Cloning and characterization of genes involved in polyhydroxyalkanoates synthesis in *Bacillus spp.*

A Thesis

By
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Submitted to the
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For the award of Degree of
Doctor of Philosophy
In
Biotechnology

Under the guidance of
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CERTIFICATE

It is certified that the thesis entitled "**Cloning and characterization of genes involved in polyhydroxyalkanoates synthesis in *Bacillus spp"*** which is submitted to the University of Mysore, Mysore, for the award of **Doctor of Philosophy (Ph. D.) degree in BIOTECHNOLOGY** is the result of research work carried out by **Mr. P. K. Anil Kumar,** under my guidance during the period from June 2002 to June 2007 at the Department of Food Microbiology, Central Food Technological Research Institute, Mysore, India. The candidate received research fellowship from Council of Scientific and Industrial Research, New Delhi, India, during the above-mentioned period.

Place: Mysore
Date: 25 June 2007

T. R. Shamala
(Guide)

DECLARATION

I, Mr. P. K. Anil Kumar, declare that the data presented in the thesis entitled "**Cloning and characterization of genes involved in polyhydroxyalkanoates synthesis in *Bacillus spp*"** which is submitted to the University of Mysore, Mysore, for the award of **Doctor of Philosophy (Ph. D.) degree in BIOTECHNOLOGY** is the result of research work carried out by me, under the guidance of **Dr. Mrs. T. R. Shamala**, Scientist, Department of Food Microbiology, Central Food Technological Research Institute, Mysore, India as a Research Fellow of Council of Scientific and Industrial Research, New Delhi, India during the period June 2002 to June 2007.

I further declare that the work presented in the thesis has not been submitted previously for the award of any degree or diploma or any other similar titles.

Place: Mysore
Date: 25th June 2007

P. K. Anil Kumar
Research Fellow

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(Anil Kumar, P.K)

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LIST OF ABBREVIATIONS

PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
P(HB-co-HV)	Polyhydroxybutyrate-co-polyhydroxyvalerate
scl-PHA	Short chain length PHA
mcl-PHA	Medium chain length PHA
scl-co-mcl PHA	Short chain length-co-medium chain length PHA
phaA	β-Ketothiolase gene
phaB	Acetoacetyl CoA reductase gene
phaC	PHA polymerase gene
phaJ4	(R) specific enoyl CoA hydratase gene
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
IPTG	Isopropyl-β–D–thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
LB	Luria- Bertani (medium)
mM	millimole(s)
OD	Optical density
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate-EDTA buffer
TAPS	(N-Tris-[hydroxymethyl] methyl -3- aminopropane sulfonic acid
TE	Tris-EDTA buffer
Tris	Tris (hydroxymethyl) amino methane
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
β	Beta
µl	Micro liter
GC	Gas Chromatography
IR	Infra red spectroscopy
NMR	Nuclear Magnetic Resonance spectroscopy
SEM	Scanning Electron microscopy
RSM	Response surface methodology
Taq	*Taq* DNA polymerase
CCRD	Central composite Rotatable Design

INTRODUCTION

Plastics, often described as one of the greatest inventions of modern age, are occupying a unique position in the world of materials. They have molded the modern world and transformed the quality of life. Plastics play a key role, in the manufacture of materials pertaining to clothing, shelter, transportation, communication, entertainment, health care etc. Plastics possess many attractive properties, such as lightweight, high strength and ease of processing and hence they meet a large share of the material needs of man. The ever-growing need and consumption of average plastic is more than 150 million tones per year and we are truly living in a plastic age.

The highly durable property of the plastics, their presence in the environment are regarded as environmental hazard, due to their biologically inert character. The rapid increase in production and consumption of plastics has led to the serious plastic waste problem so-called 'white pollution', and landfill depletion due to their high volume to weight ratio and resistance to degradation (Ren, 2003). The average earnings on plastic industry in the US alone are $ 50 billions per year. According to an estimate, more than 100 million tones of plastic is produced every year all over the world and the non degradable plastics are accumulating in the environment at the rate of 25 million tones per year. In India the plastic production is 2 million tones and the use of plastics is 2kg/ person per year. Because plastic is available at cheaper price it gets discarded easily and its persistence in the environment causes great harm. Several hundred tones of plastics are discarded in to marine environment leading to the death of several marine animals.

The solutions to plastic waste management include reduction of source, recycling and bio or photo degradation and incineration (Table 1). However there are problems associated with most of these methods. Process of sorting out of wide variety of discarded plastic materials is time consuming. Combustion of plastic waste causes the release of various poisonous compounds such as hydrogen cyanide into the environment. Moreover the additives like pigments, coatings, fillers etc. present in the plastic restrict the use of recycled material.

Table 1: Pros and cons of major waste treatment technologies (Ren, 2003)

TECHNOLOGY	PROS	CONS
Recycling	• Reduce amount of wastes for disposal • Save resources and energy in virgin production • Extend product's lifetime, conserve resources	• Not everything economically recyclable • Recycling consume energy, emit pollutants • Recycled product inferior in quality, thus only lower grade application, limited market
Composting	• Reduce load of landfill by digesting organics • End product useful for soil amendment • Need less energy than recycling, incineration	• Economics still unfavorable • Risk of odor and pest problem • No reliable market for end product (compost)
Incineration	• Reduce waste substantially by volume/weight • Generate energy • Need small space, reduce burden of landfill	• High capital and operational costs • Emission of hazardous substances (Dioxin, etc.) • More stringent in operation and control
Land filling	• Final and indispensable disposal of wastes, • Final and indispensable disposal of wastes, • Relatively easy to build and operate	• Suitable sites become scarce worldwide • Cost is increasing significantly due to higher environmental and sanitary requirement • Leachate and gas emission problems

In such a circumstance, biodegradable plastics offer the best solution to the environmental hazard posed by the conventional plastics. In the recent past, there has been growing public and scientific interest regarding the use and development of biodegradable polymer material as an ecologically useful alternative to plastics, which must still retain the derived physical and chemical properties of synthetic plastics.

As an alternative to synthetic plastics, biodegradable polymers are a newly emerging field. Recently a vast number of biodegradable polymers have been synthesized and some microorganisms and their enzymes capable of degrading them have been

identified (Lee, 1996). The biodegradable plastics are expected to solve the problems such as: -

a). Biodegradable plastics replace the bulky plastic waste materials from the land filling and prevent soil pollution.

b). Reduce the cost of recycling and environmental impact of cleaning the highly contaminated food service products.

c). The renewable source of biodegradable plastics will conserve the non-renewable sources of fossil fuels for a more sustainable society.

Biodegradable polymers

Numerous definitions are available for biodegradable polymers and according to ISO 472: 1988 – It is a plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in loss of some properties that may vary as measured by standard test methods appropriate to the plastics and application in a period of time that determines its classification. The change in chemical structure results from the action of naturally occurring microorganisms.

Biodegradable polymers have got versatile applications in medical, agriculture, drug release and packaging fields. The different members of the biopolymer family are mentioned in Table 2.

Table 2: Different members of biopolymer family

Biopolymer Family	
Polyesters	Polysaccharides (plant/algal)
< PLA (polyactic acid)	< Starch
< PHAs ((polyhydroxyalkanoates)	< Cellulose
	< Agar
Proteins	< Alginate
< Silks	< Carrageenan
< Collagen/gelatin	< Pectin
< Elastin (found in cows and pigs)	< Various gums (e.g., guar)
< Reslin	
< Adhesives	Polysaccharides (animal)
< Soy, zein from corn, wheat gluten, casein	< Chitan/chitosan
< Serum albumin	< Hyaluronic acid
Polysaccharides (bacterial)	Lipds/Surfactants
< Cellulose (bacterial)	< Acetoglycerides, waxes, surfactants
< Xanthum	< Emulsan
< Dextran	
< Gellan	Polyphenols
< Levan	< Lignin
< Curdlan	< Tannin
< Polygalactosamine	< Humic acid
	Specialty polymers
Polysaccharides (fungal)	< Shellac
< Pullulan	< Poly-gamma-glutamic acid
< Elsinan	< Natural rubber
< Yeast glucans	< Synthetic polymers from natural fats and oils

Natural biodegradable polymers

These are polymers of natural origin, obtained from living organisms during the various stages of the life cycle due to certain environmental conditions. They play a vital role in the living organisms, leading to protective mechanism or serving as reserve food material. Biopolymers are often known as natural polymers and the *in vivo* synthesis of these polymers and their degradation in the environment are linked with the enzymatic reactions. They are formed from the chain growth polymerization reactions of activated monomers from various complex metabolic processes. This includes microbial polysaccharides, starch, cellulose, pectin etc.

Source: STRATEGIC MARKET MANAGEMENT SYSTEM BIOPLASTICS, CANADA

Starch is formed of glucose residues and occurs widely in plants (Fig. 1). Starch is produced in the form of granules in principle crop plants with variable sizes. In general, the starch contains amylose (α-1, 4-) and amylopectin (α-1, 4-). Amylose is soluble in water, while amylopectins are insoluble in nature. Due to the increase in prices and non-availability of conventional film-forming resins, starch has been widely used as a raw material in film production. The physical characteristics such as low permeability of the starch have made it attractive for food packaging applications.

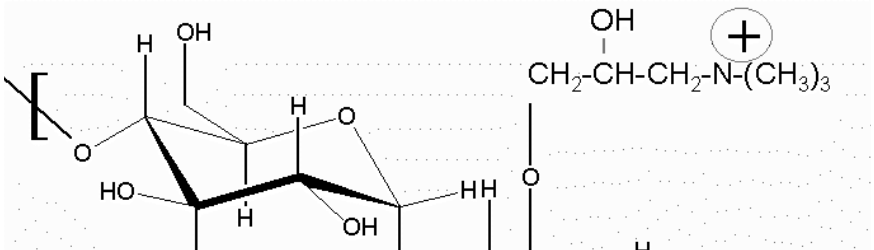


Fig. 1: Structure of starch

Starch is also useful for making agricultural mulch films because it degrades into harmless products when placed in contact with soil microorganisms. Starch is either physically mixed with its native granules or melted and blended on a molecular level with appropriate polymers or biodegradable plastics for making films. In either form, the fraction of starch in the mixture, which is accessible to enzymes, can be degraded by either, or both, amylases and glucosidases.

b. *Cellulose*

Cellulose is the most abundant natural polymer on earth and is an almost linear polymer of cellobiose residues (Fig. 2). It tends to form strongly hydrogen bonded crystalline microfibrils and fibers due to its regular structure and array of hydroxyl groups, and is most familiar in the form of paper. The cellulose is mostly of plant origin. It is the major component of the plants and they are present abundantly in the cell wall for protecting the cells from the external environment. Cellulose is an inexpensive raw material, but due to

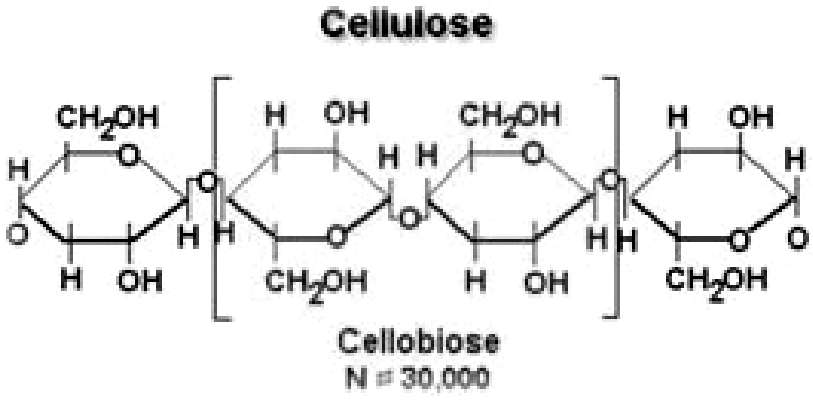


Fig. 2: Cellulose structure

its hydrophilic nature, insolubility and crystalline structure it is difficult to utilize it for various applications. The cellophane is a product from cellulose, which is a hydrophilic material and it has good mechanical properties.

c. Chitin

Chitin is a naturally occurring second most abundant polysaccharide resource that is present in the exoskeleton of invertebrates. It consists of 2-acetamide-2-deoxy-glucose with the β-(1-4)-glycoside linkage (Fig. 3). In general, chitosan, a product derived from chitin, has numerous uses as flocculant, clarifier, thickener, gas selective membrane, plant disease resistance promoter, wound healing promoting agent and antimicrobial agent and they are very likely be used as coatings for other biobased polymers. Chitin is insoluble in its native form but chitosan, the partly deacetylated form, is water-soluble.

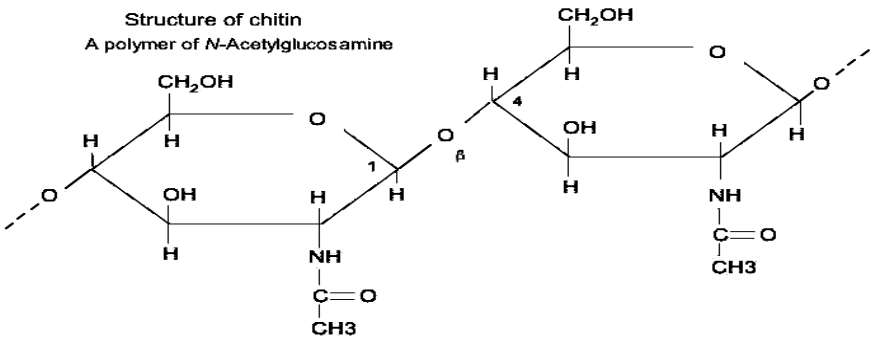


Fig. 3: Chitin structure

d. Microbial polysaccharides

The principal polysaccharides of interest for materials applications are cellulose and starch. Microbial polysaccharides such as xanthan, curdlan, pullulan etc. are also getting much attention due to their regular branched structures and novel rheological properties.

Curdlan

Curdlan, is an insoluble microbial exopolymer which is composed almost exclusively of β-(1,3)-glucosidic linkages (Fig. 4). The aqueous suspensions of curdlan can be thermally

induced to produce high-set gels and this property of curdlan has attracted the attention of various food industries. Curdlan also is used as immune stimulatory agent during vaccination.

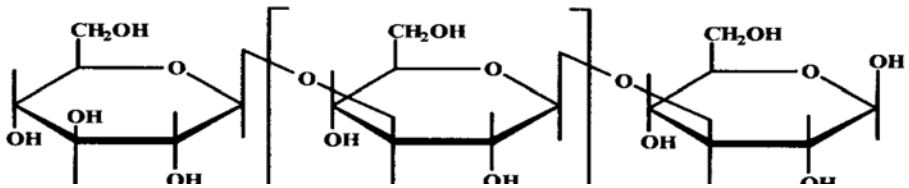


Fig. 4: Curdlan structure

e. Film forming polymer from chemical synthesis obtained from biobased monomers

Polylactic acid (PLA)

To date, polylactic acid (PLA) (Fig. 5) is the only polymer coming under this category. PLA has a high potential for the production of renewable packaging material, which can

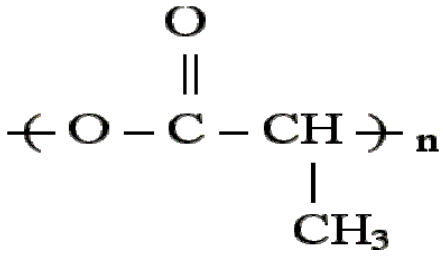


Fig. 5: Structure of polylactic acid

be produced at commercial scale. Lactic acid, the monomer of polylactic acid (PLA) can be easily produced by fermentation of carbohydrate feedstock such as agricultural waste products. PLA is polyester with a high potential for packaging applications and is water resistant. The properties of the PLA material depend on the ratio between the two L and D mesoforms of the lactic acid monomer.

Several distinct forms of polylactide exist due to the chiral nature of lactic acid : poly-L-lactide (PLLA) resulting from polymerization of lactic acid in the L form. PLLA has a crystallinity around 37%, a glass transition temperature between 50-80 °C and a melting temperature between 173-178° C. The polymerization of a mixture of both L and D forms of lactic acid leads to the synthesis of poly-DL-lactide (PDLLA) which is not crystalline but amorphous. Polylactic acid can be processed like most thermoplastics into film. PDLA and PLLA are known to form a highly regular stereo-complex with increased crystallinity. The physical blend of PDLA and PLLA are widely applicable for several uses. Currently PLA is used in a number of biomedical applications, such as sutures, dialysis media, drug delivery devices and it is also evaluated as a material for tissue engineering. It can also be employed in the preparation of bioplastics. The material with 100 % L-PLA has a very high melting point and high crystallinity. The temperature sensitivity is the drawback of this polymer, it cannot withstand at high temperature ($>55^0$ C). The degradation rate is very slow.

Pullulan

Pullulan is a neutral glucan, which can be drawn into film, and its chemical structure depends on the carbon source, microorganism (different strains of *Aureobasidium pullulans*) and fermentation conditions. The basic structure is a linear α-glucan, containing α-1, 4 maltotriose units that are linked by α-1, 6 linkages (Fig. 6). The structure may contain, up to 10 % maltotetrose units and α-1, 3 branch linkages. Depending on biosynthesis and purification procedure, a pullulan product sometimes contain heteropolysaccharides or acid polysaccharides as impurities.

