstructed from a replication start of pAYCYC184, chloramphenicol resistant gene of Tn9 and β-galactosidase gene including pUC18/19 multicloning site. Since this vector contains replication start of pAYCYC184 that allows the co-presence of this plasmid into the host cell containing a different plasmid. Competant cells of pSVA10 were prepared as described in Chapter 2 and transformation of the same was done with plasmid DNA from pSRAB. The recombinants were screened by plating them on LB plate containing both chloramphenicol and ampicillin. Further, the recombinants were checked for PHB accumulation in basal media with glycerol (1%) as the carbon source.

6.2.3 In vitro detection of PHB by fluorescent microscopy

Heat fixed bacterial smear was stained with 1.0% aqueous solution of Nile blue A at 55°C for 10 min. The slide was washed with tap water to remove excess stain and then with 8% aqueous acetic acid for 1 min. The stained smear was again washed with water and blotted dry. Before examination, the slide was remoistened with water and a

cover slip was placed on the smear (Ostle and Holt 1982). The slides were observed under the oil immersion lens of a Leica fluorescent microscope at an excitation wavelength of 460 nm.

6.3 RESULTS AND DISCUSSION

6.3.1 Open Reading Frame analysis

6.3.1.1 Open Reading Frames

The 4787 bp insert (Accession no. DQ000291) from the pSVA10 was found to have a G+C content of 35.51 mol%. The sequence was found to be A+T rich as in other *Bacillus* species.

The ORF analysis of the sequence was done using pDRAW32. The sequence contained 6 ORFs. The minimum ORF length was considered to be 400 bp. Restriction endonuclease and the ORF map of pSVA10 containing the 4,787 bp genomic DNA fragment from *Bacillus thuringiensis* R1 is presented in Figure 6.1. The ORFs and the respective deduced amino acid sequences obtained are listed in Figure 6.2.

ORF 1 was identified on the negative strand (Frame -1) and it extends from nucleotide 540 to nucleotide 18 (522 bp length). ORF 2 was also present on the negative strand (Frame -1) and extends from nucleotide 1035 to nucleotide 585 (450 bp length). ORF 3 extended between nucleotides 1188 and 1716 (528 bp) on the positive strand (Frame +3). Similarly, ORF 4 spanned nucleotides 1870 and 2611 (741 bp) on the positive strand (Frame +1), while ORF 5 extended between nucleotides 2696 to 3779 (1083 bp) on the positive strand (Frame +2). An incomplete ORF (ORF 6) was found to extend from nucleotide 3877. The ORFs 3, 4 and 5 are found to be in the opposite orientation with regards to ORFs 1 and 2.

The sequence homology search with NCBI BLAST showed that the putative genes encoded by ORF 1, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6 corresponded to *pha*P, *pha*Q, *pha*R, *pha*B, *pha*C and the partial sequence of an oxidoreductase. The different genes in the *pha* gene cluster are separated by intergenic regions: *pha*P and *pha*Q – 45 bp; *pha*Q and *pha*R –153 bp; *pha*R and *pha*B –164 bp; *pha*B and *pha*C –85 bp.

Fig. 6.1 Restriction endonuclease and ORF map of pSVA10 containing a 4,787 bp genomic DNA fragment from *Bacillus thuringiensis* R1.

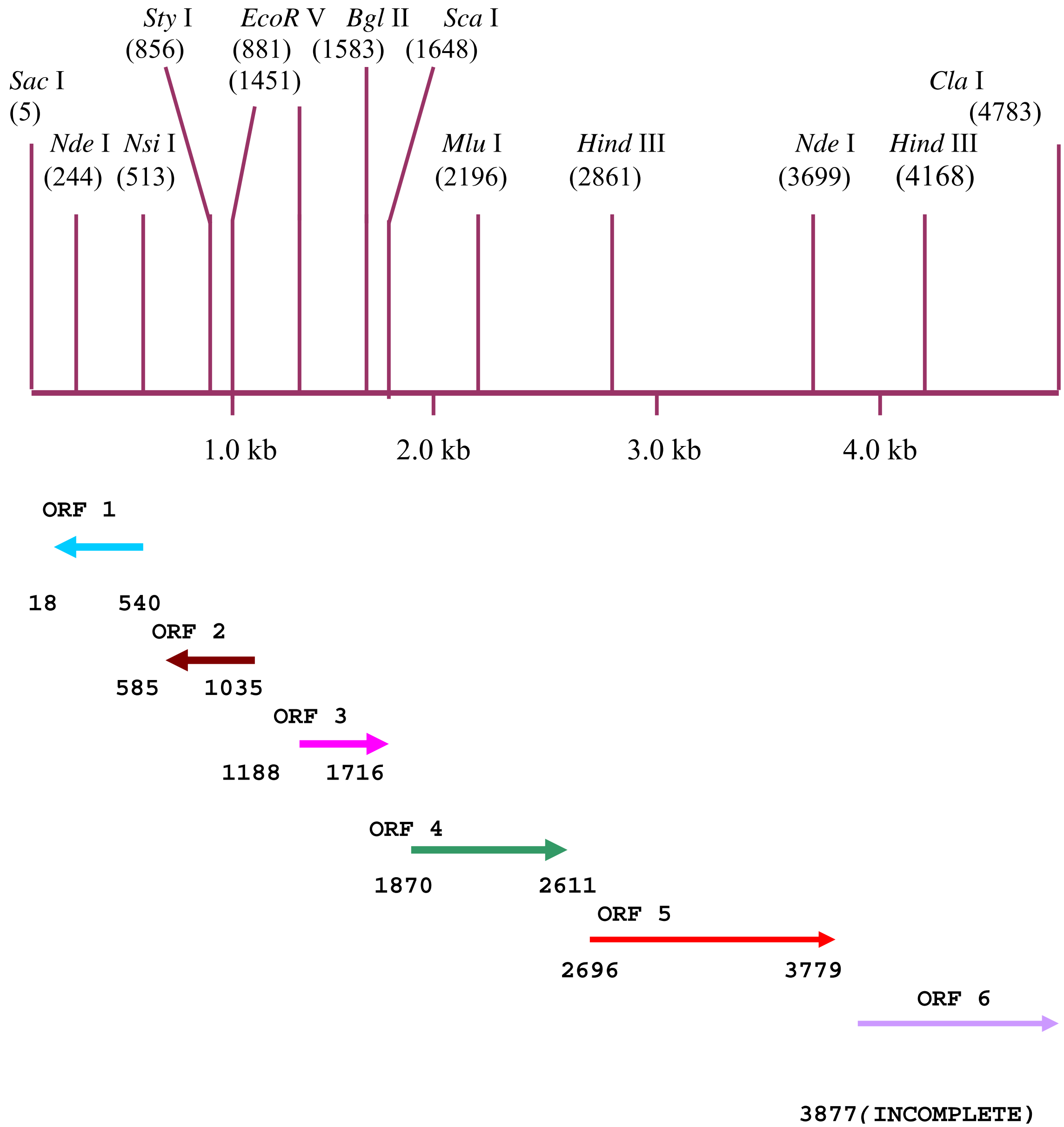


Fig. 6.2 Open Reading Frames and the amino acid sequences of the respective ORFs present in the 4,787 bp genomic DNA fragment from *Bacillus thuringiensis* R1

```
Orf#   =     1
Frame  =    -1
Start  =   542
End    =    20
Length =   522 bp
Amino acid residues = 174
METKPYELVDAFWKNWSQSLSLFSSAGKQLEQLTLETLKQQQDALHKLTS 50
GVDELEKELQQFTAQFNNQYTDYVKQLTGNSLNDQINEWQEKWKELSAHM 100
QQLTVSPTKTSLSILTQTSGQFEETTKQFIEQQQLQREEAQKQLEGFLEE 150
FKLKQLELAKKFEENSKNLFTSIK*
```

```
Orf#   =     2
Frame  =    -1
Start  =  1037
End    =   587
Length =   450 bp
Amino acid residues = 150
MLHNEPEQRESLENNQAKQPNSMPKNFLVPFLLLCLKDWSLHGYKLIQML 50
MDIGFSSVDQGNVYRTLRKLEKENLISSTWDTSDGGPAKRIYSLTEYGEQ 100
YLTTCATSFEHYQNMLRTFFTLYTNAFFPFSTSPEKDEKDSSSSPGDTAE 150
*
```

```
Orf#   =     3
Frame  =    +3
Start  =  1188
End    =  1716
Length =   528 bp
Amino acid residues = 176
MNRLNCFKNEKGSVGKVIDQKFDPLQAWKNAYEQTETFWGKALNETIKTE 50
EYSAWMGSVLDLNLFYQKALNDTTKNYLEQVNVPTKEDIARVATLVINLE 100
NKVDNIEEFLEEKVESVGQAPTLKRDVTKVKQDLRTLETKVDQILELLEK 150
QNAVLAKLQEPVKEEVKPANKPENKK*
```

Contd.

```
Orf#   =     4
Frame  =    +1
Start  =  1870
End    =  2611
Length =   741 bp
Amino acid residues = 247
MVQLNGKVAIVTGGAKGIGKAITVALAQEGAKVVINYNSSKEAAENLVNE 50
LGKEGHDVYAVQADVSKVEDANRLVEEAVNHFGKVDILVNNAGITRDRTF 100
KKLNREDWERVIDVNLSSVFNTTSAVLPYITEAEEGRIISISSIIGQAGG 150
FGQTNYSAAKAGMLGFTKSLALELAKTNVTVNAICPGFIDTEMVAEVPEE 200
VRQKIVAKIPKKRFGQADEIAKGVVYLCRDGAYITGQQLNINGGLYM*


Orf#   =     5
Frame  =    +2
Start  =  2696
End    =  3779
Length =  1083 bp
Amino acid residues = 361
MTTFATEWEKQLELYPEEYRKAYRRVKRASEILLREPEPQVGLTPKEVIW 50
TKNKTKLYRYIPKQEKTQRVPILLIYALINKPYIMDLTPGNSLVEYLVDR 100
GFDVYMLDWGTFGLEDSHLKFDDFVFDYIAKAVKKVMRTAKSDEISLLGY 150
CMGGTLTSIYAALHPHMPIRNLIFMTSPFDFSETGLYGPLLDEKYFNLDK 200
AVDTFGNIPPEMIDFGNKMLKPITNFVGPYVALVDRSENERFVESWRLVQ 250
KWVGDGIPFPGESYRQWIRDFYQNNKLVKGELVIRGQKVDLANIKANVLN 300
ISGKRDHIALPCQVEALLDHISSTDKQYVCLPTGHMSIVYGGTAVKQTYP 350
TIGDWLDERSK*


Orf#   =     6
Frame  =    +1
Start  =  3877
End    =  4783
Length =   912 bp
Amino acid residues = 303
MNLITGKLFWNRGVSVPCYPPLENDMICDVLVVGSGEAGAHIAYFLAKIG 50
MSVMLIEKREIACGSTFANVGLLQFFHDKSLTSLIHTFGEEKGVRAYKLC 100
YEALRTMEKVVSTLDIEPQFIPRNSLYYASKSEDVSSLQEEYNTLQKYGF 150
PVEYFTEVDIKERYSFTKQAALYTHGDAEVNPYLLAHSLLHKANQMGAII 200
HEHTEALHIKKRQNDLICYTKTGNQIVAKNIIMATGYEALFGMKEKNTPV 250
ETSYAVVTNEIDQFEGWHERSLIWETARPYLYFRTYKNRIMVGGLDEAMQ 300
IQS??
```

6.3.1.2 Promoter regions

The putative promoter regions for the different ORFs were identified by SoftBerry Bprom Bacterial promoter prediction software. The genes encoded by ORFs 1 (*pha*P) and 2 (*pha*Q) were possibly under the control of same promoters regions. Putative promoter sequences showing similarity to –10 (^{1099}**CACATTTAA**^{1091}) and –35 (^{1119}**AACTGA**^{1114}) motifs were identified upstream of the genes. The *pha*R (ORF 3) gene had a putative promoter sequence showing similarity to –10 (^{1143}**AAATAAAAT**^{1151}) and –35 (^{1125}**TTGTTA**^{1130}). ORFs 4 and 5 encoding *pha*B and *pha*C are probably under the control of the promoter regions at –35 (^{1765}**TTTCTA**^{1770}) and –10 (^{1784}**AAACATAAT**^{1792}) regions. Promoter sequences showing similarity to –10 (^{3854}**AATTATATT**^{3862}) and –35 (^{3831}**ATAACA**^{3836}) motifs were identified for ORF 6 encoding the partial oxidoreductase gene. The putative promoter regions for the genes are summarized in Table 6.1.

6.3.1.3 Ribosome Binding Sites (RBS)

Using the Genemark version 2.5 putative Ribosome Binding Sites (RBS) were identified. For the *pha*P and *pha*Q putative ribosome binding sites ^{556}**TTGGGG**^{551} and ^{1053}**GAGGTG**^{1048} were identified. Similarly, *pha*R, *pha*B and *pha*C three independent ribosome binding sites ^{1171}**CAGAAT**^{1176}; ^{1856}**AAGGGG**^{1861}; ^{2682}**AAGGAG**^{2687} were identified. For ORF 6 encoding the incomplete gene for an oxidoreductase a putative RBS ^{3864}**TAGGAG**^{3869} was identified. The putative ribosome binding sites for the ORFs are summarized in Table 6.2.

6.3.1.4 Putative transcription termination signals

Putative transcription termination signals (inverted repeats) have been identified for *pha*C, *pha*B and *pha*R as depicted in figures 6.3, 6.4 and 6.7 respectively.

Table 6.1 Putative promoter regions of the six Open Reading Frames in 4,787 bp genomic DNA fragment from *Bacillus thuringiensis* R1 in pSVA10.

ORF	GENE	PUTATIVE PROMOTERS	
		-35 region	**-10 region**
ORF 1	***pha*P**	^{1119}**AACTGA**^{1114}	^{1099}**CACATTTAA**^{1091}
ORF 2	***pha*Q**	^{1119}**AACTGA**^{1114}	^{1099}**CACATTTAA**^{1091}
ORF 3	***pha*R**	^{1125}**TTGTTA**^{1130}	^{1143}**AAATAAAAT**^{1151}
ORF 4	***pha*B**	^{1765}**TTTCTA**^{1770}	^{1784}**AAACATAAT**^{1792}
ORF 5	***pha*C**	^{1765}**TTTCTA**^{1770}	^{1784}**AAACATAAT**^{1792}
ORF 6	**oxidoreductase**	^{3831}**ATAACA**^{3836}	^{3854}**AATTATATT**^{3862}

Table 6.2 Putative RBS of the six Open Reading Frames in 4,787 bp genomic DNA fragment from *Bacillus thuringiensis* R1 in pSVA10.

ORF	GENE	RBS
ORF 1	***pha*P**	^{556} **TTGGGG**^{551}
ORF 2	***pha*Q**	^{1053}**GAGGTG**^{1048}
ORF 3	***pha*R**	^{1171}**CAGAAT**^{1176}
ORF 4	***pha*B**	^{1856}**AAGGGG**^{1861}
ORF 5	***pha*C**	^{2682}**AAGGAG**^{2687}
ORF 6	**oxidoreductase**	^{3864}**TAGGAG**^{3869}

6.3.2 Putative PHA synthase (phaC$_{Bt}$) from Bacillus thuringiensis R1

ORF 5 (1083 bp) was the longest gene sequence in the 4787 bp cloned fragment and encoded for the PHA synthase and was composed of 361 amino acids with a predicted molecular mass of 41.7 kDa. PHA synthases from different organisms vary in their given nucleotide size from 1068 bp in *Ectothiorhodospora shaposhnikovii* (GenBank Acc. No. AF307334) to 2011 bp in *Caulobacter crescentus* (Qi and Rehm 2001). Correspondingly, the apparent molecular mass of PHA synthases varies from ~40 kDa to ~73 kDa. Sequence homology studies using NCBI BLAST of the translation product of ORF 5 showed 100% sequence similarity to PHA synthases from several *Bacillus* sps. (*Bacillus anthracis*; *Bacillus thuringiensis serovar konkukian*; *Bacillus cereus*; Bacillus sp. INT005) and 71.5% sequence similarity to PHA synthase of *Bacillus megaterium*. Among the reports published earlier *pha*C from *Bacillus megaterium* was reported to be composed of 362 amino acids with a molecular mass of 41.5 kDa (McCool and Cannon 2001) and *pha*C from *Bacillus* sp. INT005 was composed of 361 amino acids with molecular mass of 41.7 kDa (Satoh *et al.* 2002). The PHA synthases from *Bacillus* sps. are grouped into a new class (class IV), however, class IV *pha*C sequences can be further divided into two subgroups on the basis of the extent of nucleic acid matches: *Bacillus cereus* subgroup (including *B. anthracis, Bacillus thuringiensis* and the Bacillus INT005 isolate) and the *Bacillus megaterium* subgroup (including B. *spaericus*, B. *circulans* and B. *brevis*) (Solaiman and Ashby 2005). *Bacillus thuringiensis R1* PHA synthase can be placed in the *Bacillus cereus* group of PHA synthases based on the sequence homology study.

Sequence analysis of the *pha*C$_{Bt}$ gene showed a putative RBS, ^{2682}**AAGGAG**^{2687}, eight bases upstream of the translation start codon, ^{2696}**ATG**^{2698} (Fig. 6.3). As described earlier the putatitive promoter sequences corresponding to –35 and –10 regions are found upstream of the *pha*B$_{Bt}$, which indicates that both *pha*B$_{Bt}$ and *pha*C$_{Bt}$ may be under the control of the same promoters. PHA synthases are members of prokaryotic lipase superfamily (Jia *et al*. 2000) which in turn is a member of α / β hydrolase family (Heinkinheimo *et al*. 1999). A conserved lipase box, **G-X-C-X-G-G**, wherein cysteine serves as the active nucleophile is a special feature of the

lipases (Steinbüchel and Hein 2001). The deduced amino acid sequence of the *pha*C_{Bt} gene showed the presence of the conserved lipase box, **^{149}G-Y-C-A-G-G^{154}** (Fig. 6.3). Mutagenic analysis studies have revealed that the cysteine residue in lipase box **(G-X-C-X-G-G)** of *A. vinosum pha*C plays an important role in the catalytic cycles (Müh *et al.* 1999). In addition, it has been demonstrated that histidine at position 331 is required to activate the active site of cysteine for catalysis and aspartic acid at position 302 is required to activate the hydroxyl group of 3HBCoA or a covalently bound 3HB for nucleophilic attack during the priming and elongation steps in *A. vinosum* phaC. In *pha*C_{Bt} histidine and aspartic acid residues are found at positions 335 and 306 respectively, similar to that of *Bacillus* INT 005 (Satoh *et al.* 2002). The deduced amino acid composition of putative *pha*C_{Bt} gene product is present in Table 6.3.