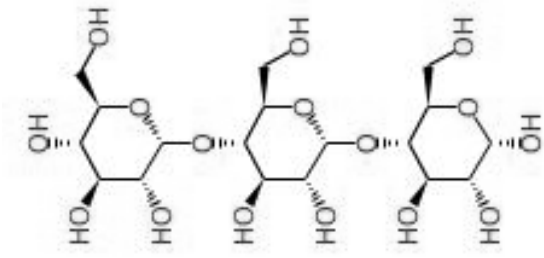


Fig 6: Structure of pullulan

Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are linear polyesters of various 3-hydroxy fatty acids monomers having the basic structural formula shown in Fig. 7. The molecular mass of PHA is generally of the order of 50,000 to 1,000,000 Daltons (Da). Numerous gram positive and gram-negative bacteria synthesize and accumulate PHA as carbon energy source material in the form of discrete granules under the condition of limiting nutrient in the presence of excess carbon source (Anderson and Dawes, 1990). PHA can be degraded by intracellular depolymerases. The number of PHA granules per cell can vary among different species. *Ralstonia eutropha* is known to contain 8-13 granules of PHA per cell with a diameter of 0.2 to 0.5 μM (Byrom, 1994). Under certain environmental conditions the polymer may be accumulated at a level of 90% of the cell dry weight. The PHA granule present in the microbial cell can be easily identified by staining with sudan black (Schlegel *et al* 1970) and nile blue (Ostle *et al*, 1982) and the monomer composition and quality of the PHA can be determined using gas chromatography with methanolysed PHA samples.

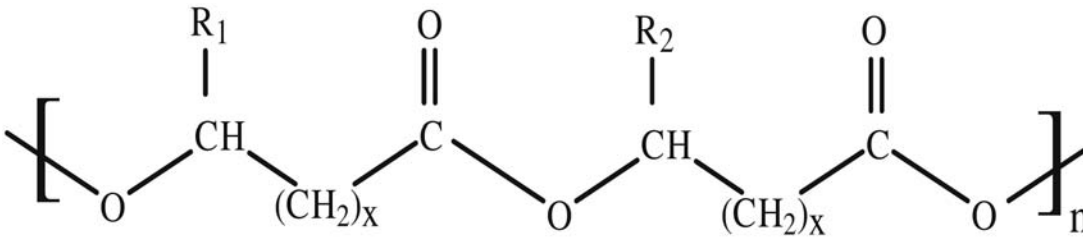


Fig. 7: Structure of polyhydroxyalkanoate

PHAs are optically active and easily biodegradable thermoplastics with a melting point temperature around 180 ^{0}C and have the same material property as that of polypropylene. The polymers of poly 3-(HB) and poly-3 (HB-co-3HV) have been used for the manufacture bottles, films and fibers for biodegradable packaging material and agricultural mulch (Hocking and Marchessault, 1994). But the putative applications of PHAs are not restricted to this area. The PHAs are widely used in the synthesis of osteosynthetic materials such as bone plates, surgical sutures and other materials of medical use. One of the possible areas for the application of PHA is as a matrix in retardant materials for the slow release of drugs, hormones, herbicides, insecticide and flavours and fragrance in medicine, pharmacy, agriculture and food industry (Stienbuchel and Fuchtenbusch, 1998).

Biodegradation

Under suitable environmental conditions such as moisture, temperature, and oxygen availability, bacteria, fungi and actinomycetes can depolymerize and utilize the polymer material as a source of nutrient. Microorganisms involved in the biodegradation are listed in Table 3.

Types of bioplastics

At present different kinds of biodegradable plastics are synthesized by different companies (Table 4**)** under different trade names using various biopolymers as substrates. The different polymeric properties of the biopolymers, are classified as five types of degradable plastics with different physical and chemical properties:-

- Biodegradable,
- Compostable,
- Hydro-biodegradable,

• Photo-degradable

• Bio erodable.

Biodegradable plastic: In biodegradable plastic the degradation is due to the action of naturally occurring microorganisms (Table 3) over a period of time (up to 2-3 years in a landfill). Eg: Polyhydroxyalkanoates.

Compostable plastic: These plastics undergo biological degradation during the composting process (up to 2-3 months) to yield carbon dioxide, water, inorganic compounds and biomass.

Photodegradable plastic: These are oil-based plastics blended with a chemical additive. Up on degradation the chemical structure of the additive undergoes changes and causes the break down in to smaller particles. The degradation is induced only when material is exposed to specific environmental conditions such as UV, moisture and heat. The break down products of these plastics is not biodegradable or compostable.

The range of degradable plastics now available includes: (Biodegradable plastics 2006)

a) Starch-based products including thermoplastic starch, starch and synthetic aliphatic polyester blends, and starch.

b) Naturally produced polyesters.

c) Renewable resource polyesters such as PLA.

c) Synthetic aliphatic polyesters.

d) Aliphatic-aromatic (AAC) co polyesters.

e) Hydro-biodegradable polyester such as modified PET.

f) Water soluble polymer such as polyvinyl alcohol and ethylene vinyl alcohol.

g) Photo-degradable plastics.

h) Controlled degradation additive master batches.

Table 3: Microorganisms capable of degrading biopolymers

Acidovorax facilis	*Penicillium daleae*
Acidovorax delafeildii	*Penicillium funiculosum*
Acremonium sp.	*Penicillium janthinellum*
Alcalienes faecalis	*Penicillium orchrochlorom*
Alteromonas haloplanktis	*Penicillium restrictum*
Arthrobacter aurescens	*Penicillium simplicissimum*
Artrobactor viscosus	*Polyporus circinatus*
Aspergillus sp.	*Pseudomonas sp.*
Aspergillus fumigatus	*Pseudomonas cepacia*
Aspergillus penicilloides	*Pseudomonas chlororaphis*
Bacillus megaterium	*Pseudomonas fluorescence*
Bacillus polymyxa	*Pseudomonas lomigenei*
Cladosporium sp.	*Pseudomonas picketii*
Clavibctor michiganence	*Pseudomonas stutzeri*
Comamonas testosteronii	*Pseudomonas syringae*
Comamonas acidovorans	*Pseudomonas vesicularis*
Cryptophage johnsonae	*Staphylococcus epidermidis*
Eupenicillium sp.	*Streptomymyces sp.*
Iylobactor delafeildii	*Variovorax paradoxus*
Mucor sp.	*Verticillium leptobactrum*
Paecilo marquandii	*Vibrio ordalii*
Penicillium adametzii	*Xanthomonas matophilia*
Penicillium chermisinum	*Zoogloea vamigera*

Source: Dieter 2001

Environmental benefits of biodegradable plastics (Biodegradable plastics 2006)

- Compared to conventional petroleum-based plastics, the use of biodegradable plastics has several identifiable environmental benefits.
- Biodegradable plastics can increase the organic content of the soil and can retain water and nutrients by forming compost in the soil.
- Manufacture of most of the biodegradable plastics requires much lower energy than for non-biodegradable plastics. PHA biopolymers are exception, which consume similar energy inputs to polyethylene.
- The other environmental benefits offered by biodegradable plastics is, it gives an opportunity to use the renewable energy resources and thereby reducing the emission of greenhouse gas**.**

Applications of biodegradable polymers

The biodegradable polymers are commercially available (Table 4) and are used in three major areas such as medical, agricultural, and packaging. Because of their unique physical and chemical characteristics there is an increase in the usage of the biopolymers in the medical field.

Most of the members of the biodegradable plastics are biocompatible; they do not cause any allergic reactions to humans. Biodegradable plastics are widely used as surgical implants in vascular and orthopedic surgery as implantable matrices for controlled release of drugs inside the body. Biomaterials in general are used for the following medical purposes (Chandra and Rustgi 1998): -

(a) To replace tissues that is diseased or otherwise nonfunctional, as in-joint replacements, heart valves and arteries, tooth reconstruction and intraocular lenses.

(b) To assist in the repair of tissue, including the obvious sutures but also bone fracture plates, ligament and tendon repair devices.

(c) To replace all or part of the function of the major organs, such as in haemodialysis

(Replacing the function of the kidney), oxygenation (lungs), left ventricular or whole heart assistance, perfusion (liver), and insulin delivery (pancreas).
(d) To deliver drugs to the body, either to targeted sites (e.g. directly to a tumor) or sustained delivery rates (insulin).

Biomaterials are used for agricultural applications such as greenhouse coverings, fumigation, mulching etc. All major classes of synthetic polymers are currently utilized in agricultural applications, which include the controlled release of pesticides and nutrients, soil conditioning, seed coatings, gel plantings and plant protection. On the other hand, biodegradable plastics are also of interest as agricultural mulches and agricultural planting containers. Eventual biodegradability, as in composting, permits the degradable plastics to be blended with other biodegradable materials and to be converted into useful soil-improving materials.

Packaging applications of the biodegradable polymers are determined by physical parameters such as the nature of the item to be packed and the environmental conditions for storage etc. Special packaging is needed for materials stored under frozen conditions. The blending of different polymers can result in materials with newer characteristics. For example the addition of pullulan to Poly (3-hydroxy butyrate-Co-3-hydroxyvalerate) may reduce oxygen permeability and increase biodegradability of the blend due to the increased surface area of PHBV exposed following the rapid removal of pullulan due to its water solubility.

There is a vast scope for the development, production and utilization of biopolymers, which are cost competitive and ecofriendly in nature. The thesis examines several aspects of one the biopolymer of microbial origin namely polyhydroxyalkanoate.

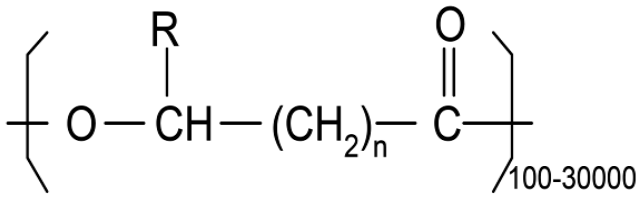
Table 4: Commercially available biopolymers

Trade name	Raw material	Manufacturing company
Biobag	Starch (materbi)	Agronne National Laboratary, Agronne Illinois USA
Swirl	Starch / Ploycapralactone	Milleta (Biotech div. Germany)
Bionelle 1000	Bionelle, starch & cellulose synthetic polyester.	Showa high polymer Co. Japan.
Biopar	Starch	BIOP Biopolymer GmbH Germany
Bioplast	Starch, cellulose & synthetic polyester	Biotech GmbH Germany.
bioskg	Starch and PVA corn	PlastirollOY
Clean Green	Starch (wheat)	Clean Green Packages Minneapolis USA
Ecopla	PLA	Cargill Dow Polymers
Ecoware	Starch	Nisser japan
Envirofill	Starch/PVA	Enpac
Evercorn	Starch (corn)	Michigan Biotechnology &Jjapan corn starch Co Ltd.
Flopakbio 8	Starch (corn/wheat)	Marfrd Industries/USA
Greenfill	Starch/PVA	Greenlight Products Ltd. London UK
Green pol	Aliphatic polyesters and starch	Greenpol Co. Dajeon Korea
Lacea	PLA from fermented glucose	Mitsui Chemicals Japan
Wood Based		
Cellotherm T	Regenerated cellulose film	UCB Films
Enviroplast	Cell acetate & polyethylene succinate	Planet Polymer Technologies Sandiago USA
Microbial Polymers		
Biomer	PHBs	Biomer Germany
Biogreen	PHB	Mitsubishi gas chemicals Japan
Biopol	PHB/PHV	Metabolix Cambridge Massachusetts
Nodax	Aliphatic polyesters primarily PHA	Tekeda Chemical Industries
Biomax	Polyethylene traphthalate	Dupont USA
Cell green	Polycaprolactone and acetyl cellulose resin	Diacel Kagaku Japan
Poval	Polyvenylalcahol	Shi Etsu Chemicals.

Ref: Friendlypackaging.org

POLYHYDROXYALKANOATES

Polyhydroxyalkanoates (PHAs) are biodegradable biopolymers that are produced by various bacteria due to nutrient depletion conditions and they are stored intracellularly as energy reserve (Abe *et al,* 1990, Dawes and Senior 1973; Doi, 1990; Steinbuchel and Valentin 1995). Polyhydroxybutyrate P (HB), which is one of the PHA homopolymer, is commonly found in various bacterial genera and it was discovered first by Lemoigne in 1926 in *Bacillus sp* (Lemoigne, 1926). Later several gram positive and gram-negative bacterial species have been identified as PHA producers (Table 5). PHAs are polyesters formed by polycondensation of carboxylic acids with hydroxyl alcohol (Fig. 8). More than 100 different monomer units have been found as constituents of PHAs (Steinbuchel and Valentin 1995). PHAs produced by bacteria are broadly classified in to two groups: 1) short-chain-length PHAs (scl -PHA) that mainly consists of monomers containing 4 to 5 carbon atoms, 2) medium-chain-length PHAs (mcl-PHA) which contain 6 to 14 carbon atoms (DeKonig, 1993). Commercially PHA is available as scl-PHA-which is a copolymer of hydroxybutyrate and hydroxyvalerate- P (HB-co-HV).



n =1	R =	hydrogen	poly (-3-hydroxypropionate)
		methyl	poly (-3-hydroxybutyrate)
		ethyl	poly(3-horoxyvalerate)
		propyl	poly (-3-hydroxyhexanoate)
		pentyl	poly (-3-hydroxyoctanoate)
		nonyl	poly (-3-hydroxydodecanoate)
n = 2	R =	hydrogen	poly (-4-hydroxybutyrate)
n = 3	R =	hydrogen	poly (-5-hydroxyvalerate)

Fig. 8: Polyhydroxyalkanote structure and monomers

Table 5: Accumulation of PHA in various microorganisms (Kim *et al* 2000).

Genus	PHA wt%	Substrate
Acinetobactor	<1	Glucose
Aquasprullum	ND	NS
Azosprullum	57	3-hydroxybutyrate
Axobactor	73	Glucose
Bacillus	25	Glucose
Beggiatoa	57	Acetate
Caulobactor	36	Glucose
Chloroflexus	<1	yeast extract
Chromatium	20	Acetate
Chrombacterium	37	Glucose/ peptone.
Clostridium	13	Tryptone
Ectothiorhodospira	ND	NS
Halobacterium	38	Glucose
Leptothrix	67	Pyruvate
Methylobacterium	47	Methanol
Methylocystis	70	Methane
Methylosinus	25	Methane
Micrococcus	28	Pentone/tryptone.
Norcardia	14	Butane
Pseudomonas	67	Methanol
Ralstonia	96	Glucose
Rhizobium	57	Methanol
Rhodobactor	60	Acetate
Rhodospirullum	47	Acetate
Sphaerotilus	45	Glucose/Peptone
Spirullum	40	Lactate
Streptomyces	4	Glucose
Syntrophomonas	30	Crotanate
Thiocaspa	36	Acetate
Thiocystis	83	Acetate

P (HB), which is commonly produced by bacteria, is crystalline, brittle, and has poor film forming and mechanical properties (Hocking and Marchessault, 1994). Only mcl-PHAs are amorphous, sticky and have limited applications. Hence, production of PHAs consisting of copolymers of scl-PHA and scl-co-mcl-PHA has been examined to overcome problems of melt stability, brittleness and amorphousness.

PHA production by wild bacterial strains

More than 200 bacteria belonging to gram+ve and gram–ve group are reported to accumulate PHA intracellularly under defined environmental conditions and many of them have been characterized at molecular level. Some bacteria such as *Ralstonia eutropha* accumulate PHA up to 80% of the cell biomass. Natural organisms produce PHA from a variety of substrates including industrial wastes.

Ralstonia eutropha

R. eutropha (earlier known as *Alcaligenes eutrophus*) has been used for the commercial scale production of P(3HB-co-3HV) by Imperial Chemical Industries (ICI). It grows well in minimal medium at 30°C on a multitude of carbon sources but not on glucose. ICI produced P(3HB-co-3HV) at a scale of 300 tons per year using a glucose-utilizing mutant (Byrom**,** 1992). ICI (now ZENECA Bio Products, UK) is currently producing P(3HB) and P(3HB-co-3HV) from glucose and a mixture of glucose and propionic acid, respectively, by fed-batch culture of *R. eutropha* on a moderately large scale. Initially *R. eutropha* is grown in a minimal medium (glucose-salts medium containing only the restricted amount of phosphate) to monitor the cell growth. Cells encounter phosphate limitation after about 60 h, and accumulate P(3HB) during the next 40 to 60 h from supplied glucose. P(3HB-co-3HV) is similarly produced except that both glucose and propionic acid are fed during the polymer accumulation phase. It is known that the concentration of PHB : PHV in P(3HB-co-3HV) can be controlled by varying the ratio of glucose to propionic acid in the feed (Byrom, 1992).

A number of strategies have also been developed for the efficient production of PHA employing *R. 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 of 164 g/l, 121 g/l, and 76%, respectively, could be obtained in 50 h, resulting in the highest productivity of 2.42 g P(3HB)/l/h (Kim *et al* 1994). Following the same fermentation strategy, 110 g/l of P (3HB-co-3HV) has been produced by feeding a mixture of glucose and propionic acid (Kim *et al,* 1994). Other than glucose, several carbon sources have also been used as substrates for PHA production. As an alternative, ethanol was used for the production of P(HB) using a mutant strain of *R. eutropha,* but the maximum concentration of P(HB) obtained was 47 g/l. P(3HB-co-3HV) can also be produced by feeding ethanol with propanol or pentanol (Alderete *et al,* 1993; Parkand Damodaran, 1994).

Alcaligenes latus

Alcaligenes latus is considered as a good candidate for the production of PHA since it grows fast and accumulates PHA during the growth phase. *A. latus* can produce PHA efficiently by utilizing sucrose as the sole carbon; which suggests that cheap beet or cane molasses can be used for the production of PHA using this organism.

Methylotrophs

Imperial Chemical Industries has used methylotrophs for the production of P(HB) from methanol. Later fed-batch fermentation of *Protomonas extorquens* was used to obtain very high concentration (149 g/l) of P(HB) (Suzuki *et al,* 1986). The average PHA content obtained with methylotrophs is 56- 60% of dry cell mass, which needs further improvement for the efficient recovery of polymers.