6.3.3 Putative acetoacetyl –CoA-reductase (phaB$_{Bt}$) from Bacillus thuringiensis R1

ORF 4 of 741 bp coding for the putative *pha*B_{Bt} gene, encodes a protein of 247 amino acids with a predicted molecular mass of 26.5 kDa. The deduced amino acid sequence showed significant homology (100%) to acetoacetyl-CoA-reductase of different *Bacillus* sps (*Bacillus anthracis*; *Bacillus thuringiensis serovar konkukian*; *Bacillus cereus*; Bacillus sp. INT005) and 70.0% homology to acetoacetyl-CoA-reductase of *Bacillus megaterium*.

Sequence analysis of the *pha*B_{Bt} and *pha*C_{Bt} genes revealed promoter regions at –35 (**^{1765}TTTCTA^{1770}**) and –10 (**^{1784}AAACATAAT^{1792}**) regions. Like other NADPH dependent acetoacetyl CoA reductases wherein the NADPH binding motif is present at the N-terminal of the protein, the *pha*B_{Bt} also showed presence of the conserved NADPH binding motif, **^{11}V-T-G-G-A-K-G-G^{18}** (Fig. 6.3) (Ploux *et al.* 1988; Oldenburg *et al.* 2000). The deduced amino acid composition of putative *phaB$_{Bt}$* gene product is present in Table 6.4.

Fig. 6.3 Putative *pha*C_{Bt} with nucleotide and amino acid sequence. The non coding region is indicated with lower case. Putative RBS and Lipase box are underlined and in bold letters. Aspartic acid and Histidine involved in triad formation are shown in black letters on grey background. The arrows show the inverted repeats.

```
agtaagaaaaaagtgcatatccatagcaggaatgcacttctttttttagaaggaaattgac
   Putative RBS
 caaaaaggagatagaaaaATGACTACATTCGCAACAGAATGGGAAAAGCAATTAGAGCTA
                    M  T  T  F  A  T  E  W  E  K  Q  L  E  L
 TACCCAGAAGAGTATCGAAAAGCATACCGCCGAGTGAAAAGGGCGAGTGAAATTTTATTA
  Y  P  E  E  Y  R  K  A  Y  R  R  V  K  R  A  S  E  I  L  L
 CGTGAACCAGAGCCGCAAGTCGGATTAACGCCGAAAGAGGTTATTTGGACGAAGAATAAG
  R  E  P  E  P  Q  V  G  L  T  P  K  E  V  I  W  T  K  N  K
 ACGAAGCTTTATCGCTACATTCCAAAACAAGAAAAAACACAAAGAGTTCCAATTCTATTA
  T  K  L  Y  R  Y  I  P  K  Q  E  K  T  Q  R  V  P  I  L  L
 ATATATGCTCTTATTAATAAACCATATATTATGGACTTAACTCCTGGAAATAGTTTAGTG
  I  Y  A  L  I  N  K  P  Y  I  M  D  L  T  P  G  N  S  L  V
 GAATATCTAGTGGACCGTGGTTTTGATGTGTATATGCTTGATTGGGGCACATTTGGTTTA
  E  Y  L  V  D  R  G  F  D  V  Y  M  L  D  W  G  T  F  G  L
 GAAGATAGTCATTTGAAATTTGATGATTTCGTGTTTGATTATATTGCAAAAGCAGTGAAA
  E  D  S  H  L  K  F  D  D  F  V  F  D  Y  I  A  K  A  V  K
 AAAGTAATGCGAACTGCAAAATCGGACGAGATTTCTTTACTTGGTTATTGCATGGGTGGA
  K  V  M  R  T  A  K  S  D  E  I  S  L  L  G  Y  C  M  G  G
                                        Putative lipase box
 ACGTTAACTTCTATTTATGCGGCACTTCATCCGCACATGCCAATTCGCAACTTAATCTTT
  T  L  T  S  I  Y  A  A  L  H  P  H  M  P  I  R  N  L  I  F
 ATGACAAGTCCTTTTGATTTCTCTGAAACAGGATTATATGGTCCTTTACTAGATGAAAAA
  M  T  S  P  F  D  F  S  E  T  G  L  Y  G  P  L  L  D  E  K
 TATTTTAACTTAGATAAAGCAGTTGATACATTTGGTAATATCCCGCCAGAAATGATTGAT
  Y  F  N  L  D  K  A  V  D  T  F  G  N  I  P  P  E  M  I  D
 TTCGGAAACAAAATGTTAAAACCAATTACAAACTTTGTGGGACCATACGTTGCTTTAGTA
  F  G  N  K  M  L  K  P  I  T  N  F  V  G  P  Y  V  A  L  V
 GATCGTTCAGAGAATGAGCGTTTCGTTGAAAGCTGGAGGTTAGTTCAAAAGTGGGTTGGT
  D  R  S  E  N  E  R  F  V  E  S  W  R  L  V  Q  K  W  V  G
 GATGGCATTCCGTTCCCAGGTGAATCATACAGACAGTGGATTCGTGATTTTTATCAAAAT
  D  G  I  P  F  P  G  E  S  Y  R  Q  W  I  R  D  F  Y  Q  N
 AATAAACTCGTTAAGGGTGAACTCGTTATTCGTGGACAAAAGGTAGACCTTGCAAATATT
```

```
 N  K  L  V  K  G  E  L  V  I  R  G  Q  K  V  D  L  A  N  I
AAGGCGAATGTCTTAAATATTTCCGGGAAACGTGATCATATCGCTCTGCCATGCCAAGTA
 K  A  N  V  L  N  I  S  G  K  R  D  H  I  A  L  P  C  Q  V
GAAGCATTACTAGATCATATTTCTAGCACAGATAAACAATATGTATGTTTACCAACGGGG
 E  A  L  L  D  H  I  S  S  T  D  K  Q  Y  V  C  L  P  T  G
CATATGTCTATCGTTTACGGTGGAACAGCTGTAAAACAAACGTATCCGACGATTGGAGAT
 H  M  S  I  V  Y  G  G  T  A  V  K  Q  T  Y  P  T  I  G  D
TGGCTTGACGAGCGTTCAAAGTAAaaatgaaaaatccaactagcgttgtgttagttggatttttt
                              ------------->          <-------------
 W  L  D  E  R  S  K  *                 inverted repeats
ttatggaaaataacaaaacatagaaaggagaaaattatattgtaggagtataatt
```

Table 6.3 Deduced amino acid composition of the putative *phaC*$_{Bt}$ gene product

Number of amino acids: 361
Molecular weight: 41.7 kDa
Theoretical pI: 7.02
Amino acid composition:

Amino acid	Number	%
Ala (A)	16	4.4%
Arg (R)	18	5.0%
Asn (N)	14	3.9%
Asp (D)	23	6.4%
Cys (C)	3	0.8%
Gln (Q)	11	3.0%
Glu (E)	23	6.4%
Gly (G)	23	6.4%
His (H)	6	1.7%
Ile (I)	24	6.6%
Leu (L)	36	10.0%
Lys (K)	28	7.8%
Met (M)	10	2.8%
Phe (F)	16	4.4%
Pro (P)	21	5.8%
Ser (S)	16	4.4%
Thr (T)	21	5.8%
Trp (W)	7	1.9%
Tyr (Y)	20	5.5%
Val (V)	25	6.9%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 46
Total number of positively charged residues (Arg + Lys): 46
Total number of AT rich residues (F,Y, M, I, N, K) : 31.0%
Total number of GC rich residues (G, A, R, P, V) : 28.5%