Pseudomonas

Pseudomonas spp are known to produce medium-chain-length PHA (Brandl *et al* 1988). *Psuedomonas* PHA synthase can gather PHA via fatty acid metabolism. *Pseudomonas*

spp accumulate PHA monomers of different chain length of C_4 to C_{12}. One of the most extensively studied species is *P. oleovorans*, this organism has the ability to synthesize a wide range of monomer units incorporated into PHA, apart from the accumulation of substantial amount of PHA. Mcl-PHAs have been produced by fed-batch and continuous culture of *P. oleovorans* to achieve high concentration of PHA and high productivity of 11.6 g/l and 0.58 g PHA/l/h, in a continuous mode of operation (Preusting *et al,* 1993[abc]). P *oleovorans* was cultivated using n-octane in a bioreactor specially designed for high oxygen transfer and cell mass, the PHA productivity was increased up to 0.32 g PHA/g/l/h with a P (3HHx-co-3HO) of 12.1 g/l.

Bacillus spp

P(HB), the best characterized PHA, was first identified and isolated from *Bacillus megaterium* (Lemoigne, 1926). Macrae and Wilkinson (1958) reported conditions under which P(HB) was formed and degraded intracellurarly by *Bacillus cereus* and *Bacillus megaterium* in washed suspensions. Members of the genus *Bacillus* accumulate co-polymers of HB when grown on different substrates (Table 6). *Bacillus* strains such as *B. cereus* DSM 31 is reported (Chen *et al,* 1991) to accumulate co-polymers of P(HB-co-HV) when the bacterium is fed with odd chain- length n-alkanoic acids such as propionic acid, valeric acid and heptanoic acid. *B. cereus* ATCC 14579 grown on caproate or octanoate resulted in the production of terpolymer containing 3HHx and 3-hydroxyoctanoate (3HO) units (Caballero *et al*, 1995). *Bacillus cereus* UW85 accumulated a tercopolymer P(HB-co-HV-co-HHx) using e-caprolactone as the sole carbon source (Labuzek and Radecka, 2001). *Bacilli* are able to produce PHA from various simple, complex and cheap carbon substrates such as sucrose, caprolactone, molasses etc. There are many *Bacillus spp* that are able to produce co-polymers of P(HB-co-HV) and some are reported to produce mcl-PHA (Labuzek and Radecka, 2001; Caballero *et al*, 1995).

Table 6: Accumulation of PHA in *Bacillus spp.* (Valappil *et al* 2006[a]).

Bacteria (*Bacillus spp*.)	**PHA content (% dcy wt.)**	**Monomeric units**	**Carbon source**
Bacillus sp. JMa5	35.0	3HB	Molasses
B. circulans DSM 1529	43.7 6.80	3HB 3HB 3HV	Acetate & 3-hydroxybutyrate Propionate
B. sphaericus	30.2	3HB	Sucrose
B. sphaericus DSM 28	4.50	3HB, 3HV	Valerate
B. brevis	32.1	3HB	Sucrose
B. licheniformis DSM394	25.8	3HB	Acetate & 3-hydroxybutyrate
B. amyloliquefaciens DSM7	17. 5.10	3HB 3HB, 3HV	Acetate & 3-hydroxybutyrate Propionate
B. laterosporus DSM335	29.5 5.00	3HB 3HB, 3HV	Acetate & 3-hydroxybutyrate Propionate
B. macerans DSM7068	40.5 4.70	3HB 3HB, 3HV	Acetate& 3-hydroxybutyrate Propionate
B. subtilis DSM10	33.5 6.60	3HB 3HB, 3HV	Acetate & 3-hydroxybutyrate Valerate
B. thuringiensis DSM2046	47.6 7.20	3HB 3HB,3HV	Acetate & 3-hydroxybutyrate Propionate
B. mycoides DSM 2048	44.7 7.50	3HB 3HB, 3HV	Acetate & 3-hydroxybutyrate Propionate
B. megaterium DSM90	47.2 8.20	3HB 3HB, 3HV	Acetate & 3-hydroxybutyrate Propionate
B. cereus DSM31	41.4 7.30	3HB 3HB, 3HV	Acetate & 3-hydroxybutyrate Propionate
B. cereus UW85	8.9	3HB, 3HV, 6HHx	e-caprolactone & Glucose
Bacillus sp. INT005	18.8 32.9	3HB, 3HV 3HB, 3HHx	Valerate Hexanoate or Glucose
B. cereus ATCC14579	2.20 ND	3HB, 3HHx 3HB, 3HO	Hexanoate Octanoate

Biosynthesis of short-chain-length PHA (scl-PHA)

Several pathways are involved in the synthesis of PHA (Figs. 9-12). The scl-PHA producing bacterium biosynthesize P(HB), polyhydroxyvalerate (P(HV), and their copolymers of P(HB-co-HV) (Doi, 1990; Doi *et al,* 1986; Doi *et al,* 1987; Steinbuchel and Schlegel, 1991). P(HB), which is one of the common scl-PHA produced by bacteria, is synthesized through three-step pathway (Fig. 9). During P(HB) synthesis following enzymatic reactions take place: 1) β-Ketothiolase catalyzes the condensation of two acetyl-CoA moieties to form acetoacetyl-CoA. 2) Acetoacetyl-CoA reductase reduces acetoacetyl-CoA to hydroxybutyryl-CoA. 3) Hydroxybutyryl CoA is polymerized by PHB-polymerase to PHB. P(HB), P(HV) or P(HB-co-HV) can also be synthesized through β-oxidation during the growth of bacteria on fatty acids, or amino acids and other substrates that can first be converted to fatty acids. These fatty acids are then metabolized through β-oxidation resulting in acetyl- or propionyl-CoA. The genes that encode these enzymes are *phaA, phaB* and *phaC* (Fig. 13) and they have been cloned from bacteria such as *Ralstonia eutropha*, *Chromatium vinosum, Zoogloea ramigera*, and others to understand the polymer biosynthesis and to improve the production of the polymer (Peoples *et al,* 1987; Steinbuchel and Schlegel, 1991).

The synthesis of P(HV) also follows the same pathway, except for the variations in the substrate used for the polymer production. In P(HV) synthesis the initial substrates are acetyl CoA and propionyl CoA, which condense to form 3-ketovaleryl CoA and which is further reduced to 3-hydroxyvaleryl CoA. The PHA synthase can polymerize this in to the growing chain of PHA (Fig. 9). In *R. eutropha* it is reported that the first step in this pathway is catalyzed by β- ketothiolase coded by the gene *bktB,* which is not present in the PHA biosynthesis operon of *R. eutropha* (Slater *et al,* 1998). The biosynthesis of P(HV) or P(HB-co-HV) requires the formation and occurrence of propionyl CoA in the cells. Propionyl CoA is synthesized under certain physiological conditions and from only a few substrates. Propionyl CoA can be derived from propionic acid, via propionyl CoA synthase enzyme or from fatty acid degradation-β oxidation of odd chain fatty acids or from various aromatic amino acids via trans carboxylation reactions or from succinyl CoA- methylmalonyl CoA pathway (Fig. 10).

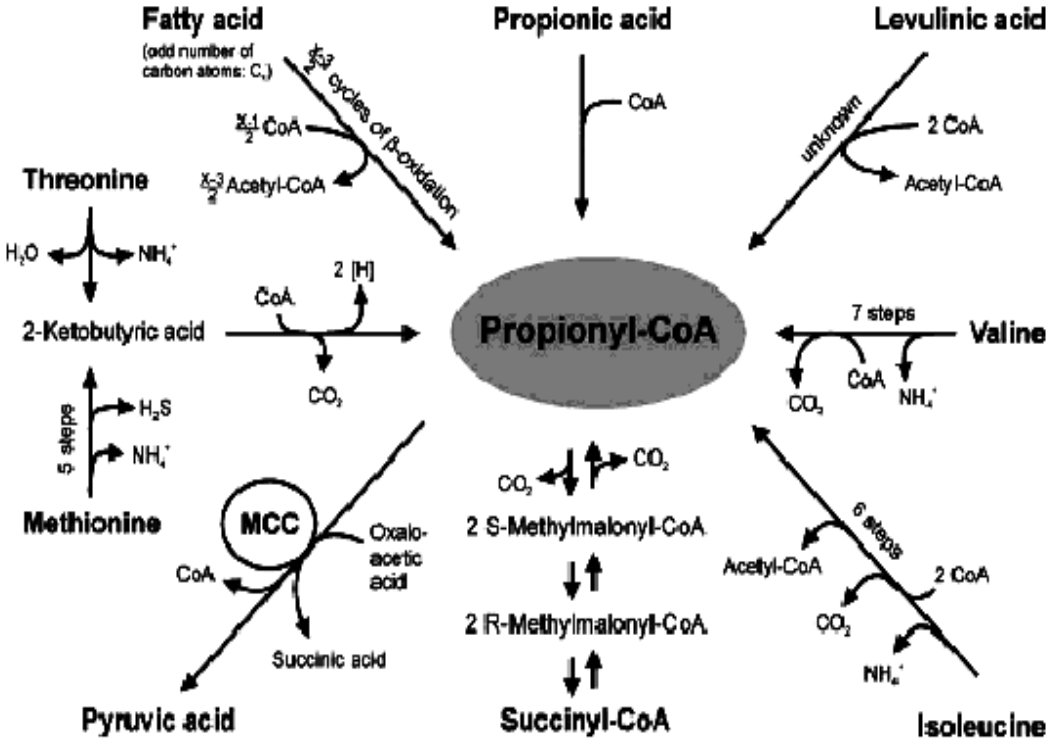


Fig. 10: Different propionogenic substrates and pathways
Ref: Steinbuchel and Eversloh, 2003

TCA cycle

TCA cycle is not directly involved in PHA biosynthesis, but various intermediates formed in the pathway can be converted in to PHA substrates. Acetyl CoA is the prime molecule for scl-PHA biosynthesis. Succinyl CoA is another molecule, which connect the TCA cycle with PHA pathway through methylmalonyl CoA. The conversion of succinyl CoA to methylmalonyl CoA is a reversible process and therefore the metabolic flux for citric acid cycle to PHA pathway is feasible in the form of acetyl CoA and succinyl CoA. Other intermediates like oxaloacetate and citric acid can also be channeled towards the scl-PHA biosynthesis pathway. Under certain conditions NADPH molecules are formed in the TCA cycle, which is an essential reducing power for scl-PHA biosynthesis.

Methylmalonyl pathway

This pathway is found in certain bacteria, which are able to produce P(HV). In addition to the methyl citric acid cycle, the co-enzyme B12 dependant methylmalonyl-CoA pathway is another important pathway for production of PHA copolymer production from propionic acid. Wild-type bacteria are known to synthesize co-polymer of poly (3HB-*co*-3HV) with very high content of 3HV from unrelated carbon substrates like glucose. In *Nocardia* and *Rhodococcus* glucose is degraded via the 2-keto-3-deoxy-6-phosphogluconate pathway to pyruvate and through reverse reactions of citric acid cycle resulting in the accumulation of succinyl CoA. The succinyl CoA is then converted in to propionyl CoA via methylmalonyl-CoA pathway for PHV synthesis (Alvarez *et al*, 1997; Valentin and Dennis, 1996). The conversion of the succinyl CoA to methylmalonyl CoA is catalyzed by the enzyme methylmalonyl CoA mutase and the methylmalonyl CoA is epimerised to its –S form in the next step. The decarboxylation of methylmalonyl CoA to propionyl CoA is catalysed by propionyl CoA decarboxyalse enzyme. The valerate specific β ketothiolase can take up this molecule and transform in to 3 hydroxyvaleryl CoA for PHV synthesis (Fig. 9).

Biosynthesis of medium-chain-length PHA (mcl-PHA)

Various *Pseudomonas spp* are known to possess ability to biosynthesize polymers containing carbon chain lengths from C_6 to C_{12} (de Smet *et al,* 1983; Huisman *et al,* 1989). This mcl-PHA is synthesized via fatty acid synthesis or fatty acid degradation pathways wherein a wide variety of substrates are utilized for the polymer production (Haywood *et al,* 1990; Huijberts *et al,* 1995; Lageveen *et al,* 1988; Timm *et al,* 1990). The precursors such as enoyl CoA, hydroxyacyl CoA, ketoacyl CoA that are generated during the fatty acid metabolism are used as substrates for PHA polymerase for their further conversion in to mcl-PHA (Kraak *et al,* 1997; Lageveen *et al,* 1988).

The β-oxidation cycle

Pseudomonas spp synthesize mcl-PHA through the intervention of β-oxidation cycle (Fig. 11). In this biosynthesis, fatty acids are activated to form the CoA thioesters, from which the reduced form of enoyl-CoA is generated. The intermediates are catalysed by enoyl-CoA hydratases and are converted to 3-hydroxyacyl-CoA. In the presence of NADH dependent 3-ketoacyl-CoA dehydrogenase the intermediate is oxidized to 3-ketoacyl-CoA and finally a shorter chain of fatty acid is formed. 3-Hydroxyacyl-CoA produced by the β-oxidation cycle is used by polymerase for the synthesis of mcl-PHA (Kraak *et al,* 1997; Lageveen *et al,* 1988).

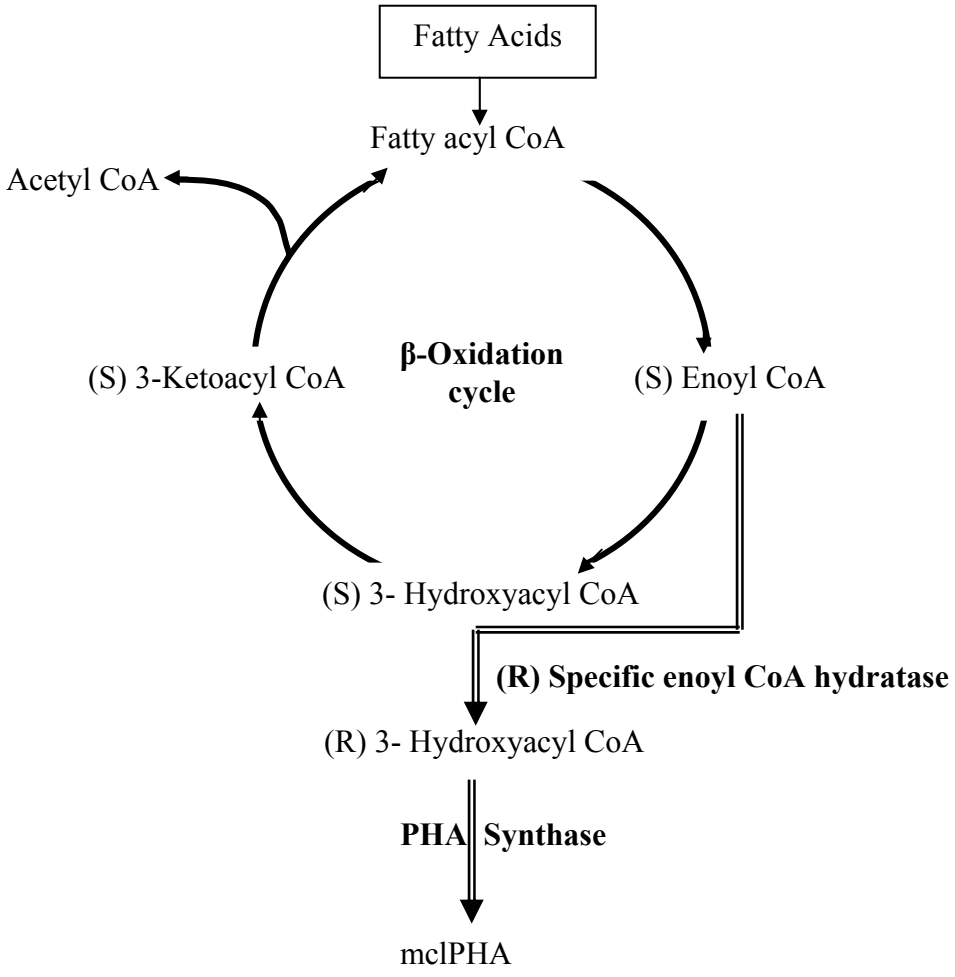


Fig 11: Link between Fatty acid β-oxidation and PHA synthesis

The fatty acid synthesis cycle

Pseudomonas spp are also known to produce mcl-PHA from unrelated carbon substrates. Instead of formation of acyl-CoA from fatty acid degradation, acyl ACP, a precursor for fatty acid synthesis is formed from unrelated substrates, which can result in mcl-PHA production (Fig. 12, Steinbuchel and Eversloh, 2003).

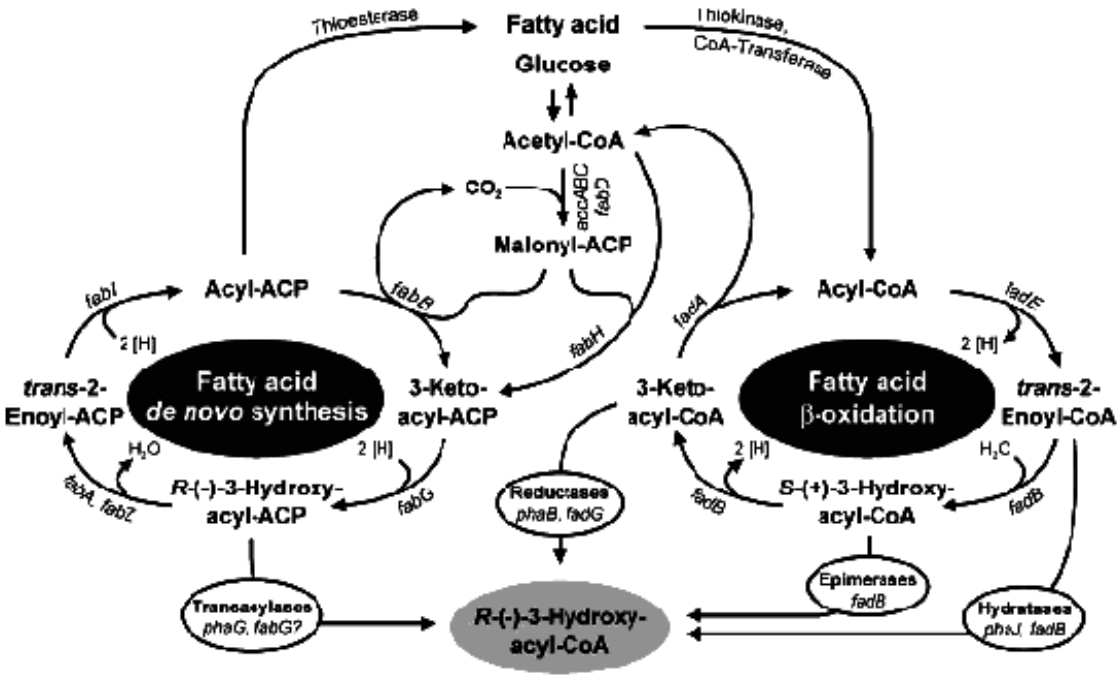


Fig. 12: Linkages between fatty acid metabolisms to synthesize R- (-)-3-Hydroxyacyl CoA for $_{MCL}$ PHA biosynthesis (Ref: Steinbuchel and Eversloh**,** 2003)

Genes essential for PHA biosynthesis

Different genes are involved in PHA biosynthesis in various pathways and in some bacteria they occur together as clusters. The organization of the genes involved in PHA biosynthesis is varied among the organisms and the difference in gene organization makes the different PHA operons. The different modes of arrangement of PHA genes are

given Fig. 13. In *Ralstonia eutropha* and many other scl-PHA producing bacteria three genes are essential for PHA synthesis and accumulation, where the synthesis is initiated with condensation of two acetyl CoA molecules. Many mcl-PHA producing bacteria possess two genes for synthesis. But *phaC* gene (PHA synthase) is very much essential for the synthesis of long chain polymer of PHA.