Fig. 6.4 Putative *pha*B$_{Bt}$ with nucleotide and amino acid sequence. The non coding region is indicated with lower case. The promoter regions and RBS are underlined and italicized. The NADPH binding motif is labeled and underlined. Stop codon is indicated by *. The arrows show the inverted repeats.

```
                                                  -35 promoter region
taatagcgcttgtttataacagataaaagagagagggagaatctcttttctatgtatatac
-10 promoter region
 agcgaaacataattatatagggactccatagtatgagtaccaaaatgattcataatagtc
              Putative RBS
 gttcacaaattcatttaaggggaaaagaaATGGTTCAATTAAATGGAAAAGTAGCAATC
                              M  V  Q  L  N  G  K  V  A  I
 GTAACAGGTGGGGCAAAAGGGATTGGTAAAGCGATTACAGTAGCGCTAGCACAAGAGGGA
  V  T  G  G  A  K  G  I  G  K  A  I  T  V  A  L  A  Q  E  G
    NADPH binding motif
GCAAAAGTTGTTATTAACTATAACAGCAGCAAAGAAGCAGCTGAAAACTTAGTAAATGAA
  A  K  V  V  I  N  Y  N  S  S  K  E  A  A  E  N  L  V  N  E
 CTAGGAAAAGAAGGACATGACGTTTATGCAGTTCAAGCGGATGTTTCTAAAGTAGAAGAT
  L  G  K  E  G  H  D  V  Y  A  V  Q  A  D  V  S  K  V  E  D
GCAAACCGACTTGTAGAAGAAGCAGTGAATCATTTTGGTAAAGTTGATATTCTTGTTAAT
  A  N  R  L  V  E  E  A  V  N  H  F  G  K  V  D  I  L  V  N
 AATGCTGGTATTACAAGAGATCGTACATTTAAAAAGTTAAATCGTGAAGATTGGGAACGC
  N  A  G  I  T  R  D  R  T  F  K  K  L  N  R  E  D  W  E  R
 GTAATTGACGTGAACTTAAGCAGTGTGTTTAATACGACAAGCGCGGTACTTCCATACATA
  V  I  D  V  N  L  S  S  V  F  N  T  T  S  A  V  L  P  Y  I
ACGGAAGCAGAAGAAGGTAGAATTATTAGCATTTCTTCTATTATTGGTCAAGCGGGTGGA
  T  E  A  E  E  G  R  I  I  S  I  S  S  I  I  G  Q  A  G  G
TTTGGACAAACAAACTACTCAGCAGCAAAAGCAGGTATGCTAGGATTTACAAAATCATTA
  F  G  Q  T  N  Y  S  A  A  K  A  G  M  L  G  F  T  K  S  L
 GCATTAGAGCTTGCAAAAACAAATGTAACTGTAAACGCTATTTGCCCAGGATTTATTGAT
  A  L  E  L  A  K  T  N  V  T  V  N  A  I  C  P  G  F  I  D
 ACTGAAATGGTAGCAGAAGTACCAGAAGAAGTACGTCAAAAAATCGTTGCAAAAATCCCG
  T  E  M  V  A  E  V  P  E  E  V  R  Q  K  I  V  A  K  I  P
 AAAAAACGTTTTGGTCAAGCAGATGAAATTGCAAAAGGTGTAGTATACCTATGCCGTGAC
  K  K  R  F  G  Q  A  D  E  I  A  K  G  V  V  Y  L  C  R  D
 GGTGCTTATATTACAGGTCAGCAATTAAACATTAACGGCGGATTATATATGTAAtgaagt
  G  A  Y  I  T  G  Q  Q  L  N  I  N  G  G  L  Y  M  *
```

```
aagaaaaaagtgcatatccatagcaggaatgcacttctttttttagaaggaaattgacca
             ------->       <-------
              inverted repeats
aaaaggagatagaaaa
```

Table 6.4 Deduced amino acid composition of the putative *pha*B_{Bt} gene product

Number of amino acids: 247

Molecular weight: 26.7 kDa

Theoretical pI: 5.84

Amino acid composition:

Amino acid	Number	%
Ala (A)	27	10.9%
Arg (R)	9	3.6%
Asn (N)	17	6.9%
Asp (D)	10	4.0%
Cys (C)	2	0.8%
Gln (Q)	9	3.6%
Glu (E)	19	7.7%
Gly (G)	24	9.7%
His (H)	2	0.8%
Ile (I)	20	8.1%
Leu (L)	16	6.5%
Lys (K)	18	7.3%
Met (M)	4	1.6%
Phe (F)	7	2.8%
Pro (P)	4	1.6%
Ser (S)	11	4.5%
Thr (T)	13	5.3%
Trp (W)	1	0.4%
Tyr (Y)	7	2.8%
Val (V)	27	10.9%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 29
Total number of positively charged residues (Arg + Lys): 27
Total number of AT rich residues (F,Y, M, I, N, K) : 29.5%
Total number of GC rich residues (G, A, R, P, V) : 36.7%

6.3.4 Putative phaP$_{Bt}$ (Phasins) from Bacillus thuringiensis R1

ORF 1 (*pha*P$_{Bt}$) present in the complementary strand of 522 bp, encodes a protein of 174 amino acids (Fig. 6.4) with a predicted molecular mass of 20.4 kDa (Table 6.5).

In recent years, phasins have gained in significance. It has been demonstrated that defective or absence of phasin genes substantially effect PHA synthesis (Pötter and Steinbüchel 2005). McCool and Cannon (1999) demonstrated that PhaP is an extremely hydrophilic storage protein localized to inclusion bodies in the living cells. Deletion experiments of *pha*P gene demonstrated that the amount of PHB under a defined set of growth conditions was reduced to 50% with respect to wild type *W. eutropha* (York *et al.* 2002).

The occurrence of four genes for phasin homologues in *W. eutropha* raised the question whether the three additionally detected genes are intact as it was previously shown for PhaP1 or some of them are silent genes, which are not transcribed and translated into functionally active proteins. However, reverse transcription and polymerase chain reaction (RT-PCR) analysis clearly demonstrated that PhaP2, PhaP3, and PhaP4 were also transcribed under conditions permissive for PHB biosynthesis and accumulation (Pötter *et al.* 2004). When one- and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis was used and followed by N-terminal amino acid sequence and matrix-assisted laser desorption ionization time-of-flight analysis, PhaP3 and PhaP4 could be identified (Pötter *et al.* 2004). Although PhaP2 could not be localized *in vivo* at the PHB granules, *in vitro* experiments clearly demonstrated binding of PhaP2 to these granules (Pötter *et al.* 2004). These new and unexpected findings will affect the current models of the structures of PHA granules and on the mobilization of PHA *in vivo* and may also have severe impacts on the establishment of heterologous production systems for PHAs (Pötter and Steinbüchel 2005).

6.3.5 Putative phaQ$_{Bt}$ from Bacillus thuringiensis R1

ORF 2 encoding the *pha*Q$_{Bt}$ is present on the complementary strand. This 450 bp ORF encodes a protein of 150 amino acids with a predicted molecular mass of 17.3 kDa (Table 6.6). Sequence analysis revealed that *pha*P$_{Bt}$ and *pha*Q$_{Bt}$ could be transcribed by the same promoter regions (Fig. 6.6). Lee *et al.* (2004) have reported that *pha*Q gene, which is located upstream of the *pha*P, codes for a new class of transcriptional regulator that negatively controls the expression of both *pha*Q and *pha*P. McCool and Cannon (1999) reported that *pha*P and *pha*Q are transcribed by different promoters. However, Lee *et al.* (2004) have reported that *pha*P and *pha*Q together constitute a bicistronic operon and have experimentally proved the absence of any promoter in the intergenic region between *pha*P and *pha*Q. Experiments conducted by Lee and coworkers (2004) in *Bacillus megaterium* showed *pha*Q encodes a new class of transcriptional regulator that can regulate *pha*P expression by binding to the PHB granules as well as DNA *in vitro* and sense the presence of PHB *in vivo*. Their findings also suggested that phaQ is a PHB-responsive repressor, and PHB can act as an inducer for *pha*P expression in phaQ-mediated regulatory system.

6.3.6 Putative phaR$_{Bt}$ from Bacillus thuringiensis R1

The 528 bp ORF 4 encodes a protein of 176 amino acids (Fig. 6.5) with the predicted molecular mass of 20.3 kDa (Table 6.7). This ORF encodes the PhaR protein. It is the transcriptional repressor that regulates the expression of *pha*P1 (one of the homologue of *pha*P) by binding upstream of it. PhaR negatively regulates PhaP accumulation across a range of growth conditions in *W. eutropha*. Derepression of phaP requires biosynthesis and accumulation of PHB (Pötter *et al.* 2002). In *Bacillus megaterium pha*RBC operon the PhaR and or PhaC are in active form only in PHA accumulating cells. *Pha*R is instrumental in producing an unknown product required to activate *pha*C or is required along with *pha*C for synthase activity (McCool and Cannon 1999).

Figure 6.5 Putative *pha*P_{Bt} with nucleotide and amino acid sequence. The non coding region is indicated with lower case. The RBS sequence is underlined and italicized. Stop codon is indicated by *.

```
                                 Putative RBS
  tctgcgttcacaatatgtaaaaaaacatttggggtcatacAATGGAAACTAAACCATAC
                                              M  E  T  K  P  Y
GAATTAGTCGATGCATTTTGGAAAAACTGGTCTCAATCACTTTCCCTTTTCTCTTCAGCTGGG
 E  L  V  D  A  F  W  K  N  W  S  Q  S  L  S  L  F  S  S  A  G
AAACAATTAGAGCAACTTACTTTAGAAACATTAAAACAACAACAAGATGCTTTGCATAAATTA
 K  Q  L  E  Q  L  T  L  E  T  L  K  Q  Q  Q  D  A  L  H  K  L
ACATCAGGAGTAGATGAACTAGAAAAAGAACTGCAACAATTCACTGCTCAGTTCAATAATCAA
 T  S  G  V  D  E  L  E  K  E  L  Q  Q  F  T  A  Q  F  N  N  Q
TATACAGATTACGTGAAGCAATTAACTGGAAACTCCTTAAATGATCAAATTAATGAGTGGCAA
 Y  T  D  Y  V  K  Q  L  T  G  N  S  L  N  D  Q  I  N  E  W  Q
GAGAAATGGAAGGAACTTTCTGCTCATATGCAGCAGCTTACTGTTTCTCCTACGAAAACATCT
 E  K  W  K  E  L  S  A  H  M  Q  Q  L  T  V  S  P  T  K  T  S
TTGTCTATTCTTACTCAAACAAGCGGCCAATTTGAAGAAACAACAAAACAATTTATTGAACAA
 L  S  I  L  T  Q  T  S  G  Q  F  E  E  T  T  K  Q  F  I  E  Q
CAACAATTACAACGTGAAGAGGCTCAAAAACAGTTAGAAGGTTTTTTGGAAGAGTTCAAGTTA
 Q  Q  L  Q  R  E  E  A  Q  K  Q  L  E  G  F  L  E  E  F  K  L
AAGCAGTTGGAACTCGCAAAAAAGTTCGAGGAAAACTCAAAAAATCTATTTACTTCCATCAAG
 K  Q  L  E  L  A  K  K  F  E  E  N  S  K  N  L  F  T  S  I  K
TAAgaaaaatgtgggagctc
 *
```

Table 6.5 **Deduced amino acid composition of the putative *pha*P$_{Bt}$ gene product**

Number of amino acids: 174

Molecular weight: 20.4 kDa

Theoretical pI: 5.00

Amino acid composition:

Amino acid	Number	%
Ala (A)	7	4.0%
Arg (R)	1	0.6%
Asn (N)	8	4.6%
Asp (D)	5	2.9%
Cys (C)	0	0.0%
Gln (Q)	25	14.4%
Glu (E)	21	12.1%
Gly (G)	5	2.9%
His (H)	2	1.1%
Ile (I)	4	2.3%
Leu (L)	24	13.8%
Lys (K)	18	10.3%
Met (M)	2	1.1%
Phe (F)	10	5.7%
Pro (P)	2	1.1%
Ser (S)	14	8.0%
Thr (T)	15	8.6%
Trp (W)	4	2.3%
Tyr (Y)	3	1.7%
Val (V)	4	2.3%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 26
Total number of positively charged residues (Arg + Lys): 19
Total number of AT rich residues (F,Y, M, I, N, K) : 25.7%
Total number of GC rich residues (G, A, R, P, V) : 10.9%

Figure 6.6 Putative *pha*Q_{Bt} with nucleotide and amino acid sequence. The non coding region is indicated with lower case. The promoter regions and RBS are underlined and italicized. Stop codon is indicated by *.

```
   tattttctaatattctgaataaaacttcaagtctatatttattttttctattttc

     -35 promoter region  -10 promoter region
taacaaatgtattgactttaaaaacagataggtgtaaattgcacttataagcaaaaaatt
                   putative RBS
ccatcttactgattcatgaggtgatgcacaatcATGCTACATAATGAACCAGAACAACGC
                                              M  L  H  N  E  P  E  Q  R
GAAAGTTTGGAGAACAACCAAGCAAAACAACCAAACTCCATGCCAAAAAACTTCTTAGTT
 E  S  L  E  N  N  Q  A  K  Q  P  N  S  M  P  K  N  F  L  V
CCCTTCTTACTTCTCTGTCTTAAAGACTGGAGTCTTCATGGTTACAAACTTATTCAAATG
 P  F  L  L  L  C  L  K  D  W  S  L  H  G  Y  K  L  I  Q  M
CTAATGGATATCGGCTTCTCTTCTGTTGACCAAGGTAATGTGTACAGAACACTACGCAAA
 L  M  D  I  G  F  S  S  V  D  Q  G  N  V  Y  R  T  L  R  K
TTAGAAAAAGAAAACCTTATTTCTTCTACTTGGGATACAAGCGACGGAGGGCCAGCCAAA
 L  E  K  E  N  L  I  S  S  T  W  D  T  S  D  G  G  P  A  K
AGAATTTATTCTTTAACTGAATATGGAGAGCAATATTTAACAACATGTGCGACTTCTTTT
 R  I  Y  S  L  T  E  Y  G  E  Q  Y  L  T  T  C  A  T  S  F
GAACATTATCAAAATATGTTGCGGACGTTTTTCACGTTATACACCAATGCATTCTTTCCA
 E  H  Y  Q  N  M  L  R  T  F  F  T  L  Y  T  N  A  F  F  P
TTTTCTACTTCTCCAGAAAAGGATGAAAAAGATTCTTCATCTTCACCTGGTGATACAGCA
 F  S  T  S  P  E  K  D  E  K  D  S  S  S  S  P  G  D  T  A
GAGTAAtctgcgttcacaatatgtaaaaaaacatttgggggtcataca
 E  *
```

Table 6.6 Deduced amino acid composition of the putative *pha*Q_{Bt} gene product

Number of amino acids: 150

Molecular weight: 17.3

Theoretical pI: 5.07

Amino acid composition:

Amino acid	Number	%
Ala (A)	5	3.3%
Arg (R)	5	3.3%
Asn (N)	9	6.0%
Asp (D)	8	5.3%
Cys (C)	2	1.3%
Gln (Q)	7	4.7%
Glu (E)	12	8.0%
Gly (G)	7	4.7%
His (H)	3	2.0%
Ile (I)	4	2.7%
Leu (L)	17	11.3%
Lys (K)	9	6.0%
Met (M)	5	3.3%
Phe (F)	9	6.0%
Pro (P)	8	5.3%
Ser (S)	16	10.7%
Thr (T)	12	8.0%
Trp (W)	2	1.3%
Tyr (Y)	7	4.7%
Val (V)	3	2.0%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 20
Total number of positively charged residues (Arg + Lys): 14
Total number of AT rich residues (F,Y, M, I, N, K) : 28.7%
Total number of GC rich residues (G, A, R, P, V) : 18.6%

Figure 6.7 Putative $pha\mathrm{R}_{Bt}$ with nucleotide and amino acid sequence. The non coding region is indicated with lower case. The promoter regions and RBS are underlined and italicized. Stop codon is indicated by ***.** The arrows show the inverted repeats.

gattgtgcatcacctcatgaatcagtaagatggaatttttgcttataagtgcaattt

-35 promoter region -10 promoter region

acacctatctgtttttaaagtcaatacat***ttgtta***gaaaatagaaaa***aaataaaa***tata

Putative RBS

gacttgaagttttatt***cagaat***attagaaaata**ATG**AATAGGTTGATTGTTTCAAAAAC

M N R L N C F K N

GAGAAAGGGAGTGTAGGTAAAGTGATTGATCAAAAATTCGATCCACTACAAGCATGGAAA

E K G S V G K V I D Q K F D P L Q A W K

AATGCTTATGAACAAACCGAAACATTTTGGGGAAAAGCGCTCAATGAAACAATTAAAACA

N A Y E Q T E T F W G K A L N E T I K T

GAAGAATATTCTGCTTGGATGGGCAGCGTTCTAGATTTGAATTTGTTTTATCAAAAAGCA

E E Y S A W M G S V L D L N L F Y Q K A

TTAAATGATACGACAAAAAATTATTTAGAGCAGGTGAATGTGCCTACGAAAGAGGATATC

L N D T T K N Y L E Q V N V P T K E D I

GCTAGAGTGGCTACGCTTGTTATTAACTTAGAAAATAAAGTGGATAACATTGAGGAGTTT

A R V A T L V I N L E N K V D N I E E F

CTAGAAGAGAAGGTAGAGTCAGTAGGACAAGCTCCTACATTAAAGCGTGATGTTACGAAA

L E E K V E S V G Q A P T L K R D V T K

GTAAAACAAGATCTTCGCACATTAGAAACGAAGGTTGATCAAATTTTAGAATTGCTAGAA

V K Q D L R T L E T K V D Q I L E L L E

AAGCAAAATGCAGTACTAGCGAAACTACAAGAACCTGTAAAGGAAGAAGTAAAACCTGCG

K Q N A V L A K L Q E P V K E E V K P A

AATAAGCCAGAAAATAAAAAG**TGA**taatagcgcttgtttataacagat**aaaagaga**gagg

N K P E N K K *****

inverted

gagaa**tctctttt**ctatgtatatacagcgaaacataattatatagggactccatagtatg

repeats

agtaccaaaatgattcataatagtcgttcacaaattcatttaaggggaaaagaa

Table 6.7 Deduced amino acid composition of the putative $phaR_{Bt}$ gene product

Number of amino acids: 176

Molecular weight: 20.3 kDa

Theoretical pI: 5.31

Amino acid composition:

Amino acid	Number	%
Ala (A)	11	6.2%
Arg (R)	4	2.3%
Asn (N)	15	8.5%
Asp (D)	9	5.1%
Cys (C)	1	0.6%
Gln (Q)	10	5.7%
Glu (E)	21	11.9%
Gly (G)	5	2.8%
His (H)	0	0.0%
Ile (I)	6	3.4%
Leu (L)	19	10.8%
Lys (K)	23	13.1%
Met (M)	2	1.1%
Phe (F)	5	2.8%
Pro (P)	6	3.4%
Ser (S)	4	2.3%
Thr (T)	12	6.8%
Trp (W)	3	1.7%
Tyr (Y)	4	2.3%
Val (V)	16	9.1%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 30
Total number of positively charged residues (Arg + Lys): 27
Total number of AT rich residues (F,Y, M, I, N, K) : 31.2%
Total number of GC rich residues (G, A, R, P, V) : 23.8%

6.3.7 Codon usage

Each organism has its preferred choice of nucleotide usage to encode any particular amino acid, called as codon usage. The importance of considering the variation in amino acid codon usage comes from the degenerate nature of the genetic code. Patterns of nucleotide usage are of great importance in the definition and functional investigation of coding regions. In addition, intra- and intercodon variations may determine the extent of dissimilarity for divergent but structurally and functionally conserved genes, and influence the outcome of phylogenetic analyses. Genes in an organism or in related species generally show the same pattern of codon usage.