Different strategies have been applied to identify PHA synthase genes and other genes involved in PHA biosynthesis. Homologous and heterologous gene probes have been synthesized from a well characterized organism like *R. eutrpoha* to screen the genomic libraries of various PHA producing microorganisms and a number of pha operons have been isolated.

***PhaC* (PHA synthase)**

This is the prime gene in the pha operon and it is wide spread in all PHA producing organisms. Based on the mode of action, subunit structure and substrate specificity, the PHA synthases are divided in to four different classes (Table 7).

Type I PHA synthase is represented by the enzyme of *R. eutropha* and the molecular weight of the subunits is 64.3 k Da. Pseudomonas group represents the type II PHA synthase. The molecular weight of the PHA synthase subunit is 62.3 k Da. Type III PHA synthase is represented by the enzyme of *Chromatium vinosm*. It consist of two different subunits coded by two genes –*phaC* and *phaE* exhibiting the molecular weight of 39.7 and 40.5 k Da, respectively.

McCool and Cannon reported (1999) PHA synthase of *Bacillus megateruium* as a unique one and are considered as the newer type (type IV). The molecular weight of the PHA synthase is reported as 40kDa and another protein associated with this phaR protein (22k Da), coded by the gene *phaR*, regulate the PHA synthase activity upon binding.

The size of the gene varies from 1 –2 kb. In *Bacillus megaterium* the size of *phaC* is 1089 base pairs (McCool and Cannon, 1999). The PHA synthase (encoded by *PhaC*), use the CoenzymeA thioesters of hydroxyalkanoic acids (HAs) as substrate and catalyze the polymerization of various HAs in to PHA with the release of CoA. After cloning *Pha* operon from *Ralstonia eutropha* (Schuburt *et al,* 1988; Slater *et al,* 1988; Peoples and

Sinskey, 1989) many other PHA synthase were cloned from various microorganisms and primary structure of 44 different PHA synthases and their nucleotide sequences are available (Table 8).

Table 7: Classes of PHA synthases (Valappil *et al* 2006[a])

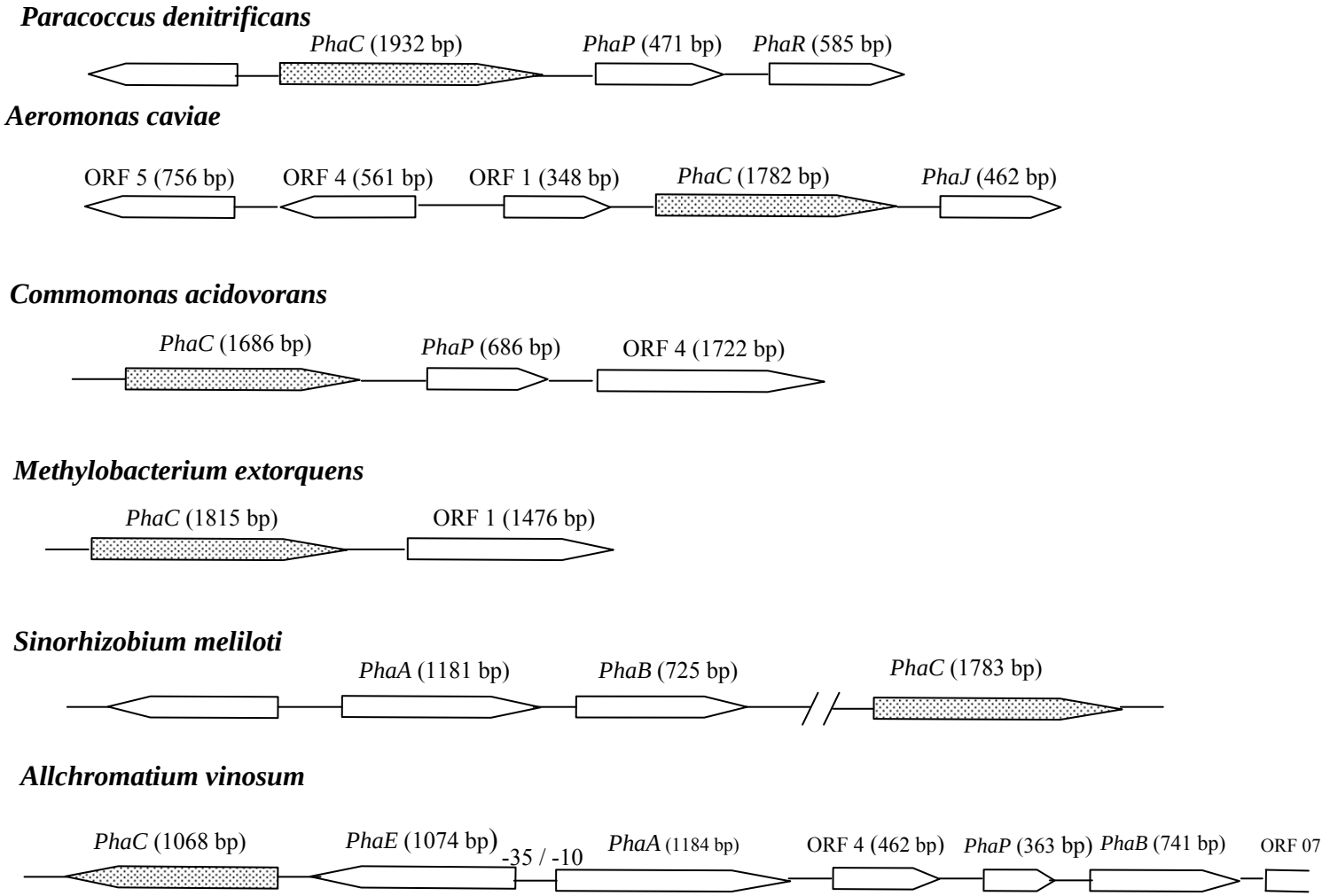
I	PhaC ~ 60 – 73 kDa	***Ralstonia eutropha*** + most other bacteria except those listed below	$3HA_{SCL}$-CoA [~ C_3-C_5] also: $4HA_{SCL}$-Co $5HA_{SCL}$-CoA, $3MA_{SCL}$-
II	PhaC ~ 60 – 65 kDa	***Pseudomonas oleovorans*** + all *Pseudomonas* sp. belonging to rRNA homology group I	$3HA_{MCL}$-CoA [~≥C_5]
III	PhaC ~ 40 kDa, PhaE ~ 40 kDa	***Allochromatium vinosum*** ***Thiocapsa pfenningii*** +other sulphur purple bacteria +all cyanobacteria	$3HA_{SCL}$-CoA [~ C_3-C_5] ($3HA_{MCL}$-CoA [~ C_6-C_8] also: 4HA-CoA, 5HA-C
IV	PhaC ~ 40 kDa, PhaR ~22kDa	***Bacillus megaterium***	$3HA_{SCL}$-CoA [~ C_3-C_5]

Table 8: PHA synthase genes cloned and characterized from bacterial strains

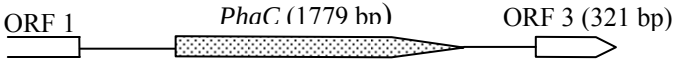
Bacterium	Gene bank Accession number
Acinetobactor sp	L37761
Aeromonas caviae	D88825
Alcaligenes latus	AF078795
Alcaligenes sp. SH-69	U78047
Azorhizobium coulinodans	AJ006237
Bacillus megaterium	AF109909
Burkholderia cepacia	-
Chromatium vinosum D	L01112
Commomonas acidovorans	AB009273
Ectothiorhodospira shaposhnikovii N1	-
Lamprocytis roseopersicina 3112	-
Methylobacerium extorquens IBT6	L07893
Nocardia corallina	AF019964
Paracoccus dentrificans D43784	-
Pseudomonas acidophila	-
Pseudomonas aeruginosa	*X66592*
Pseudomonas citronellolis	-
Pseudomonas fluorescence	-
Pseudomonas mendocina	-
Pseudomonas putida KT2442	-
Pseudomonas sp DSMZ 1650	-
Pseudomonas sp. GP4BHI	-
Pseudomonas sp. 61-3	AB014757/
Pseudomonas sp. Z80158	AB014758
Pseudomonas oleovorans	M58445
Ralstonia eutropha H16	M64341
Rhizobium etli	U30612
Rhodobactor capsulatus	-
Rhodobactor sphaeroides	L17049
Rhodococcus rubber PP2	X66407
Rhodospirullum rubrum HA	-
Rhodospirullum rubrum ATCC25903	-
Sinorhizobium meliloti 41	X93358
Synechocystis sp PCC6803	SlR 1830/29
Syntrophomonas wolfei	-
Thicystis violacea 2311	L01113
Thiocapsa pfennigii 9111	-
Zoogloea ramigera	U66242

Source *Microbial Polyesters*; VCH Publishers: New York

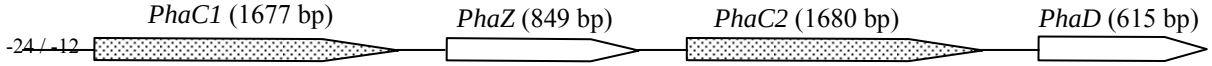
Fig. 13: Organization of genes involved in PHA synthesis in various bacteria



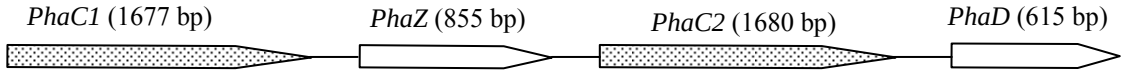
Rhodospirullum rubrum



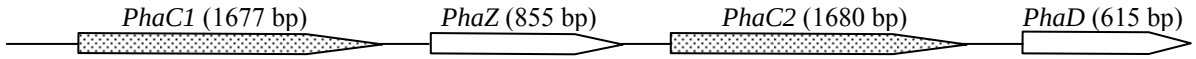
P. oleovorans



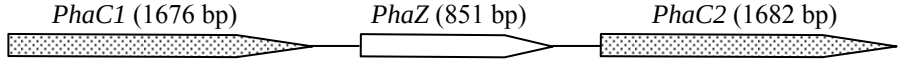
P. aeroginosa



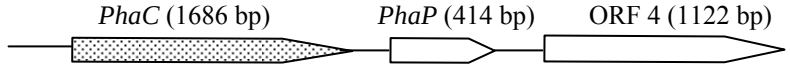
Pseudomonas sp 61-3



Pseudomonas putida U



Rhodococcus ruber



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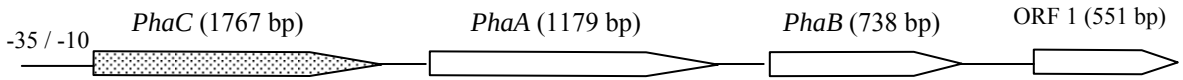

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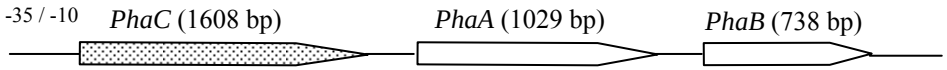
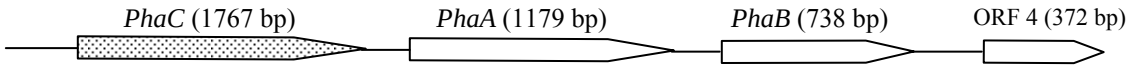
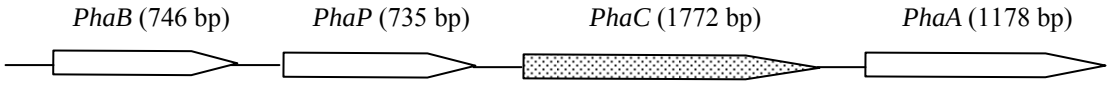


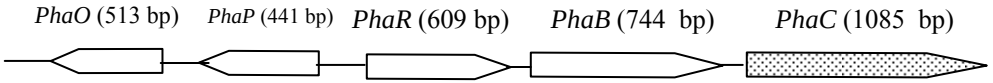
Burkholderia sp. DSMZ 9242



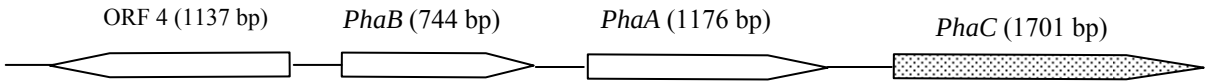
Acinetobactor sp. RA 3849



Bacillus meaaterium



Pseudomonas sp. 61-3



***PhaA* gene (β Ketoacyl CoA thiolase)**

The gene *phaA* codes for the enzyme β-Ketoacyl CoA thiolase, which catalyzes the first step in the PHB synthesis. It is a member of a family of enzyme involved in the thiolytic cleavage of substrates in to acyl CoA and acetyl CoA. They are found in higher eukaryotes, yeasts, prokaryotes, and are divided in to two groups based on their substrate specificity. First group consists of thiolases with broad specificity for β-ketoacyl CoA ranging from C_4 - C_{16}. This class of enzyme is mainly involved in the degradation of fatty acids. The second class of β-ketoacyl CoA thiolase is considered biosynthetic and has a narrow range of chain length specificity (C_3-C_5). These biosynthetic thiolases are specialized for a variety of roles, such as ketone body formation, steroid and isoprenoid biosynthesis and P (HB) synthesis (Masamune *et al,* 1989).

In *R. eutropha,* two β ketothiolases take part in PHA biosynthesis (enzyme A and enzyme B). These enzymes differ in their substrate specificity. The enzyme A is a homotetramer of 44 kDa subunits and convert acetyl CoA into acetoacetyl CoA. In contrast, the enzyme B is a homotetramer of 46 kDa subunits, has broader substrate specificity and cleaves acetoacetyl CoA, 3-ketopentanyl CoA, 3-ketohexanoyl CoA, 3 ketoheptanoil CoA etc. Earlier it was thought that the enzyme B is involved mainly in fatty acid degradation and enzyme A plays a primary role in biosynthesis of PHA (Haywood *et al,* 1988). In *R. eutropha* the size of the β-ketothiolase is approximately 1.7 kb, and one of the ketothiolase gene (*phaA*), is associated with the *pha CAB* operon. In *Bacillus* the gene is ~1.2 kb, and it is not present in the operon of PHA biosynthesis.

***PhaB* (NADPH dependant acetoacetyl CoA reductase)**.

This gene is present in most of the scl-PHA-producing organisms and in many bacteria *phaB* is associated with PHA synthase. In *R. eutropha* it is positioned between *phaA* and *phaC* genes and forms the *phaCAB* operon (Schuburt *et al,* 1988), while in *Bacillus sp* it forms *phaRBC* operon, wherein it is flanked by the *phaR* and *PhaC* (McCool and Cannon, 1999). The enzyme coded by the *phaB*-NADPH dependant acetoacetyl CoA

reductase is a (R)-3 hydroxyacyl CoA dehydrogenase and it catalyzes second step in PHB biosynthesis pathway by converting acetoacetyl CoA in to 3-hydroxybutyryl CoA.

***PhaG* (R) 3 hydroxyacyl ACP CoA transferase)**

The *phaG* gene was identified as a metabolic link between *de novo* fatty acid biosynthesis and PHA biosynthesis from *Pseudomonas putida KT2440* (Rehm *et al* 1998). This enzyme catalyses the conversion reaction of (R) –3 hydroxy ACP intermediates of fatty acid synthesis to the corresponding CoA derivatives, the resultant (R)-3 hydroxyacyl CoA can be utilized as a substrate for PHA synthesis.

***PhaJ* (R)-Enoyl CoA Hydratase**

This enzyme was identified in *A.. caviae* as a molecular converter of β-oxidation intermediate to (R)-3 hydroxyacyl CoA monomer unit of PHA synthesis (Fukui *et al,* 1998). The over expression of *phaJAC* forms a homodimer and shows (R) specific hydration activity towards trans 2 enoyl CoA with 4–6 carbon atoms. The *phaJ* gene from *R. rubrum* expressed in *E. coli* showed a similar substrate specificity to $phaJ_{AC,}$ but an extra C terminal extension of amino acids were present, which help the enzyme to form a homotetramer in the native state (Reiser *et al* 2000). Two *P. aeruginosa* genes homolgus to the $phaJ_{AC}$, referred to the *phaJ1* and *phaJ2* with different substrate specificities were also cloned and their role in PHA production was investigated (Tsuge *et al,* 2000).

Cloning and characterization of genes for PHA synthesis

The introduction of molecular techniques has boosted the biopolymer research that has enabled to understand various regulations in the PHA synthesis in vivo to produce PHA in an efficient way. The isolation of the genes involved in PHA biosynthesis resulted in

heterologous expression of the cloned PHA genes in suitable host organisms and metabolic engineering for higher and safer productivity. Different strategies have been applied to identify and isolate PHA biosynthesis genes from various natural microorganisms. The PHA operon was first characterized in *R. eutropha* (Peoples and Sinskey, 1989) that made available the use of heterologous probe to isolate PHA genes from a number of organisms. Most of the PHA genes reported are isolated from various bacteria through constructing genomic DNA library and screening with probes. Genomic DNA library strategy involves construction of genomic library in *E. coli* and screens the library with heterologous probe. Staining method or complementation studies in PHA negative mutants will monitor the expression of the DNA fragment.

R.. eutropha is a gram-negative bacterium, which is used widely used at industrial level for PHA production from glucose and propionic acid as substrates. PHA operon of *R. eutropha* was isolated by genomic DNA library method and the genes were used for complementation analysis in PHA negative mutants of *R. eutropha*, which is developed by transposon mutagenesis. The results demonstrated that the three enzymes of polyhydroxybutyric acid pathway are organized as *PhbC, PhbA* and *PhbB* down stream to a single promoter. The expression of these genes in *E. coli* resulted in the accumulation of PHA in significant level (Peoples and Sinskey, 1989). Plasmid harboring 5.5 kb DNA insert of *A. latus* DSM1124 was isolated from the PHB producing *E. coli* colony. The insert was sequenced partially and three Open Reading Frames (ORF) were found representing PHA synthase, β ketothiolase and acetoacetyl CoA reductase. The sequences were similar to *R. eutropha* PHA operon; *phaC* 71%, *PhaA* 77% *phaB* 80%. The recombinant *E.coli* accumulated PHA of 20% (Genser *et al*, 1998).

From a genomic library of *Chromatium vinosum* strain D in lambda L47, a 16.5-kbp *EcoRI*-restriction fragment was identified by hybridization with a DNA fragment harboring the operon for *R. eutropha* PHA synthesis (Liebergesell, 1992). The DNA fragment restored the ability to synthesize and accumulate PHA in PHA-negative mutants of *R. eutropha*. The partial sequencing of 6997 bp long fragment revealed the presence

of seven open reading frames (ORFs) which represented the coding regions; six of these are most probably relevant for PHA biosynthesis in *C. vinosum*. The structural genes for biosynthetic acetyl-CoA acyltransferase (β-ketothiolase; $phbA_{Cv}$, 1188 bp) and NADH-dependent acetoacetyl-CoA reductase ($phbB_{Cv}$, 741 bp) were separated by ORF4 (462 bp) and ORF5 (369 bp). The functions of ORF4, ORF5, and ORF7 were not known. The amino acid sequences of β-ketothiolase and acetoacetyl-CoA reductase deduced from *phbACv* and *phbBCv*, exhibited a similarity of 68.2% and 56.4% identical amino acids, respectively, to the corresponding enzymes of *R. eutropha*. The ORF2 (1074 bp), whose function is not known but its presence in *E. coli* was essential for expression of PHA synthase activity. The amino acid sequence of PHA synthase deduced from $phbC_{Cv}$ exhibited 24.7% similarity with the PHA synthase of *R. eutropha*.

PHA operon of *Alcaligens latus* is reported to posses' gene organization similar to that of *R. eutropha* (Choi *et al,* 1998). Using the *Alcaligens latus* strain genomic DNA a plasmid library was constructed in *E. coli* XL Blue with pUC19. A 6286 bp genomic fragment carrying the three ORFs was isolated by probe hybridization. The ORF 1 (1608 bp, 563 aa) showed a sequence similarity of 62% with *R. eutropha phaC* gene. Subsequently ORF2 and ORF 3 were found down stream to *phaC* gene and had amino acid sequence homology of 67% and 75% to β-ketothiolase and acetoacetyl CoA reductase of *R. eutropha,* respectively. The recombinant colony was opaque in nature due to the accumulation of PHA and was able to produce PHA in higher level compared to *R. eutropha*.

A 7,917-bp fragment of DNA was cloned from *Bacillus megaterium* carrying a 4,104-bp cluster of 5 pha genes, *phaP*, -*Q*, -*R*, -*B*, and –*C* (McCool 1999). The *phaP* and -*Q* genes were found to transcribe in one orientation, each from a separate promoter, while immediately upstream, *phaR*, -*B*, and -*C* were divergently transcribed as a tricistronic operon. Transfer of this gene cluster to *E. coli* and to a *PhaC2* mutant of *Pseudomonas putida* gave a Pha1 phenotype in both strains. Transnational fusions to the

green fluorescent protein localized *PhaP* and *PhaC* to the PHA inclusion bodies in living cells. The data presented are consistent with the hypothesis that the extremely hydrophilic protein PhaP is a storage protein and suggests that PHA inclusion bodies are not only a source of carbon, energy, and reducing equivalents but are also a source of amino acids.

Similar strategy was used to isolate PHA biosynthesis genes from *Aeromonas caviae*. A 5.5 kbp *EcoRI-EcoRV* restriction fragment from the genomic library of *A. caviae* was isolated and analysed (Fukui, 1997). The nucleotide sequence of this region showed the presence of three ORFs and a 1782 bp PHA synthase gene was identified by sequence homology with *R. eutropha* PHA operon. The third ORF in the DNA fragment that was downstream to the PHA synthase was identified as enoyl CoA hydratase by checking the enzyme activity.

The cloning of *Thiocystis violacea* was achieved by constructing a genomic DNA library in *E.coli* (Leibergesell and Steinbuchel, 1993). The genomic DNA was digested with *EcoRI* and ligated into λL47 cosmid vectors. A cosmid carrying 5361 bp, which showed signal to the hybridization, was selected and analysed for sequence. This anoxigenic purple, sulfur-reducing bacterium exhibited occurrence of PHA synthase and β-ketothiolase genes as ORF3 and ORF1, respectively. The *phaC* was similar to *C. vinosum* while the *phaB* was absent in the operon. The complementation study using the DNA fragment in PHB mutant *R. eutropha* resulted in the PHA accumulation in significant level.

Using λDASHII replacement vector *Rhodspirullum rubrum*-ATCC 25903 genomic library was constructed in *E. coli* XL Blue1 strain and screened using a probe prepared from the *R. eutropha phaC* gene (Clemente *et al,* (2000). It was found that in *R. rubrem phaC* gene was present independently and lacked *phaA* or *phaB* genes as flanking ORFs. The *phaC* gene was of 1179 bp long and the amino acid sequence similarity was 45% with *Methylobacterium extorquens* and 29% with *R. eutropha*. The *phaC* gene expressed in PHA negative strain of *R. eutropha* in complementation studies.

Chromobacterium violaceum PHA biosynthesis genes were isolated from a 6.3 kb genomic fragment-using DIG labeled *phaC* probe (Kolibachuk *et al,* 1999). The study found that there are three ORFs in a same orientation out of which ORF1 did not show resemblance to any of the PHA genes reported. ORF2 was matching *R. eutropha* PHA synthase by 48% and ORF3 had 69% similarity to *R. eutropha phaA* gene. The gene coding for acetoacetyl CoA reductase was absent in the operon. The *phaC* was expressed in mutant *R. eutropha* and found to produce PHA.

Approximately 4.9 kb Sau3A genomic DNA fragment from *Streptomyces aerofaciens* NRRL 2209 was analysed and found to contain 2 kb long *phaC* gene with a conserved lipase box and 79% GC content (Ramachander and Rawal, 2005). The *phaC* was expressed in *E. coli* (63.5 kDa) and observed to accumulate intracellular PHA.

Engineered pathways

The PHAs are of diverse origin and functions and they have a wide range of uses. Using genetic engineering techniques, qualitative and quantitative PHA production for specific end uses has been optimized. Recombination involving PHA synthesis genes of required characters has been undertaken to obtain specific functions (Preusting *et al,* 1993[a]; Rehm *et al,* 1998; Ren, 1997). A number of PHA biosynthesis genes have been cloned from various organisms, which facilitate the construction of recombinant *E.coli* with engineered pathways to enhance PHA production.

Recombinant DNA technique has enabled to introduce new metabolic pathways (metabolic engineering) to broaden the consumable substrate range, to enhance PHA synthetic capacity and to produce novel PHAs. A recombinant *K. aerogenes* harboring the *R. eutropha* PHA biosynthesis genes was also developed and used for the production of PHB from molasses. This is one of the examples for the introduction of the heterologous PHA biosynthetic pathway form PHA producing bacteria, which would not normally produce PHA. This strategy can be extended to virtually any bacterium, which

possesses metabolic advantages over those currently in use. As an altered pathway to produce novel polyester of poly (3-hydroxypropionate-*co*-3-hydroxybutyrate), *prpE* gene coding for the enzyme propionyl-CoA synthetase from *Salmonella enterica* was expressed in *E. coli.* The recombinant is reported to accumulate a novel PHA, containing poly (3-hydroxypropionate) (Valentin, *et al,* 2000). To increase the availability of acetyl CoA towards PHA biosynthesis pathway a knock out mutant of isocitratelyase in the glyoxylate pathway was developed from *P. putida* KT2442 (Klinke *et al,* 2000). The mutants were superior in growth and accumulation of PHA than the wild strain, which produced 20-30% of PHA after 48 hrs growth. The lack of isocitrate lyase activity resulted in the increased level of acetyl CoA for fatty acid synthesis and PHA accumulation. In order to exploit methylmalonyl CoA pathway and to develop propionate independent pathway for PHV production, genes coding for methylmalonyl CoA mutase and methylmalonyl CoA decarboxylase from *E. coli* was expressed in *pprC* strain of *Salmonella enterica* (which lacks propionate metabolism) along with *Acinetobactor* PHA biosynthesis genes. Recombinant *Salmonella* is reported to produce high amount of PHA with high PHV fraction in the polyester (Ilana *et al,* 2002). In an attempt to construct metabolic link between pentose phosphate pathway and PHA biosynthesis pathway, recombinant *R. eutropha,* which carry two pentose phosphate pathway genes from *E.coli,* was developed (Jim *et al* 2003). Linkage of these two pathways was expected to increase the availability of glyceraldehyde-3 phosphate and NADPH towards PHA production. For this purpose gene coding for the enzymes 6-phosphogluconate dehydrogenase (*6PGDH*) and transketolase (*tktA*) were cloned from *E. coli* and introduced in to *R. eutropha*. The recombinant *R. eutropha* accumulated PHB about 1.7 fold compared to control bacterium at the accumulation phase.

The first successful metabolic production of mcl-PHA in *E. coli* was achieved by the utilization of fatty acid oxidation deficient strain, *E. coli fadB* mutant (Langenbach *et al*, 1997). It is known that in mcl-PHA synthesizing organisms PHA biosynthesis is associated with fatty acid metabolism and for efficient channeling of fatty acid metabolic intermediates to PHA biosynthesis pathway many fatty acid mutants were developed. A

fadA mutant of *E.coli* strain *WA101* showed increased accumulation of 3 hydroxydecanoate monomers up to 93% from sodium decanoate (Park *et al,* 2002). The *fadA* mutant of *E.coli* strain was transformed with *E.coli fabG* gene and *Pseudomonas aerogenosa rhlG* gene, encoding 3-ketoacyl carrier protein reductase to enhance the mcl-PHA production. The strain was able to redirect the 3-ketoacyl CoA thiolase from fatty acid β- oxidation.

The addition of enoyl CoA by cloning acyl-CoA dehydrogenase gene from *E.coli* enhanced the PHA production (Lu *et al,* 2003). Acyl-CoA dehydrogenase gene (yafH) of *E coli* was expressed together with PHA synthase gene ($phaC^{Ac}$) and (R)-enoyl-CoA hydratase gene ($phaJ^{Ac}$) from *Aeromonas caviae* in *E coli* strains JM109, DH5K and XL1-blue. All recombinant *E. coli* strains harboring *yafH*, $phaC^{Ac}$ and $phaJ^{Ac}$ accumulated at least four times more poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHB-co-HHx) compared with the strains harboring only $phaC^{Ac}$ and $phaJ^{Ac}$. It was observed in the study that the over expression of acyl-CoA dehydrogenase gene (yafH) enhanced the supply of enoyl-CoA which is the substrate of (R)-enoyl-CoA hydratase. With the enhanced precursor supply, the recombinants accumulated more PHB-co-HHx. Cloning of *phaJ,* gene coding for the enzyme (R)-specific enoyl CoA hydratase, also enhanced the metabolic link between fatty acid metabolism and PHA production. This enzyme can convert the (S) specific intermediates of fatty acid metabolism to (R) form, which can easily be utilized by the PHA biosynthesis system (Tsuge *et al,* 2003). The presence of enoyl CoA hydratase in recombinant *E. coli* led to the high levels of PHA accumulation (as high as 36-41 wt.% in dry cells) consisting of mainly short- (C_4-C_6) and medium-chain-length (C_6-C_{10}) 3HA units.

PHA production by recombinant *E. coli*

E. coli has been used as the workhorse for the production of PHA since the successful expression of PHA biosynthesis genes from *R. eutropha* and production of PHA using the recombinant *E. coli* strain (Slater *et al* 1998). Some of the major achievement in PHA production using recombinant *E. coli* is listed (Table. 9).

The advantages of employing recombinant *E. coli* as PHA production host is given below (Fidlerand Dennis, 1992).

- Fast growth to a high cell density
- Accumulation of large amount of PHAs
- Ability to use several inexpensive carbon sources
- Easy recovery of PHA
- The lack of intracellular depolymerases

Unlike other PHA producing microorganisms *E. coli* does not require limitation of a specific nutrient for the synthesis of P(3HB), it depends on the availability of acetyl-CoA.

Table 9: Comparison of several PHA production process in recombinant *E. coli*

Strain	Culture mode	PHAs biosynthesis genes source	Type of PHA	Major substrate	Cell conc. (g/l)	PHA content (%)	Producti-vity (g/l/h)
E. coli XL-Blue 1	Fed-batch	*Alcaligenes latus*	P(3HB)	Glucose	194.1	73	4.63
E. coli HMS174	Fed-batch	*Ralstonia eutropha*	P(3HB)	Molasses	39.5	80	1
E. coli GCSC 4401	Fed-batch	*Alcaligenes latus*	P(3HB)	Whey (lactose)	194	87	4.6
E. coli XL1-Blue 1	Fed-batch	*Alcaligenes latus*	P(3HB co-3HV)	Glucose propionic; oleic acid	203.1	78.2	2.88
E. coli XL1-Blue 1	Fed-batch	*Ralstonia eutropha, Clostridium kluyveri*	P(4HB)	Glucose, 4-hydroxy butyrate	12.6	36	0.07
E. coli RS3097	Fed-batch	*Pseudomonas aeruginosa*	mcl-PHA	Decanoic acid	2.6	38	0.06

(**Li *et al*, 2006)**

The synthesis of intracellular PHA is significantly increased by the addition of various complex nitrogen sources (Lee and Chang, 1994[a]). The importance of the presence of acetyl CoA and NADPH, a cofactor of the reductase in the PHA synthetic pathway, for PHA biosynthesis is also authenticated by the raise in PHA level upon adding amino acid or oleic acid (Lee and Chang, 1995. The amino acid and oleic acid are thought to be the sources of acetyl CoA and NADPH) generally. Filamentation of cells is a very common phenomenon during polymer synthesis in *E. coli*. This adversely affects the polymer yield in many fermentation trials. The cell filamentation is due to the inactivation of major cell division protein called *FtsZ*. The expression of this protein along with PHA biosynthesis genes was reported to increase the PHA yield (Lee, 1994[b]). The filamentation suppressed *E. coli* strain allowed production of PHB to a high concentration of 104g/l with P(HB) content of 70% of cell dry weight and productivity of 2g/l/h in a defined medium. *E. coli* normally uses glucose for PHA accumulation. The development of a recombinant *E. coli* strain for the production of P(3HB) from sucrose has also been reported (Zhang *et al*, 1994). A number of fermentation trials have been carried out in the past for the improved production of PHA at economic level (Table: 9). Several efforts have been made to increase the PHA yield since the first report of fed-batch culturing of recombinant *E. coli* harboring the *R. eutropha* PHB biosynthetic genes with a high productivity of 2.08 g/l/h (Lee *et al*, 1994[c]). *E. coli* harboring the *A. latus* PHB biosynthesis genes accumulated PHB more efficiently than those of harboring the *R. eutropha* genes (Choi *et al*, 1998).With a pH-stat fed-batch culture, a high-productivity of 4.63 g/l/h was obtained. During the fed batch culture of recombinant *E. coli*, a large amount of oxygen was necessary to maintain the dissolved oxygen concentration. As a solution to this problem *Vitreoscilla* globin gene (*vgb*) was cloned and expressed in *E. coli* (Yu *et al*, 2002). The expression of *vgb* induced the cell growth and PHB accumulation, especially under low dissolved oxygen concentration conditions during the fermentation process. This methodology was again facilitated by the use of a mutant strain of *E. coli*, arcA mutant, which conferred high respiratory capacity of the host under micro aerobic conditions, and gave rise to higher growth rate and polymer accumulation (Nikel *et al*, 2006). PHA

production from economically cheap substrates using recombinant *E. coli* has gained much attention recently. PHA has been produced using cheese whey, a co-product of dairy industry, as a sole carbon substrate has been reported (Solaiman *et al*, 2006). *E. coli* accumulated PHB at a productivity of 4.6 g/l/h, when it was supplied with whey as a substrate (Wang and Lee, 1998). Recombinant *E. coli* is also reported to use molasses, a co-product of sugarcane refining industries, as a substrate for PHB production. Employing beet molasses as a sole carbon source, *E. coli* accumulated PHB at a productivity of 1 g/l/h (Liu *et al*, 1998).