Organisms which are AT-rich have gene sequences encoded by AT-rich codons including the phenylalanine (F), tyrosine (Y), methionine (M), isoleucine (I), asparagine (N) and lysine (K). The same are correspondingly poor in amino acids coded for by GC-rich codons: glycine (G), alanine (A), arginine (R) and proline (P) (Sueoka 1961; Jukes and Bhushan 1986; D'Onofrio 1991; Collins and Jukes 1993; Jermiin *et al*. 1994; Porter 1995; Anderson and Sharp 1996). However, some genes may not conform to the above bias (Hashimoto *et al*. 1995). Table 6.8 shows the codon usage data calculated using the gene sequences available for *Bacillus thuringiensis serovar konkukian* (http://www.kazusa.or.jp/codon/). The codon usage tables are useful to increase the expression levels in the target organisms by codon optimization.

Codon optimization is useful to:

- Match codon frequencies in target and host organisms to ensure proper folding
- Bias GC content to increase mRNA stability or reduce secondary structures
- Minimize tandem repeat codons or base runs that may impair gene expression
- Customize transcriptional and translational control regions
- Remove/add post translation modification sites in encoded protein
- Insert or delete restriction sites; Modify ribosome binding sites and mRNA degradation sites
- Adjust translational rates to allow the various domains of the protein to fold properly

Table 6.8 Codon usage table for *Bacillus thuringiensis serovar konkukian str. 97-27* [gbbct]: 5117 CDS's (1465093 codons)

fields: [triplet] [frequency: **per thousand**] ([number])

UUU 32.9(48131)	UCU 15.4(22552)	UAU 27.9(40919)	UGU 6.2 (9091)
UUC 14.1(20686)	UCC 3.2(4690)	UAC 9.3(13562)	UGC 2.1 (3067)
UUA 49.6(72644)	UCA 14.5(21266)	UAA 2.4(3560)	UGA 0.5 (765)
UUG 9.5(13875)	UCG 4.6(6732)	UAG 0.5(792)	UGG 10.1(14761)
CUU 17.9(26296)	CCU 9.1(13373)	CAU 16.5(24118)	CGU 13.8(20221)
CUC 4.2(6209)	CCC 1.1(1570)	CAC 4.8(6970)	CGC 4.8(6983)
CUA 10.5(15397)	CCA 16.3(23870)	CAA 30.1(44137)	CGA 5.4(7869)
CUG 3.3(4780)	CCG 7.5(11028)	CAG 6.7(9845)	CGG 1.4(1998)
AUU 50.6(74061)	ACU 12.4(18219)	AAU 33.0(48334)	AGU 14.4(21044)
AUC 13.2(19388)	ACC 2.6(3814)	AAC 13.1(19174)	AGC 5.7(8362)
AUA 17.3(25307)	ACA 27.6(40467)	AAA 55.9(81826)	AGA 9.2(13545)
AUG 27.8(40715)	ACG 13.6(19904)	AAG 17.8(26136)	AGG 2.4(3543)
GUU 25.5(37422)	GCU 20.7(30363)	GAU 37.3(54637)	GGU 24.7(36168)
GUC 5.7(8331)	GCC 4.0(5877)	GAC 8.7(12675)	GGC 8.4(12234)
GUA 30.7(44995)	GCA 29.2(42729)	GAA 56.5(82715)	GGA 24.4(35776)
GUG 10.7(15739)	GCG 13.0(18989)	GAG 18.4(26957)	GGG 9.5(13890)

Coding GC 36.00% 1st letter GC 48.06% 2nd letter GC 33.77% 3rd letter GC 26.16%

Table 6.9 Amino acid usage table for *Bacillus thuringiensis serovar konkukian*

Letter	Amino Acid	Frequency	Count	Group
A	**Ala**	5.10%	**1,028**	Aliphatic
V	**Val**	6.48%	**1,304**	Aliphatic
L	**Leu**	8.53%	**1,717**	Aliphatic
I	**Ile**	7.60%	**1,530**	Aliphatic
G	**Gly**	5.44%	**1,095**	Structural
P	**Pro**	2.92%	**587**	Structural
F	**Phe**	4.59%	**925**	Aromatic
H	**His**	1.49%	**300**	Aromatic
W	**Trp**	0.87%	**175**	Aromatic
Y	**Tyr**	4.49%	**905**	Aromatic
C	**Cys**	0.88%	**177**	Sulfur
M	**Met**	2.67%	**537**	Sulfur
K	**Lys**	9.57%	**1,927**	+
R	**Arg**	4.07%	**820**	+
D	**Asp**	5.59%	**1,126**	-
E	**Glu**	8.07%	**1,625**	-
N	**Asn**	6.40%	**1,289**	Polar
Q	**Gln**	3.96%	**798**	Polar
S	**Ser**	6.25%	**1,258**	Polar
T	**Thr**	5.04%	**1,015**	Polar

Total number of AT rich residues (F,Y, M, I, N, K) : 35.32%
Total number of GC rich residues (G, A, R, P, V) : 23.99%

Codon usage for putative *pha*C_{Bt}, *pha*B_{Bt}, *pha*P_{Bt}, *pha*Q_{Bt} and *pha*R_{Bt} are presented in Tables 6.10; 6.11; 6.12; 6.13; 6.14.

Table 6.10 Codon usage table for putative *pha*C_{Bt} gene *from Bacillus thuringiensis* R1

fields: (amino acid) (triplet) (number)(frequency: per thousand)

Phe	UUU	10(1.25)	Ser	UCU	5(1.88)	Tyr	UAU	14(1.40)	Cys	UGU	1(0.67)
	UUC	6(0.75)		UCC	1(0.38)		UAC	6(0.60)		UGC	2(1.33)
Leu	UUA	20(3.33)		UCA	3(1.12)	TER	UAA	1(3.00)	TER	UGA	0(0.00)
	UUG	1(0.17)		UCG	1(0.38)	TER	UAG	0(0.00)	Trp	UGG	7(1.00)
	CUU	7(1.17)	Pro	CCU	3(0.57)	His	CAU	5(1.67)	Arg	CGU	8(2.67)
	CUC	2(0.33)		CCC	0(0.00)		CAC	1(0.33)		CGC	3(1.00)
	CUA	5(0.83)		CCA	12(2.29)	Gln	CAA	10(1.82)		CGA	3(1.00)
	CUG	1(0.17)		CCG	6(1.14)		CAG	1(0.18)		CGG	0(0.00)
Ile	AUU	19(2.38)	Thr	ACU	4(0.76)	Asn	AAU	10(1.43)	Ser	AGU	4(1.50)
	AUC	4(0.50)		ACC	0(0.00)		AAC	4(0.57)		AGC	2(0.75)
	AUA	1(0.12)		ACA	10(1.90)	Lys	AAA	19(1.36)	Arg	AGA	2(0.67)
Met	AUG	10(1.00)		ACG	7(1.33)		AAG	9(0.64)		AGG	2(0.67)
Val	GUU	10(1.60)	Ala	GCU	4(1.00)	Asp	GAU	18(1.57)	Gly	GGU	10(1.74)
	GUC	2(0.32)		GCC	0(0.00)		GAC	5(0.43)		GGC	2(0.35)
	GUA	6(0.96)		GCA	9(2.25)	Glu	GAA	15(1.30)		GGA	9(1.57)
	GUG	7(1.12)		GCG	3(0.75)		GAG	8(0.70)		GGG	2(0.35)

Table 6.11 Codon usage table for putative *pha*B_{Bt} gene *from Bacillus thuringiensis* R1

Phe	UUU	7(2.00)	Ser	UCU	3(1.64)	Tyr	UAU	4(1.14)	Cys	UGU	0(0.00)
	UUC	0(0.00)		UCC	0(0.00)		UAC	3(0.86)		UGC	2(2.00)
Leu	UUA	8(3.00)		UCA	2(1.09)	TER	UAA	1(3.00)	TER	UGA	0(0.00)
	UUG	0(0.00)		UCG	0(0.00)	TER	UAG	0(0.00)	Trp	UGG	1(1.00)
	CUU	4(1.50)	Pro	CCU	0(0.00)	His	CAU	2(2.00)	Arg	CGU	5(3.33)
	CUC	0(0.00)		CCC	0(0.00)		CAC	0(0.00)		CGC	1(0.67)
	CUA	4(1.50)		CCA	3(3.00)	Gln	CAA	8(1.78)		CGA	1(0.67)
	CUG	0(0.00)		CCG	1(1.00)		CAG	1(0.22)		CGG	0(0.00)
Ile	AUU	16(2.40)	Thr	ACU	2(0.62)	Asn	AAU	8(0.94)	Ser	AGU	1(0.55)
	AUC	3(0.45)		ACC	0(0.00)		AAC	9(1.06)		AGC	5(2.73)
	AUA	1(0.15)		ACA	9(2.77)	Lys	AAA	17(1.89)	Arg	AGA	2(1.33)
Met	AUG	4(1.00)		ACG	2(0.62)		AAG	1(0.11)		AGG	0(0.00)
Val	GUU	9(1.33)	Ala	GCU	4(0.59)	Asp	GAU	7(1.40)	Gly	GGU	12(2.00)
	GUC	0(0.00)		GCC	0(0.00)		GAC	3(0.60)		GGC	1(0.17)
	GUA	15(2.22)		GCA	18(2.67)	Glu	GAA	17(1.79)		GGA	9(1.50)
	GUG	3(0.44)		GCG	5(0.74)		GAG	2(0.21)		GGG	2(0.33)