OBJECTIVES OF THIS THESIS

The review presented above indicated that diversity exists among bacteria with regard to synthesis and accumulation of PHA. A need was felt to explore indigenous bacterial cultures for PHA production. *Bacillus spp* have adapted successfully in harsh environmental conditions, to the stress and nutrient imbalance etc. Hence isolation of *Bacillus sp* and its utilization for PHA synthesis and cloning and characterization of the genes involved in PHA synthesis was undertaken. Development of a recombinant strain harboring PHA synthesis genes from *Bacillus sp* and also a combination of *Bacillus sp* and *Pseudomonas sp* genes for PHA copolymer production containing scl-co-mcl PHA was envisaged.

Based on the above, the main objectives of the thesis were:

1. Isolation of PHA producing *Bacillus* strains.
2. Cloning of the genes involved in PHA biosynthesis from selected species of *Bacillus* and to study the gene organization and polymorphism.
3. Cloning the operon in different heterologus systems such as *E. coli*, *Bacillus subltiis etc*.

The objectives were worked out in detail under five chapters.

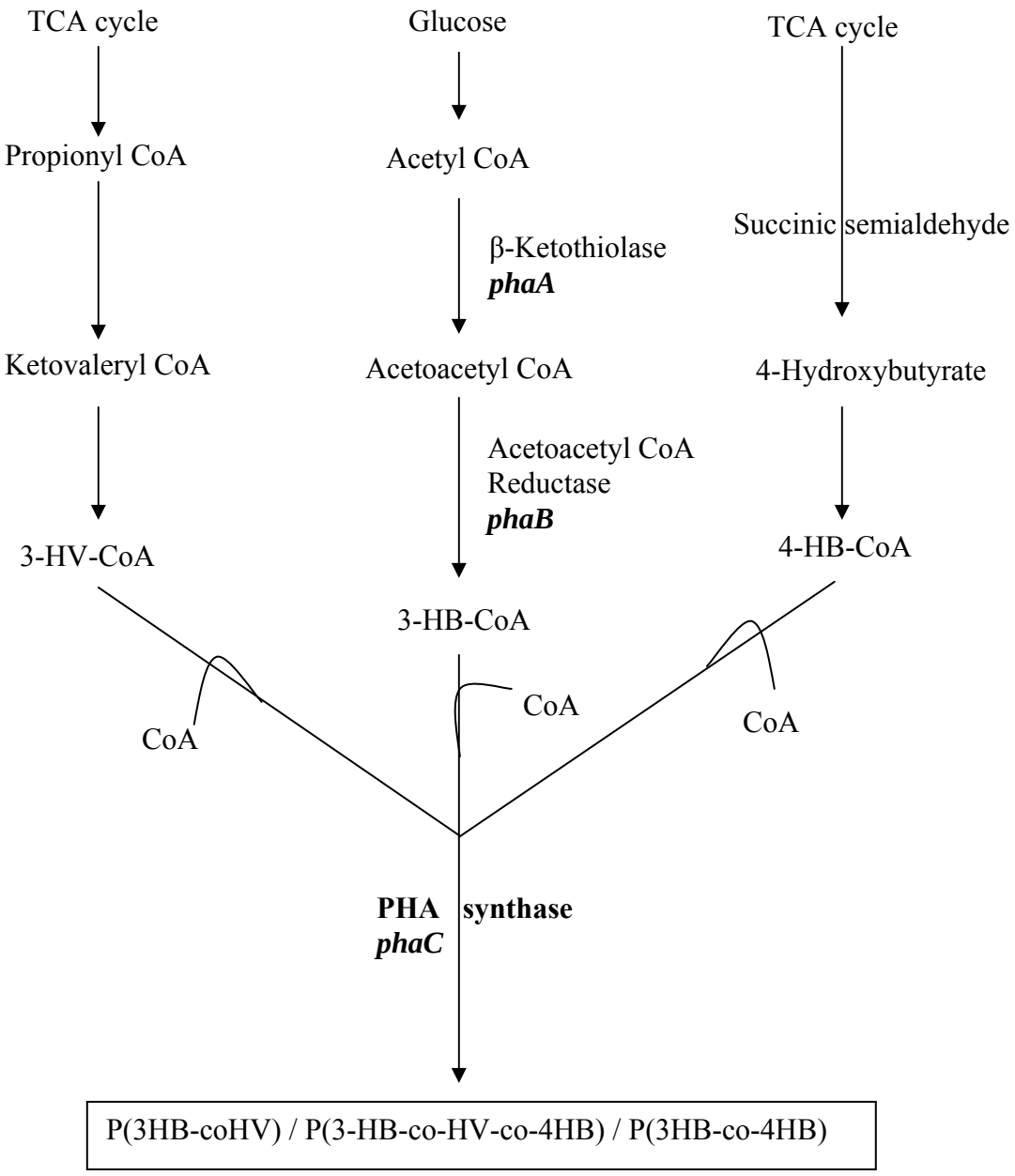


Fig 9: Pathways involved in scl PHA biosynthesis

MATERIALS AND METHODS (GENERAL)

This chapter deals with the general materials and methods used in various experiments that are described in chapters 1-5. Specific methods used are dealt under relevant chapters.

Materials used

Chemicals (General): Most of the chemicals were obtained from known Indian companies such as SRL, Qualigenes, Himedia, Nice and SISCO. Chemicals imported from E Merck (Germany), Sigma (USA) and Sigma Aldrich (USA) was also used. The list of chemicals used are as follows:

Peptone, NaCl, Beef extract, Yeast extract, Agar, Na_2HPO_4 2 H_2O, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$ 7 H_2O, Sucrose, Citric acid, $FeSO_4.7H_2O$; CaCl2 $2H_2O$; $ZnSO_4$ $7H_2O$; $MnSO_4$ $4H_2O$; $CuSO_4$ $5H_2O$; $(NH_4)_6Mo_7O_{24}$ $4H_2O$; $Na_2B_4O_7$ $10H_2O$. KNO_3, NH_4NO_3, $(NH_4)_2HPO_4$, $NaNO_3$, rhamnose, lactose, inositol, sorbitol, dextrin, mannitol, starch, glucose, arabinose, fructose, sucrose, pectin, galactose, sodium hypochlorite, butyric acid, valeric acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, polyhydroxybutyrate; polyhydroxybutyrate-co-hydroxyvalerate (5 mol%), polyhydroxybutyrate-co-hydroxyvalerate (8 mol%), benzoic acid; HCl, H_2SO_4, dintrosalicylic acid, sodium potassium tartarate, NaOH.

Solvents: These were obtained from reputed companies such as SRL, SISCO, E Merck. The solvents used were: chloroform, acetone, ethanol, methanol, hexane.

Equipments:

a) pH meter (Control dynamics)
b) Gas chromatograph
c) Spectrophotometer: Shimadzu, UV 160, Japan
d) Fourier Transform Infrared Spectrophotometer

e) Fermentor: Bio flo 110, New Brunswick Scientific Co. USA; 3 l jar fermentor
f) Incubator shaker
g) Scanning Electron Microscope (LEO-435).
h) PCR machine
i) Gel documentation system
j) NMR: AMX 400 spectrophotometer
k) Electrophoresis system
l) Phase contrast microscope
m) Autoclave
n) Mettler balance
o) Incubator shaker
p) Air flow drier
q) Deep freeze
r) Refrigerators
s) Laminar hood
t) Electroporator machine

Sterilization of the media

Liquid and solid media used in various experiments were autoclaved in flasks or tubes that were plugged with absorbent cotton, at 15-lbs of steam pressure and 121^{o}C for 20 min.

Glass ware sterilization

Glassware such as petriplates, flasks, pipettes and test tubes used for experiments were sterilized in a hot air oven, by dry heat at 180 ^{o}C for 1.5 h. Pipettes were lightly plugged at pipetting end with cotton and loaded into copper container for sterilization.

Microorganism

The bacterium studied in detail in the present study (*Bacillus sp* 256) was isolated from soil sample collected from agricultural land in Mathura (India), which experiences very hot summers (reaching up to 45 ^{0}C). Soil sample was diluted and spread on nutrient agar plate. Out of the several cultures screened for PHA production, *Bacillus sp* 256 was selected for further studies.

Maintenance of microorganisms

The purified culture of *Bacillus sp* 256 was maintained at 4^0 C on nutrient agar slants. The culture was sub cultured once a month for maintenance.

Recombinant strains were maintained on Luria Bertani agar (Himedia, Mumbai, India) slants as stab cultures and on agar plate. The medium contained ampicillin sodium salt (100 mg/l) for sustenance of the plasmids. The culture was sub cultured on monthly basis and stored at 4 ^{0}C.

Inoculum

Inoculum of *Bacillus sp* 256 was prepared by transferring 1-2 loop full of 24 h old slant culture into 10 ml of nutrient broth. The culture was allowed to grow at 30 ^{0}C, 250 rpm for 18-20 h and then transferred to 100 ml sterile nutrient broth contained in 500 ml capacity Erlenmeyer flasks. The inoculated flasks and tubes were incubated at 30 ^{0}C and 250 rpm for 18 h. The inoculum hence obtained contained about 250 mg % biomass (w/v) on dry weight basis. The viable cell count (cell forming unit) was estimated on agar plate and it was $2x10^3$ cfu/ml.

Inoculum of recombinant *E. coli* was prepared as mentioned using Luria Beratni medium with 100 mg/l of ampicillin sodium salt. The culture was allowed to grow for 8 h at 37 ^{0}C and the culture broth contained 13 x 10^4 cfu/ml.

PHA production in shake flasks

PHA production by *Bacillus sp* 256 was carried out in triplicate in 500 ml Erlenmeyer flasks in liquid medium (100 ml). The medium contained (g/l): Na_2HPO_4 2 H_2O, 4.4; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.5; $MgSO_4$ 7 H_2O, 0.2 and sucrose 20. The flasks were inoculated with 10% (v/v) of inoculum and incubation was performed at 250 rpm and 30 ^{0}C for 48-72 h. Experiments were carried out using following components: 1) Propionic, acetic, malic, succinic, valeric, oleic, linoleic, palmitic and stearic acids were added at 3 g/l of medium, as neutralized and sterilized solutions in 5 ml portions of water to 24 h old growing cultures. 2) Rice bran oil, castor oil and pongamia oils were also used (3 g/l) as carbon co substrates. 3) Shake flask experiments were also carried out in triplicate, using sucrose (20 g/l) as main carbon source. After 24 h of fermentation, sucrose (5 g/l, equivalent to carbon content of 3 g of oleic acid), pongamia oil (4.3 g/l which is equivalent to 3 g oleic acid) and saponified pongamia oil (equivalent to 3 g/l oleic acid) were added individually to the flasks and fermentation was carried out up to 72 h. 4) Utilization of various carbon sources was tested by using rhamnose, inositol, sorbitol, dextrin, mannitol, glucose, arabinose, fructose and sucrose at 20 g/l concentration in the medium. 5) To assess the effect of different nitrogen sources on PHA production, KNO_3, peptone, NH_4NO_3, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, tryptone and $NaNO_3$ were added to the medium at 0.3 g N/l level. Sucrose (20 g/l) was used as carbon source. 6) Various media formulated to test the growth and PHA production are shown in Table 20-21. 7) Utilization of various economic substrates for PHA production was tested. This included molasses, cornstarch (hydrolysate) and corn steep liquor. The composition of the media containing these substrates is given in Table 18.

Recombinant strain was cultivated in shake flasks in a) LB medium with 2% (w/v) glucose and 10 mg% ampicillin and b) modified synthetic medium of Wang and Lee (1998). The medium contained (g/l): KH_2PO_4, 13; $MgSO_4$, $7H_2O$, 1.4; citric acid, 1.7; $(NH_4)_2HPO_4$, 4.0; and glucose 20 optionally with yeast extract (Himedia, Mumbai, India), 2 or casein hydrolysate, 2.0. Medium (pH 7-50 ml) contained in 250 ml Erlenmeyer flasks were inoculated (10%, v/v) with 8 h old inoculum and incubated at 30^0

C at 250 rpm for 48 h. Isopropyl-β-D-thiogalacto pyranoside (IPTG) was added (0.4 mmol) after 8 h of growth period. Sodium butyrate, valeric acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid and decanoic acid were added (0.2 g%, w/v) in 5 ml portions of neutralized and filter sterilized solutions after 24 h of growth.

PHA production in fermentor

Bacillus sp 256 inoculum was prepared in 500 ml capacity Erlenmeyer flasks as mentioned earlier. Fermentation was carried out in a jar fermentor (Bio flo 110, New Brunswick Scientific Co. USA) of 3 l capacity, containing 2 l of mineral medium and 200 ml inoculum as mentioned under shake flask experiment. Culture pH was controlled at 7 automatically, by the addition of 1 M NaOH. The dissolved oxygen was maintained above 40% of the air saturation level by varying the agitation and airflow through cascading effect automatically. Experiments conducted were as follows: 1) Control medium with 20 g/l sucrose was used for cultivation of *Bacillus sp* 256. Additional quantity of sucrose (5 g/l) equivalent to carbon content of oleic acid addition was supplied after 8 h of fermentation 2) 3 g/l of oleic acid was added after 18 h growth in the form of sterile solution (25 ml water). 3) 4.3 g of pongamia oil equivalent to 3 g of oleic acid was added to fermenting broth. 4) Fermentation was also carried out using 3 g/l of saponified pongamia oil. All the experiments were carried out for a total period of 40 h.

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

Statistical analysis

Statistical analysis of the results was carried out using computer based Microsoft excel programme with nonbiased or n-1 method.

Residual sugar

Residual sugar in the centrifuged fermented broth was estimated according to the method of Miller (1959) using Dinitrosalicylic acid reagent. Sucrose present in the culture broth was hydrolysed by the addition of 1ml of 1:1 HCl to 5 ml of culture supernatant. The mixture was kept at room temperature for 10-15 h (or in boiling water bath for 30 min) for hydrolysis. The pH of the hydrolysate was adjusted to 7.0 by using 40% NaOH solution and the volume was made up to 50 ml in a volumetric flask. 0.1 to 1 ml of this was made up to a total volume of 1 ml by the addition-distilled water, wherever applicable and used for estimation. 2 ml of DNS reagent was added to 1ml of sample and the tube was kept in boiling water bath for 5 min. The tube was then cooled and 20 ml of distilled water was added. The orange yellow color was measured at 540 nm in a spectrophotometer (Shimadzu, UV 160, Japan) against reagent blank. Glucose was used as standard (up to 2 mg). Reagent: 100 ml of reagent (DNS) contained 1g of 3.5-Dinitrosalicylic acid, 20 ml of 2 N NaOH and 30 g of Na-K-tartarate.

Viable plate counts

Total plate counts or viable cell count or cell forming units (cfu) of inoculum was estimated by plating diluted fresh growing culture on nutrient medium. One ml of the broth was serially diluted six times in test tubes containing 9 ml of sterile water and homogenized. 0.1 ml sample from individual dilutions were plated in duplicates on nutrient medium contained in petriplates. Plates were incubated at 30 ^{o}C for 24-48 h and the number of colonies developed was counted. Number of viable cells was presented as colony forming units or viable cell counts/ml.

Staining Methods

a) Gram staining

Reagents

a) One volume of saturated solution of crystal violet was added to 4 volumes of ammonium oxalate (1%) solution and filtered.
b) Potassium iodide (2g) was dissolved in 300 ml of water. Iodine (1g) was solubilised in this KI solution.
c) Absolute alcohol
d) Saturated alcohol solution of safranin (100 ml) was prepared and the volume was made up o 1litre with distilled water.

Method: A thin smear of the culture was made on a glass slide and heat fixed. Crystal violet reagent was added to the smear and allowed for 30 sec. Excess of stain was removed by rinsing with distilled water. Iodine solution was added and the slide was left for 30 sec and rinsed again with distilled water. Ethanol was added to remove excess stain and the slide was rinsed with water and finally safranin solution was added and allowed to act for 30 sec. The slide was then washed and the dry slide was observed under the microscope. Retention of crystal violet indicated that the bacterium was gram positive and uptake of pinkish safranin counter stain by cells was considered gram negative.

b) Sudan Black staining (Gerhardt *et al* 1981)**:**

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

Method: Cells were smeared on to a glass slide and heat fixed. Sudan black reagent was added to the heat fixed cells and allowed to stain for 20 min. Then the slides were washed with distilled water and counter stained with safranin (0.5% in water). The slides were washed with distilled water after 30 seconds, dried and then observed under light microscope. Presence of polyhydroxyalkanoate was visualized as blue stained granules inside the cells.

Media

Maintenance medium

Nutrient Agar

Composition	g/l of distilled water
Peptone	5.0
NaCl	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	1.5
(pH 7.0)	

PHA production medium

Composition	g/l of distilled water
Na_2HPO_4 2 H_2O	4.4
KH_2PO_4	1.5
$(NH_4)_2SO_4$	1.5
$MgSO_4$ 7 H_2O	0.2
Sucrose	20.0
(pH 7.0)	

Luria Bertani medium

Components	g/l of distilled water
Bacto-tryptone	10.0 g
Bacto-Yeast extract	5.0 g
Sodium chloride	10.0 g
(pH 7)	

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

Composition	**g/l of distilled water**
KH_2PO_4	13.0
$MgSO_4$ $7H_2O$	1.4
Citric acid	1.7
$(NH_4)_2HPO_4$	4.0
Glucose	20.0
Yeast extract (Optional)	2.0
Tryptone	2.0
Trace metal solution	1.0 ml/l

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

Biochemical tests

Catalase test

NA slants were inoculated with the culture and incubated overnight at 30 ^oC. 1ml of 3% H_2O_2 was pipetted on to the slant. The slant was examined for evolution of bubbles, the presence of which indicated a positive test.

Oxidase test

A filter paper strip was moistened in 1% solution of N,N,N^1,N^1,-tetramethyl-p-phenylenediamine-hydrochloride. Cells from the test culture was transferred on to the paper. Development of purple colour indicated that the test was positive for oxidase.

Cell size

Cell size was measured using ocular micrometer (one division =1/10mm) in the microscope. The ocular micrometer was calibrated by using the stage micrometer (1 scale

division = 1/100 mm). The cell size was calculated on the basis of 10 values recorded for length and breadth.

Growth on different carbohydrate sources

For characterization of the isolated culture of *Bacillus* sp, the culture was grown on NA in the presence of 5 mg% bromocresol dye. Carbohydrates such as fructose, mannose, cellobiose, rhamnose, maltose, glucose, raffinose, sucrose, trehalose, dulcitol, adonitol, arabinose, sorbitol, xylose, mannitol, melibiose and inulin were substituted as carbon sources in the medium individually at 2 g% (w/v) level. The culture was incubated at 30 ^{0}C up to 72 h. Change in the dye colour was observed and recorded. Formation of hay yellow colour indicated the formation of acid.

Fourier Transform Infrared Spectroscopy

a). Standard PHA, PHA from recombinant *E. coli* (5 mg each) were mixed with 100 mg of FTIR grade KBr and pelletized. The FTIR spectrum was recorded at 400-4000 cm^{-1} in FTIR Nicolet Magna 5700 spectrophotometer. b). PHA samples (10 mg) were dissolved in 200 µl of chloroform and placed on KBr window and spectrum was recorded.

Scanning Electron Microscopy

To study the morphology of the cells, culture broth was centrifuged and the cells were washed twice in phosphate buffer 0.1M (pH 6.5). The washed cells were fixed in 1-2% glutaraldehyde overnight. The cells were centrifuged and serially washed with 10-100% gradient of ethanol followed by methanol. The sample was then dried in a desiccator. The dried cells were sputter coated with gold and visualised in a scanning electron microscope (LEO-435 VP scanning electron microscope). Scanned image was captured at a magnification of 5000 X to study the morphology of the cells.

Estimation of biomass

Dry weight of the cells was estimated by centrifugation of fermented broth at 8000 rpm for 15 min followed by washing and drying of the sedimented cells at 50 ^{0}C to a constant weight.

Extraction and Quantification of PHA

Gravimetric method:

PHA content of bacterial cells was determined gravimetrically by sodium hypochlorite method (Law and Slepecky, 1961; Williams and Willkinson, 1958). Dried biomass was held in sodium hypochlorite (5% chlorine) and allowed for digestion for 1h at 37 ^{o}C. After 1h the suspension was centrifuged at 6000 rpm for 15 min. The pellet obtained was washed successfully with 5 ml each of water, acetone, diethyl ether and absolute alcohol. The final white powder obtained was dissolved in chloroform and air-dried. The dried polymer obtained was then weighed. PHA concentration was expressed as % PHA in the biomass.

Isolation of PHA by solvent extraction

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

GC method

Quantitative and qualitative estimation of PHA was carried out by GC analysis using lyophilized cells and purified polymer (Brandl *et al,* 1988). For this analysis, PHA and the cells were subjected to methanolysis in sealed test tubes with a solution consisting of 1 ml chloroform, 0.85 ml methanol and 0.15 ml conc. H_2SO_4 at 100 ^{o}C for 140 min. After cooling, seal was broken, deionized water was added, the contents were homogenized and the bottom phase was used for GC analysis. The methyl esters were analyzed by GC with flame ionization detector, in a 30 m DB-1(fused silica gel - polymethyl siloxane) capillary column (internal diameter 0.25 mm and film thickness

0.25 microns). N_2 (1 ml/min) was used as a carrier gas. The injector and the detector were maintained at 170 ^oC and 220 ^oC, respectively. Temperature program used was**:** 55 ^oC for 7 min; ramp of 4^o C per min up to 100 ^oC; 10 ^oC per min rise up to 200 ^oC and hold at 200 ^oC for 10 min. Calibration was performed using standard P(HB), P(HB-co-HV) containing 5/8 mol% of hydroxyvalerate (Sigma Aldrich, USA) with benzoic acid as internal standard.

NMR analysis

Lyophilized cells (100 mg) were suspended in ten ml of chloroform and extracted overnight at 40 ^0C. Cell sediment was separated by centrifugation at 1900 x g for 20 minutes and PHA was isolated from clear chloroform layer by the addition of 2 volumes of hexane. Precipitated polymer was air-dried at 40 ^0C. ^{1}HNMR of the polymer was carried out in deuterated chloroform at 400 MHz on an AMX 400 spectrophotometer. [P(HB), P(HB-co-HV) from Sigma Aldrich, USA] were used as standards.

MOLECULAR BIOLOGY METHODS

General chemicals

Most of the chemicals such as Tris Base, Tris HCl, EDTA, agarose, glacial acetic acid, phenol, chloroform, CH_3CooNa, ethanol, isopropanol, tritonX, SDS, NaOH, proteinase K, potassium acetate, $CaCl_2$ $7H_2O$, glucose, antibiotics, Taq polymerase, dNTPS, T4 DNA ligase, acrylamide, bis-acrylamide, ammonium per sulphate, TEMED, Tris, glycine, restriction enzymes, DNA and protein markers, were collected from Indian companies. All laboratory reagents used were of molecular biology or A.R. grade wherever necessary. Stock solutions and media were sterilized by autoclaving at 121^0C for 20 min. Reagents and buffers not suitable for autoclaving were sterilized by filter sterilization using Millipore disposable filters (0.4 μm). Standard procedures (Sambrook

and Russell, 2001) or manufacturer's instructions in the case of commercial kits were followed for all the experiments, unless specified otherwise.

Isolation of *Bacillus* genomic DNA

Bacillus genomic DNA was isolated using nucleo-Spin extraction kit (Macheri Nagel, Germany) following manufacturers instructions. The quality and quantity of the genomic DNA was analysed by agarose gel elctrophoresis.

Agarose gel Electrophoresis

Materials and solutions

1. Agarose (SRL, Mumbai, India).
2. TAE 50 X buffer: (100ml.)

   24.2 g Tris base, 5.71 ml of glacial acetic acid and 10 ml of 0.5 M EDTA (pH 8.0) were added to 80 ml of distilled water. The pH was adjusted to 7.2 and the final volume was made up to 100 ml with distilled water. The buffer was sterilized by autoclaving and stored at room temperature.
3. 100bp DNA ladder (Bangalore Genei).
4. Gel casting boat
5. Mini gel apparatus and power supply (Bangalore Genie, India).
6. Ethidium bromide stock solution (10 mg/ ml):

   10 mg of ethidium bromide (Sigma, MO, USA) was dissolved in 1 ml of distilled water. The solution was stored in a micro centrifuge tube wrapped in aluminium foil at 4 ^{0}C.

Methodology

The boat was sealed with an adhesive tape and the comb held in place for the formation of wells. 1.2 g of agarose was added to 100 ml of 1 X TAE buffer. The mixture was heated for the solubilization of agarose. The solution was cooled to 50 ^{0}C and poured into the sealed boat. The gel was allowed to polymerize. The comb and the adhesive tape were removed and the gel was placed in the electrophoresis tank with sufficient volume

of 1 X TAE buffer to cover the surface of the gel. The samples and the standard DNA size marker were loaded in the wells. Electrophoresis was carried out at 50 volts till the dye reached 75% of the total gel area. The gel was removed from the tank and stained by soaking in a solution of 0.5 μg/ml ethidium bromide for 30 min at room temperature. The gel was destained in distilled water for 10 min, examined on a UV transilluminator and documented using Gel Documentation system (Herolab, Germany).

Designing of oligonucleotides

Oligonucleotide primers for the experiments were synthesized based on the DNA sequences available in the GenBank (Table 10) using various online software.

Table 10: Gene bank nucleotide sources

gi number	*Bacillus sp*
>gi|13812266	*Bacillus megaterium*
>gi| 27348111	*Bacillus sp* INT005
>gi|30018278	*Bacillus cereus* ATCC 14579
>gi|42779081	*Bacillus cereus* ATCC 10987
>gi|52140164	*Bacillus cereus* ZK
>gi|50196905	*Bacillus anthracis* str. 'Ames Ancestor'
>gi|57596592	*Bacillus halodurans* C-125
>gi|49476684	*Bacillus thuringiensis* serovar konkukian str. 97-27
>gi|50812173	*Bacillus subtilis* subsp. subtilis str. 168

Polymerase Chain reaction

PCR reactions were carried out in a thermocycler GeneAmp PCR System 9700 (Perkin-Elmer, USA) the conditions for which described under relevant sections.

Purification of PCR products

PCR amplicons used in the study were purified using GenElute PCR Clean - Up Kit (Sigma, USA) following manufacturers instructions.

Corresponding methodologies used in cloning are dealt with in respective chapters.

A-tailing of PCR products using *Taq* DNA polymerase

A-tailing of the purified phaC amplicon was carried out by the reported method (Kobs, 1997). To 5 µl of purified PCR fragment, 1 µl of *Taq* DNA Polymerase reaction buffer (1X) and 1 µl of 25 mM $MgCl_2$ were added. dATP to final concentration of 0.2 mM and 5 Units (2 µl) of *Taq* DNA polymerase (Bangalore Genie, India) were added to the reaction. The samples were incubated at 70^0C for 20 to 30 min. To remove the residual dATP present in the reaction mixture, the PCR product was purified using GenElute PCR Clean - Up Kit (Sigma, USA). The A-tailed PCR product was ligated to T-tailed vector.

1.0 INTRODUCTION

A wide variety of microorganisms are capable of synthesizing polyhydroxyalkanoates (PHAs). The amount of PHA accumulated by microorganisms vary from 20-80% of their cellular dry weight and form intracellular granules in the cytoplasm as inclusion which can be observed under light microscope as refractive bodies. PHA was first discovered by Lemogine (1926), who reported the presence of 3-hydroxybutyric acid in the cells of *Bacillus megaterium*. The similarity in material properties of PHA with polypropylene has enabled the use of the polymer in several applications, such as packaging, pharmaceuticals, agriculture and food industry, or as raw materials for the synthesis of enantiomerically pure chemicals and the production of paints (Anderson and Dawes, 1990).

PHAs are synthesized as intracellular energy and carbon storage material, which enable the bacteria to survive under certain adverse conditions. They are biodegradable, insoluble in water, non-toxic, biocompatible, thermophilic and elastomeric. The bacterial origins of the PHAs make these polyesters a natural material, and many microorganisms have evolved the ability to degrade these macromolecules.

It took a long time to achieve the commercial production of PHA and it was produced in 1982, by ICI at commercial level under the trade name Biopol®. Among the various microorganisms identified as PHA producers, only a few have been exploited commercially for PHA production due to their higher efficiency to accumulate PHA. Some of the commercially important strains are *Ralstonia eutropha*, *Alcaligens latus*, *Azotobactor vinilandii*, *Pseudomonas spp* etc. Various *Bacillus spp* are known to produce PHA (Valappil *et al* 2007[a]; Shamala *et al*, 2003). Generally *Bacilli* produce scl - PHA, which comprises of PHA of C_4-C_6 and a few strains are reported to the ability of some members in the genera to produce mcl –PHA also (Caballero *et al*, 1995; Tajima *et al*, 2003).

There are advantages using *Bacillus spp* as PHA production hosts as they grow fast, can produce copolymers from single carbon substrate and they can utilize cheaper carbon sources for polymer synthesis (Table 6). The fermentation can result in the release of certain industrially important enzymes such as proteases and amylases, they lack lipopolysachharide which is present in gram-negative organisms that co purify with PHA and causes immunogenic reactions in certain individuals. The genome data for some *Bacilli* are available so it is easier to manipulate them genetically. A vast number of *Bacillus spp* can be isolated from the environment, which may produce varied quantity and quality of the polymer. The present chapter describes the isolation and screening of some PHA producing *Bacillus spp*, identification and characterization of a potent PHA producing strain using morphological, physiological and molecular techniques.

1.1 MATERIALS AND METHODS

Media used for maintenance of *Bacillus spp* and production of polymer using the isolated strains are describe below. *E. coli* DH5α was grown in LB medium and the composition of which is detailed under general materials and methods (page 53).

1.1.1 Nutrient Agar (Maintenance medium)

Components	**g/l of distilled water**
Peptone	5.0
NaCl	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
pH 7.0	

1.1.2 PHA production medium

Components	**g/l of distilled water**
Na_2HPO_4 2 H_2O	4.4
KH_2PO_4	1.5
$(NH_4)_2SO_4$	1.5
$MgSO_4$ 7 H_2O	0.2
Sucrose	20.0
pH 7.0	

1.1.3 Isolation of *Bacillus spp.*

Soil samples were collected from different parts of the country and stored at room temperature. The dry soil samples were heated at 60^0C for 30 min, serially diluted and plated on nutrient agar plates. The plates were incubated at 30 ^{0}C for 24-48 h. In the identification process the *Bacillus sp*p were initially selected based on the Gram reaction, morphology and catalase test. The screening of PHA producing *Bacilli* was performed by Sudan black B staining method (materials and methods page 52). The *Bacllii* showing

PHA granules were selected for further studies. The selected strains were used further for molecular detection of PHA synthase gene by polymerase chain reaction (PCR).

1.1.3.1 Inoculum

Inoculum was prepared by transferring 1-2 loop full of 24 h old slant culture in to 5 ml of nutrient broth. The cultures were allowed to grow at 30^0 C, 250 rpm for 18-20 h. The inoculum hence obtained contained 2 x 10^3 viable cells / ml as assayed on nutrient agar plates.

1.1.3.2 PHA production in shake flasks

The isolated strains were cultivated in sterile PHA production medium (50 ml) in triplicate that was contained in 250 ml capacity Erlenmeyer flasks. The above-mentioned inoculum was transferred in to production medium and the flasks were incubated at 30^0 C, 250 rpm for 72 h.

1.1.3.3 Estimation of biomass and extraction of PHA

Culture broth was centrifuged (7000 rpm, 20 min) and the washed cell sediment was dried to a constant weight at 70 ^{0}C. The dried cells were extracted by sodium hypochlorite extraction method, which is described under the materials and methods section (Page 56).

1.1.4 Characterization of *Bacillus sp*. 256

Taxonomic characteristics of the *Bacillus sp* 256 was studied by following biochemical tests (Reddy and Reddy, 2000).

1.1.4.1 Gram staining

Reagents

- ***Crystal violet***

Solution A

Crystal violet (90% dye content)	2 g
Ethyl alcohol (95%)	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80 ml

Mix the solutions **A** and **B**

- ***Gram's iodine***

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 ml

- ***Ethyl alcohol 95%***

- ***Safranin***

Safranin	0.25g
Ethyl alcohol 95%	10 ml
Distilled water	100 ml

A thin smear of the culture was made on a glass slide and heat fixed. Crystal violet reagent was added to the smear and allowed for 30 sec. Excess of stain was removed by rinsing with distilled water. Iodine solution was added and the slide was left for 30 sec and rinsed again with distilled water. Ethanol was added to remove excess stain and the slide was rinsed with water and finally safranin solution was added and allowed to act for 30 sec. The slide was then washed and then observed under the microscope. Retention of crystal violet indicated that the bacterium was gram positive and uptake of pinkish safranin counter stain by cells was considered gram negative.

1.4.1.2 Endospore staining

- A smear of *Bacillus sp* 256 was made on a clean slide, the cells were air dried and heat fixed
- A few drops of 5 % malachite green solution were placed on the smear and the slides were heated by steaming for 5 minutes. More stain was poured on to the smear from time to time.
- The slides were washed under slow running water
- Samples were air dried and counter stained with 0.5% safranin and allowed for staining for 30 second and excess stain was removed using water and blotted to remove the moisture and dried
- The slides were viewed under microscope. Bluish green spores in the cells indicated presence of endospore.

1.1.4.3 Cell size

Cell size was measured using ocular micrometer (one division =1/10mm) in the microscope. The ocular micrometer was calibrated by using the stage micrometer (1 scale division = 1/100 mm). The cell size was calculated on the basis of 10 values recorded for length and breadth.

1.1.4.4 Catalase test

NA slants were inoculated with the culture and incubated overnight at 30^{o}C. 1ml of 3%H_2O_2 was pipetted on to the slant. The slant was examined for evolution of bubbles, the presence of which indicated a positive test.

1.1.4.5 Oxidase test

A filter paper strip was moistened in 1% solution of N,N,N^{1},N^{1},-tetramethyl-p-phenylenediamine-hydrochloride. Cells from the test culture were transferred on to the paper. Development of purple colour indicated that the test was positive for oxidase.

1.1.4.6 Nitrate reduction

The basal medium was supplemented with 0.1% potassium nitrate and 0.17% agar and inoculated with fresh culture of *Bacillus sp* 256 and incubated for 24 h. To 24h old culture 1 ml of solution A (1-naphthyl ethylenediamine (0.02 g) dissolved in 100 ml of 1.5N HCl) and 1 ml of solution B (1 g of sulfanilic acid in 100 ml of 1.5N HCl) were added. The development of pink/ red colour indicated the presence of nitrite in the medium.

1.1.4.7 Hydrolysis of gelatin

12% gelatin was added to nutrient agar medium and the *Bacillus* was inoculated and incubated at 30^0C for 24h. The tubes were observed for hydrolysis or loosening of the solid medium.

1.1.4.8 Hydrolysis of casein

Skimmed milk agar was prepared by mixing sterile skimmed milk with double strength nutrient agar medium at 50^0C and plated. The plate was streaked with test culture and incubated. The clear zone around the colony indicated the hydrolysis of casein.

1.1.4.9 Hydrolysis of starch

Nutrient agar medium containing 2% starch was prepared in petriplates. The plates were streaked with the test organism and incubated at 30^0C for 24h. The plates were treated with iodine solution and observed for the clearance zone around the colony.