Table 6.12 Codon usage table for putative *pha*P_{Bt} gene *from Bacillus thuringiensis* R1

Phe	UUU	5(1.00)	Ser	UCU	6(2.57)	Tyr	UAU	1(0.67)	Cys	UGU	0(0.00)
	UUC	5(1.00)		UCC	3(1.29)		UAC	2(1.33)		UGC	0(0.00)
Leu	UUA	10(2.50)		UCA	4(1.71)	TER	UAA	1(3.00)	TER	UGA	0(0.00)
	UUG	4(1.00)		UCG	0(0.00)	TER	UAG	0(0.00)	Trp	UGG	4(1.00)
	CUU	6(1.50)	Pro	CCU	1(2.00)	His	CAU	2(2.00)	Arg	CGU	1(6.00)

	CUC	1(0.25)		CCC	0(0.00)		CAC	0(0.00)		CGC	0(0.00)
	CUA	2(0.50)		CCA	1(2.00)	Gln	CAA	20(1.60)		CGA	0(0.00)
	CUG	1(0.25)		CCG	0(0.00)		CAG	5(0.40)		CGG	0(0.00)
Ile	AUU	3(2.25)	Thr	ACU	7(1.87)	Asn	AAU	5(1.25)	Ser	AGU	0(0.00)
	AUC	1(0.75)		ACC	0(0.00)		AAC	3(0.75)		AGC	1(0.43)
	AUA	0(0.00)		ACA	7(1.87)	Lys	AAA	12(1.33)	Arg	AGA	0(0.00)
Met	AUG	2(1.00)		ACG	1(0.27)		AAG	6(0.67)		AGG	0(0.00)
Val	GUU	1(1.00)	Ala	GCU	5(2.86)	Asp	GAU	5(2.00)	Gly	GGU	1(0.80)
	GUC	1(1.00)		GCC	0(0.00)		GAC	0(0.00)		GGC	1(0.80)
	GUA	1(1.00)		GCA	2(1.14)	Glu	GAA	15(1.43)		GGA	2(1.60)
	GUG	1(1.00)		GCG	0(0.00)		GAG	6(0.57)		GGG	1(0.80)

Table 6.13 Codon usage table for putative *pha*Q_{Bt} gene *from Bacillus thuringiensis* R1

Phe	UUU	4(0.89)	Ser	UCU	10(3.75)	Tyr	UAU	4(1.14)	Cys	UGU	2(2.00)
	UUC	5(1.11)		UCC	1(0.38)		UAC	3(0.86)		UGC	0(0.00)
Leu	UUA	6(2.12)		UCA	2(0.75)	TER	UAA	1(3.00)	TER	UGA	0(0.00)
	UUG	2(0.71)		UCG	0(0.00)	TER	UAG	0(0.00)	Trp	UGG	2(1.00)
	CUU	5(1.76)	Pro	CCU	1(0.50)	His	CAU	3(2.00)	Arg	CGU	0(0.00)
	CUC	1(0.35)		CCC	1(0.50)		CAC	0(0.00)		CGC	2(2.40)
	CUA	3(1.06)		CCA	6(3.00)	Gln	CAA	7(2.00)		CGA	0(0.00)
	CUG	0(0.00)		CCG	0(0.00)		CAG	0(0.00)		CGG	1(1.20)
Ile	AUU	3(2.25)	Thr	ACU	4(1.33)	Asn	AAU	4(0.89)	Ser	AGU	2(0.75)
	AUC	1(0.75)		ACC	1(0.33)		AAC	5(1.11)		AGC	1(0.38)
	AUA	0(0.00)		ACA	5(1.67)	Lys	AAA	8(1.78)	Arg	AGA	2(2.40)
Met	AUG	5(1.00)		ACG	2(0.67)		AAG	1(0.22)		AGG	0(0.00)
Val	GUU	2(2.67)	Ala	GCU	0(0.00)	Asp	GAU	5(1.25)	Gly	GGU	3(1.71)
	GUC	0(0.00)		GCC	1(0.80)		GAC	3(0.75)		GGC	1(0.57)
	GUA	0(0.00)		GCA	3(2.40)	Glu	GAA	9(1.50)		GGA	2(1.14)
	GUG	1(1.33)		GCG	1(0.80)		GAG	3(0.50)		GGG	1(0.57)

Table 6.14 Codon usage table for putative *pha*R_{Bt} gene *from Bacillus thuringiensis* R1

Phe	UUU	3(1.20)	Ser	UCU	1(1.50)	Tyr	UAU	4(2.00)	Cys	UGU	1(2.00)
	UUC	2(0.80)		UCC	0(0.00)		UAC	0(0.00)		UGC	0(0.00)
Leu	UUA	6(1.89)		UCA	1(1.50)	TER	UAA	0(0.00)	TER	UGA	1(3.00)
	UUG	4(1.26)		UCG	0(0.00)	TER	UAG	0(0.00)	Trp	UGG	3(1.00)
	CUU	2(0.63)	Pro	CCU	4(2.67)	His	CAU	0(0.00)	Arg	CGU	1(1.50)
	CUC	1(0.32)		CCC	0(0.00)		CAC	0(0.00)		CGC	1(1.50)

	CUA	6(1.89)		CCA	2(1.33)	Gln	CAA	9(1.80)		CGA	0(0.00)
	CUG	0(0.00)		CCG	0(0.00)		CAG	1(0.20)		CGG	0(0.00)
Ile	AUU	5(2.50)	Thr	ACU	0(0.00)	Asn	AAU	12(1.60)	Ser	AGU	1(1.50)
	AUC	1(0.50)		ACC	1(0.33)		AAC	3(0.40)		AGC	1(1.50)
	AUA	0(0.00)		ACA	6(2.00)	Lys	AAA	16(1.39)	Arg	AGA	1(1.50)
Met	AUG	2(1.00)		ACG	5(1.67)		AAG	7(0.61)		AGG	1(1.50)
Val	GUU	4(1.00)	Ala	GCU	5(1.82)	Asp	GAU	9(2.00)	Gly	GGU	1(0.80)
	GUC	0(0.00)		GCC	0(0.00)		GAC	0(0.00)		GGC	1(0.80)
	GUA	7(1.75)		GCA	3(1.09)	Glu	GAA	14(1.33)		GGA	2(1.60)
	GUG	5(1.25)		GCG	3(1.09)		GAG	7(0.67)		GGG	1(0.80)

The AT rich residues were higher compared to the GC rich amino acid residues in all the gene products except for PhaB wherein the GC rich resides were higher compared to the AT rich residues (Tables 6.3-6.7). From the codon usage tables it is evident that there is a strong bias for the use of A/T in the third position of the codon.These tables would be useful to increase the expression levels if the genes are to be targeted in a different host/system e.g., plant system.

6.3.8 Complementation of phaA from W. eutropha

The low levels of PHB accumulation in *E. coli* recombinant harboring pSVA10, may be due to the absence of *pha*A gene in *E. coli* as well as in the gene cluster isolated and cloned from *Bacillus thuringiensis* R1 (McCool and Cannon 1999). To increase the PHB production in pSVA10, plasmid pSRAB that included *pha*A and *pha*B (Satoh *et al.* 2002) was introduced into pSVA10 in order to synthesize monomers. The positive clone (pSVAR10) thus obtained showed higher PHB accumulation (25.0% of dry cell mass) in basal media with glycerol as the carbon source. The cells of pSVAR10 stained with Nile blue A showed a characteristic orange fluorescence when observed fluorescence microscope at an excitation wavelength of 460 nm indicating PHA accumulation in these cells (Fig. 6.8).

Fig. 6.8 Fluorescent micrograph of Recombinant *E. coli* pSVAR 10 stained with Nile blue showing PHB accumulation

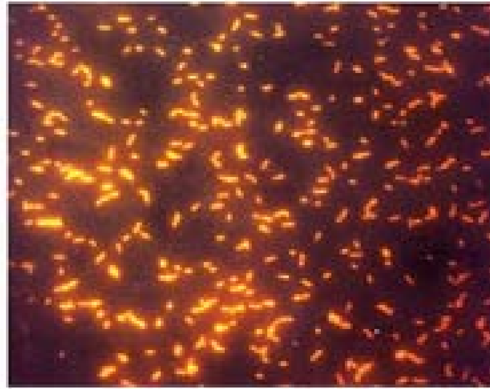
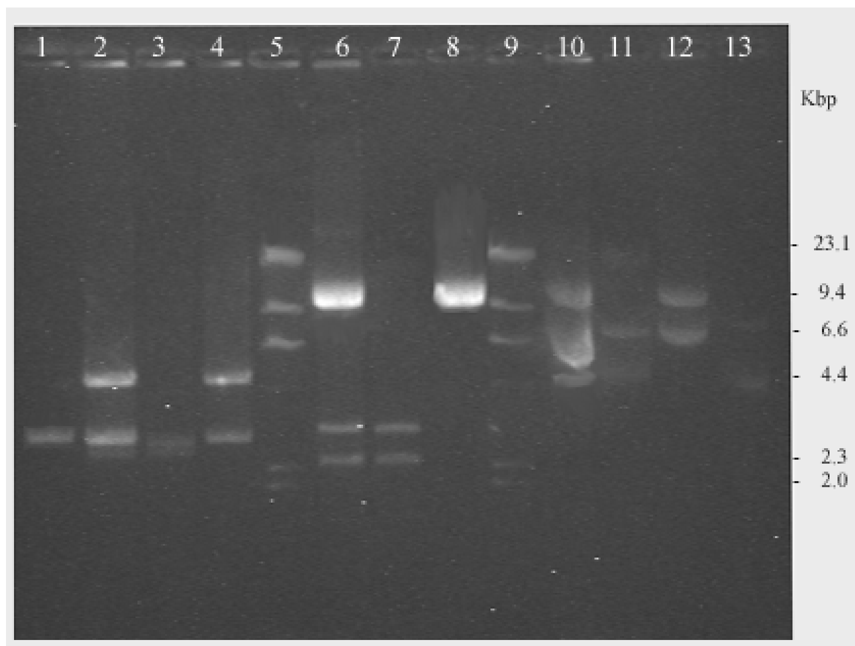


Fig. 6.9 Restriction analysis of pSVAR10



Lanes: 1. pBS linear; 2: pSVAR10/*Cla* I/*Sac* I; 3: pSRAB/*Cla* I/*Sac* I; 4: pSVA10/*Cla* I/*Sac* I; 5: λ/*Hind* III marker; 6: pSVAR10/*Pst* I; 7: pSRAB/*Pst* I; 8: pSVA10/*Pst* I; 9: λ/*Hind* III marker; 10: pSVAR10 uncut; 11: pSRAB uncut; 12: pSVA10 uncut; 13: pBS uncut.

Restriction digestion of pSVAR10 with *Pst* I and *Cla* I - *Sac* I also confirmed the presence of both the plasmids (Fig. 6.9). The restriction digestion of pSVAR10 with *Pst* I (Lane 6) released the 2.3 kb insert from the pUC18 vector similar to that

pSRAB digestion (Lane 7). As *Pst* I does not cut the pSVA10 plasmid DNA (Lane 8) it confirmed that pSVAR10 harbors both the plasmids from pSVA10 and pSRAB. Similarly, digestion of pSVAR10 (Lane 2) and pSVA10 (Lane 4) with *Cla* I/*Sac* I released the 5.0 kb insert from pBKS+ vector that confirmed the presence of both the plasmids in pSVAR10. The digestion of pSRAB with *Cla* I/*Sac* I produced a duplet of ~2.5 Kb (Lane 3) as *Sac* I cuts the insert and vector in pSRAB.

Since the accumulation of PHB (25.0% of dry weight) was observed in the recombinant (pSVAR10) with both plasmids, it could be concluded that the clone pSVA10 contained all the PHA biosynthetic genes from *Bacillus thuringiensis* R1 except *pha*A that is also confirmed by sequence analysis.

6.4 CONCLUSIONS

- The six ORFs coded for 6 putative genes *pha*P_{Bt}, *pha*Q_{Bt}, *pha*R_{Bt}, *pha*B_{Bt}, *pha*C_{Bt} and partial sequence of oxidoreductase.
- The conserved lipase box ^{149}**G-Y-C-A-G-G**^{154} and NADPH binding domain ^{11}**V-T-G-G-A-K-G-G**^{18} were identified in *pha*C and *pha*B respectively.
- The plasmid pSVA10 carries all the genes required for PHA biosynthesis in *E. coli* except *pha*A gene that resulted in low levels of PHB accumulation. The sequence analysis confirms the absence of β-ketothiolase (*pha*A) gene in the *pha* gene cluster.
- The introduction of pSRAB (with *pha*A and *pha*B from $pCAB_{re}$) into pSVA10 increased the PHB production to 25% of dry cell mass.
- The cells of pSVAR10 stained with Nile blue showed a characteristic orange fluorescence when observed fluorescence microscope indicating PHA accumulation in these cells.
- The restriction analysis of pSVAR10 confirmed the presence of both plasmids from pSVA10 and pSRAB.

CONCLUSIONS

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- A Gram-positive soil microorganism, *Bacillus thuringiensis* R1 capable of accumulating polyhydroxyalkanoates has been isolated.

- Maximum growth and PHB accumulation was obtained at 36 hrs when *Bacillus thuringiensis* R1 was grown in shake flasks.
- Maximum accumulation of PHB was found in the medium containing glycerol as the sole carbon source followed by molasses and sucrose.
- Biphasic growth cycle of *B. thuringiensis* R1 cells resulted in PHB accumulation of 64.10%.
- *Bacillus thuringiensis* R1 is found to accumulate PHB when grown on a single carbon source.
- PHB-V was accumulated when glycerol and propionic acid were used in the medium as the carbon sources.

- SEM of the recovered polymer revealed a uniform spherical shape with a stable configuration with a granule size of 5 microns.
- NMR and FTIR studies have shown the accumulated polymer to be isotactic homopolymer of PHB when *Bacillus thuringiensis* R1 was grown on a single carbon source.
- Molecular weight of the PHB synthesized and accumulated by *Bacillus thuringiensis* R1 was found to be around 1.0385×10^5 by GPC.
- DSC and TGA results show a shift in the thermal degradation temperature pattern of the polymer, which is found to be at 261°C.
- Inclusion bodies associated proteins of ~45 kDa and ~40 kDa were purified from *Bacillus thuringiensis* R1 cell lysate.
- The set I primers (*pha*C F1, *pha*C F2 & *pha*C R4) could not amplify the *pha*C gene. However, *pha*C F2 & *pha*C R4 amplified a different gene i.e., DNA-directed RNA polymerase, beta' subunit of the *Bacillus* sps. genome.
- An ~1 kb gDNA fragment amplified with set II primers (P5 and P6) coded for the *Bacillus thuringiensis* putative *pha*C gene which could be used as probe to locate the PHA gene locus.
- The putative *pha*C gene is designated as pRDC2 has the putative lipase box **(G-X-C-X-G-G)** of the PHA synthase.

- An ~5 kb *Sac* I – *Cla* I insert form *Bacillus thuringiensis* R1 confers the ability of PHA accumulation to recombinant *E. coli* designated as pSVA10. PHB accumulation, however, was in negligible amounts.
- A restriction map was generated for pSVA10.
- The complete sequence of the insert in the pSVA10 was found to be 4787 bp and the sequence was deposited with NCBI GenBank and is available under the accession number **DQ000291.**
- The six ORFs coded for 6 putative genes *pha*P_{Bt}, *pha*Q_{Bt}, *pha*R_{Bt}, *pha*B_{Bt}, *pha*C_{Bt} and partial sequence of oxidoreductase.
- The conserved lipase box ^{149}**G-Y-C-A-G-G**^{154} and NADPH binding domain ^{11}**V-T-G-G-A-K-G-G**^{18} were identified in *pha*C and *pha*B respectively.
- The plasmid pSVA10 carries all the genes required for PHA biosynthesis in *E. coli* except *pha*A gene that resulted in low levels of PHB accumulation. The sequence analysis confirms the absence of β-ketothiolase (*pha*A) gene in the *pha* gene cluster.
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RESEARCH PAPERS/ ABSTRACTS PUBLISHED/ ACCEPTED FOR PUBLICATION/ COMMUNICATED FOR PUBLICATION

1. Synthesis of PHB by recombinant *E. coli* harboring an ~5 Kb genomic DNA fragment from *Streptomyces aureofaciens* NRRL 2209. T.V.N. Ramachander, **D. Rohini**, A. Belhekar, S. K. Rawal. *International Journal of Biological macromolecules* 31(2002) 63-69.
2. A 4.3 Kb g-DNA fragment from *Streptomyces aureofaciens* supports PHB synthesis in recombinant *E. coli.* T.V. N. Ramachander, **D. Rohini Devi**, Manish Arha and S. K. Rawal. Abstract *Proceedings of the 5th National Symposium on Biochemical Engineering and Biotechnology*. BE19. 2003.
3. Low cost production of Polyhydroxybutyrate by *Bacillus* sp. using Molasses as carbon source. **D. Rohini**, T.V.N. Ramachander, Manish Arha and S.K. Rawal. Abstract published in *Proceedings of the 5th National Symposium on Biochemical Engineering and Biotechnology*. BE13. 2003.
4. Best poster award for the poster entitled "Biosynthesis of Polyhydroxybutyrate and its copolymer by *Bacillus* sp." **D. Rohini** and S.K. Rawal. Abstract of the poster published in proceedings of National Science Day held at NCL, in February'2004.
5. Best poster award for the poster entitled "Overexpression of the *Cob*A gene makes *E. coli* blush." **D. Rohini** and S.K. Rawal. Abstract of the poster published in proceedings of National Science Day held at NCL, in February'2005.
6. Synthesis and characterization of poly-β-hydroxybutyrate from *Bacillus thuringiensis* R1. **D. Rohini**, S. Phadnis and S. K. Rawal accepted in *Indian Journal of Biotechnology* in June 2005.
7. Cloning and expression of Uroporphyrinogen III methyltransferase (*cobA*) gene from *Bacillus thuringiensis* R1. **D. Rohini**, Sagar Goel, K. Deepika, M. Vineet and S. K. Rawal communicated to *International Microbiology* in August 2005.
8. Isolation, characterization and heterologous expression of PHA synthesizing genes from *Bacillus thuringiensis* R1. **D. Rohini**, M. Vineet, K. Deepika and S. K. Rawal communicated to *Archives of Microbiology* in September 2005.