1.1.4.10 Acid production from sugars

Nutrient agar medium containing 5mg% of bromo cresol purple and 2g% of sugar was prepared. Various sugars tested included arabinose, mannitol, lactose, xylose, rhamnose, cellobiose, sucrose, glucose and maltose. The bacterial culture was inoculated in the medium and incubated. The colour change from purple to yellow indicated acid production by bacteria.

1.1.4.11 MR-VP test

Requirements Nutrient broth culture, MR-VP broth tubes, methyl red, pH indicator, Barrit's reagent

Procedure

1) Took 5 ml of MR-VP broth in each tube and sterilized by autoclaving.

2) Inoculated two MR-VP broth with *Bacillus* 256, incubated at 37^{o}C for 48 h.

3) At the end of incubation period, added 1-2 drops methyl red and 2-3 drops of Barrit's reagent mixed thoroughly after removing the cap to expose optimum amount of oxygen.

4) Allowed the reaction to complete for 15-30 minutes.

The results were analysed on the basis of presence of red and yellow colur (+ve and –ve for MR respectively); presence of pink colour (VP positive and colourless for VP negative).

1.1.4.12 Growth in 3% NaCl and anaerobic growth in the presence of glucose

The strain was inculated into the nutrient broth and nutrient agar media containing 3 % NaCl and incubated overnight at 30^{0} C.

The strain was stabbed into nutrient agar medium containing 1 % glucose, the culture was overlaid with sterile glycerol and incubated overnight at 30^{0} C.

1.1.4.13 Confirmatory tests for *B. endophyticus*

Tubes containing the culture were incubated at 28^{0} C for 3-4 days on PHA agar medium. Anaerobic growth was tested in the fermentative medium under sterile mineral oil. Growth in the presence of ampicillin (100μg/ml) and lysozyme (1 mg/ml) were tested in nutrient broth for 3-4 days at 28^{0} C.

1.1.5 Molecular characterization

The molecular level characterization of the *Bacillus sp 256* was carried out by 16srRNA gene sequence homology study. One portion of the 16srRNA gene was amplified from

genomic DNA of *Bacillus sp* 256 by PCR. The primer used for PCR amplification is given below (Table 11).

Table 11: 16SrRNA gene primers

Primer	Sequence	Region
rRNAF	GCT CTA GAG CGA TTA CTA GCG ATT CCG ACT TCG	1324 –1353
rRNAR	CGA CGT CGG CTC AGG ATG AAC GTC GGC GGC	15-43

1.1.5.1 Polymerase chain reaction

The PCR reaction was carried out by combining the following components in 25µl reaction volume:

Components	Volume (µl)	Final concentration
Nuclease-free water	18.7	
10 X Reaction Buffer	2.5	1 x
dNTP mix (10 mM)	0.5	0.2 mM
Taq polymerase (3U/ µl)	0.3	0.03U/µl
Primer B1F (Forward)	1.0	0.2 µM
Primer B1R (Reverse)	1.0	0.2 µM
Template (~100 ng)	1.0	
Total reaction volume	25.0	

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

Initial denaturation: 94^0 C for 3 min
Denaturation: 94^0 C for 1 min
Annealing: 46^0 C for 1 min
Extension: 72^0 C for 2 min
35 cycles
Final extension: 72^0 C for 10 min

The 1.2 kb PCR product was cloned in sequencing vector pTZ57R/Tand sequenced partially by dideoxy method at Bangalore Genie, Bangalore, India. The sequence data was taken for further analysis.

1.1.6 Construction of phylogenetic tree

The phylogenetic relationship of the *Bacillus sp* 256 was studied by deriving phylogenetic tree from BLAST results.

1.1.7 Detection of PHA producing *Bacillus sp* by PCR

Genomic DNA was isolated from all the *Bacillus* isolates and subjected to PCR using *phaC* specific primers (Table 12).

Table 12: Primers used for PCR detection

Primer	Sequence	Amplicon size
B1F	AACTCCTGGGCTTGAAGACA	600 bp
B1R	TCGCAATATGATCACGGCTA	

1.1.7.1 Methodology

Various PCR conditions were optimized to obtain the 600 bp amplicons for *phaC* gene. Primer combination of B1F and B1R resulted in amplification of a product of 600 bp in size. The PCR was carried out by the following PCR parameters: Initial denaturation at 94^0 C for 2 min, annealing at 56^0 C for 1.5 min and the extension temperature at 72^0 C for 2 min and a final extension step at 72^0 C for 10 min. The PCR amplifications were performed using Taq DNA polymerase (Bangalore Genei, India). The PCR reactions were conducted in 25 µl volumes containing 50 ng of *Bacillus* genomic DNA, 10 mM of

dNTP mix, 10 μM each of forward and reverse primer and 1x Reaction Buffer (10 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin)

1.1.7.2 Analysis of PCR product

After the reaction, 10 μl of the sample was run on 1% agarose gel electrophoresis as described in chapter III (page 124). The size of the *Bacillus phaC* gene amplicon was checked in comparison with a 100 bp DNA ladder (Bangalore Genei, India).

1.2 RESULTS

1.2.1 Isolation of *Bacillus sp* and PHA production.

Different *Bacillus sp* were isolated from various soil samples. PHA production was carried out using 38 isolated strains. Growth and accumulation of PHA among these strains varied in the medium containing sucrose as carbon source (Table 13). Accumulation of PHA varied from 12-55% of dry biomass weight. Maximum concentration of PHA was synthesized by *Bacillus sp* 256 compared to others. This culture gave optimum growth on nutrient agar slant and dense growth in synthetic medium and was used for further studies (Figs. 14 & 15). Amongst the cultures isolated many of them gave poor yields of PHA ranging from 12% to 20% of biomass dry weight (No. 2, 3, 4, 8, 9, 11, 14, 17, 18, 19, 22, 18, 25, 31, and 36), while in others the yields ranged from 20-55%.

1.2.2 PHA production by *Bacillus sp* 256

The *Bacillus sp* 256 grew efficiently in PHA production media, containing sucrose as the carbon source, and accumulated PHA at 18- 20 h of incubation. Maximum PHA accumulation occurred during 65 to 70 h. The polymer concentration decreased after 72 h of the growth due to intracellular degradation of the polymer. The isolated strain produced 55% PHA of dry biomass weight.

1.2.3 Characterization of *Bacillus sp* 256

Bacillus sp 256 was characterized by morphological, biochemical (Table 14) as well as at molecular level. *Bacillus* 256 was gram positive, rod shaped (Fig. 16A) and non motile. The bacterium produced endospores at apical position (Fig. 16B). The cells were found in chains. The size of the cell was determined and it was 6 x 1.5µ. The *Bacillus* colonies were circular, off white dry and translucent. It showed positive reaction for catalase, nitrate reduction, and oxidase tests. It could not hydrolyse starch, gelatin and casein.

Table 13: Accumulation of PHA by various *Bacillus* isolates

Culture No.	Biomass (g/l)	PHA% of dry biomass
1	1.8	20
2	2.2	17
3	2.3	12
4	1.9	19
5	2.6	21
6	2.1	38
7	2.4	21
8	2.0	18
9	1.9	20
10	1.6	28
11	2.8	14
12	2.3	25
13	2.4	42
14	2.1	17
15	2.9	26
16	2.4	31
17	2.6	16
18	2.1	19
19	2.2	16
20	2.2	27
21	1.9	37
22	2.4	17
23	2.9	18
24	2.3	50
25	1.6	20
26	1.6	23
27	2.5	27
28	2.2	29
29	1.7	34
30	1.8	23
31	2.6	18
32	2.0	33
33	1.7	25
34	2.8	31
35	2.1	21
36	1.5	16
37	1.1	26
256	2.8	55

Fig. 14: Slant culture of *Bacillus sp* 256

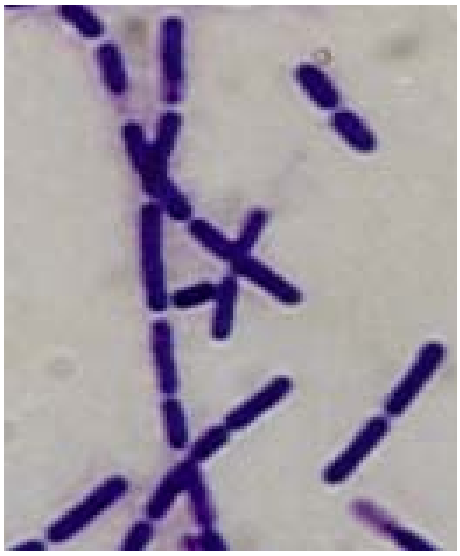
Fig. 15: Shake flask culture of *Bacillus sp* 256

The ability of the strain to utilize sugars was tested and it was found that the bacterium was able to produce acids from all the sugars provided for growth except lactose. The results of the morphological studies suggested that the isolated bacterium belonged to the genus *Bacillus.* The strain showed similarities with *B. endophyticus* because: it had ellipsoidal spore situated at terminal position (Fig. 16B); nonmotile, absence of anaerobic growth; Voges-Proskauer negative; oxidase positive; not able to hydrolyze casein, gelatin and starch; acid production from arabinose, glucose, manitol, maltose, mannose, rhamnose and xylose; etc. The strain produced pale pink pigmentation on PHA agar slants and the pigment produced was not diffusible. On nutrient agar the colonies were slimy; the cells were resistant to ampicillin and also grew in the presence of lysozyme. Further the bacterium was subjected to molecular phylogeny characterization.

1.2.4 Molecular phylogeny of *Bacillus sp 256*

This was carried out by studying the 16SrRNA sequence homology. One portion of 16SrDNA was amplified from the genomic DNA of *Bacillus sp* 256 by PCR (Fig. 17). The DNA fragmnet was cloned in to sequencing vector and sequenced partially (Table 15). The DNA sequence was analysed using various online softwares. The BLAST analysis of the 16SrDNA sequence (Table 16) showed DNA sequence similarity with *Bacillus endophyticus* (99%), *Bacillus sp*. 19490 (99%), *Bacillus sp*. GB02-16/18/20 (97%), *Bacillus sp*. MSSRF (96%) etc. Using the *Bacillus sp* 256 sequence the phylogenetic tree of *Bacillus sp* 256 was derived from BLAST results (Fig. 18). The test strain was closely related to *B. endophyticus* than with *B. megaterium* which is known to produce PHA.

A



B

Fig. 16: (A) Gram stained cells of *Bacillus sp* 256
(B) Endospore of *Bacillus sp* 256 (malachite green stained cells)

Table 14: Characterization of *Bacillus sp* 256

Morphological characters	
Gram staining	+
Presence of endospore	+
Cell size	6 x 1.5µ
Cell shape	Rod shape in chains
Motility	nonmotile
Morphological characters of the colony	
Shape	Circular
Colour	Off-white
Surface	Dry, translucent
Biochemical characters	
Catalase	+
Nitrate reduction	+
Anerobic growth with 1% glucose	+
M.R.test	+
V.P test	-
Oxidase	+
Growth in 3% NaCl	-
Starch hydrolysis	-
Hydrolysis of casein	-
Hydrolysis of gelatin	-
Anaerobic growth at 50^0C	-
Acid production	
Arabinose	+
Mannitol	+
Lactose	-
Xylose	+
Rhamnose	+
Cellobiose	+
Sucrose	+
Glucose	+
Maltose	+

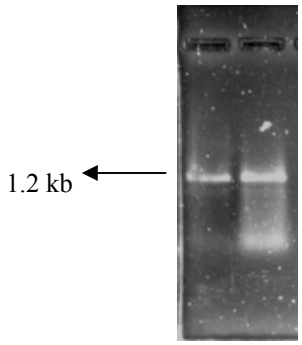


Fig. 17: PCR amplification of 16S rDNA gene of *Bacillus sp* 256

Table 15: 16S rDNA sequence from *Bacillus sp* 256

GATTCGAGCTCGGTACCTCGCGAATGCATCTAGATTGCTCTAGAGCGATTACTAGCGATTCCGACTTCGTGTA

GGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGTTTTATGGGATTGGCTCGACCTCACGGTTTTGCAGCCC

TTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCT

TCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGC

TCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCC

CGAAGGGAACCTTCTATCTCTAGAAGTAGCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAA

TTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCC

CCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTT

TACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGAC

CAGAGAGCCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACATGTGGAATTCCGCTC

TCCTCTTCTGCACTCAAGTTCCCAGTTTCCATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTA

AGGAACCCCTGCCCGCGCTTTACGCCCAATAATTCCGGAAAACGCTTGG

Table 16: BLAST results of 16SrDNA sequence

gi|93117547|gb|DQ485415.1|	*Bacillus endophyticus* strain XJU-1...	1487
gi|16973331|emb|AJ315058.1|BSP315058	*Bacillus sp*. 19490 16S rRNA	1487
gi|71648760|gb|DQ079006.1|	*Bacillus sp*. GB02-16/18/20 16S rib...	1481
gi|9930623|gb|AF295302.1|	*Bacillus endophyticus* 16S ribosomal RN	1479
gi|22121137|gb|AY046591.1|	*Bacillus sp*. SCD-2001 small subuni...	1463
gi|60678567|gb|AY941162.1|	*Bacillus sp*. MSSRF 1 16S ribosomal RN	1429
gi|7110430|gb|AF227852.1|AF227852	*Bacillus sp*. 82352 16S ribosom	1388
gi|44985592|gb|AY557616.1|	*Bacillus sp*. BA-54 16S ribosomal RNA	1376
gi|75858351|gb|DQ176423.1|	*Bacillus niabensis* strain 5T52 16S...	1372
gi|75858349|gb|DQ176421.1|	*Bacillus niabensis* strain 5M45 16S...	1372
gi|14328886|dbj|AB062678.1|	*Bacillus sp*. MK03 gene for 16S rRNA	1372
gi|116739108|gb|DQ993294.1|	*Bacillus sp*. MHS037 16S ribosomal RN	1368
gi|75707109|gb|AY998119.2|	*Bacillus niabensis* strain 4T19 16S...	1364
gi|4959926|gb|AF140014.1|AF140014	*Bacillus cohnii* 16S ribosomal	1364
gi|498797|emb|X76437.1|BC16SRRX	*B.cohnii* (DSM 6307 T) gene for 1	1364
gi|75858350|gb|DQ176422.1|	*Bacillus niabensis* strain 5M53 16S...	1364
gi|75858348|gb|DQ176420.1|	*Bacillus niabensis* strain 4T12 16S...	1364
gi|80975752|gb|DQ249996.1|	*Bacillus sp*. L41 16S ribosomal RNA ge	1364
gi|15042015|dbj|AB055095.1|	*Bacillus sp*. Y gene for 16S rRNA	1364
gi|6009580|dbj|AB023412.1|	*Bacillus cohnii* gene for 16S rRNA	1358
gi|15042016|dbj|AB055096.1|	*Bacillus sp.* SD521 gene for 16S rRNA	1358

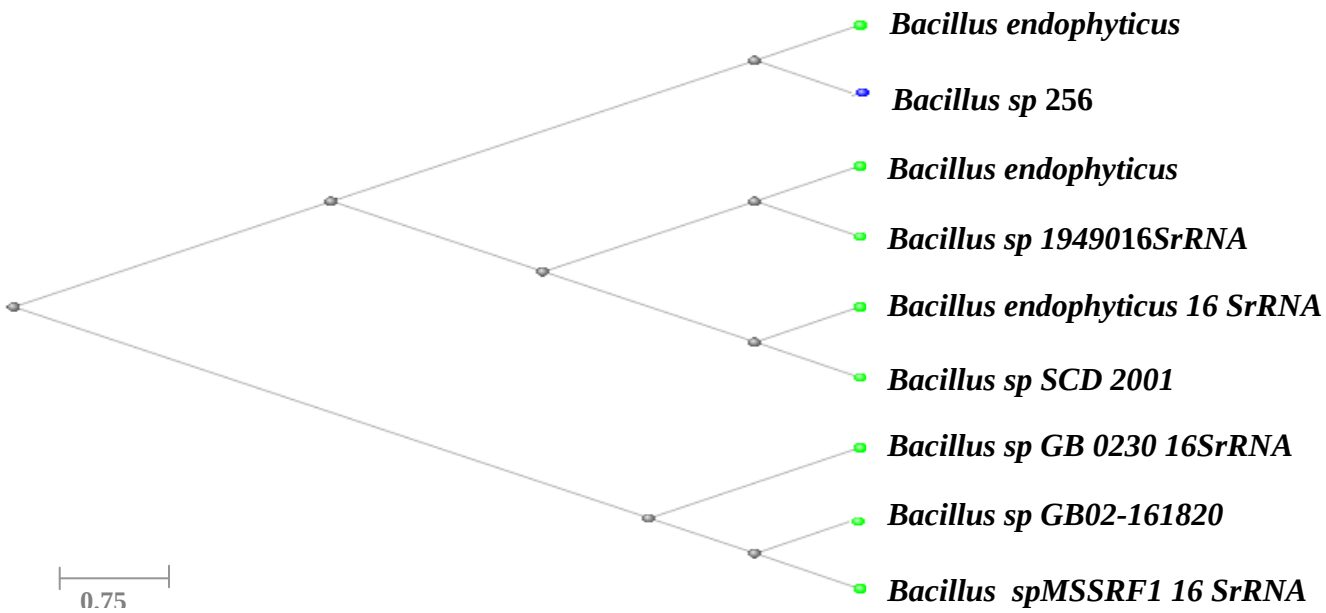


Fig.18: Phylogenetic tree of *Bacillus sp* 256

1.2.4. Molecular detection of PHA producing *Bacillus spp.*

PCR reaction was carried out for molecular detection of PHA producing *Bacillus sp*p. The genomic DNA was isolated from all the *Bacillus* isolates and using it as a template one portion of *PhaC* gene was amplified, using *PhaC* specific primers. A 600 bp fragment of *phaC* gene was amplified from the genomic DNA of some *Bacillus* isolates by PCR. The size of the fragment was confirmed by agarose gel electrophoresis using 100 bp DNA ladder (Fig. 19).

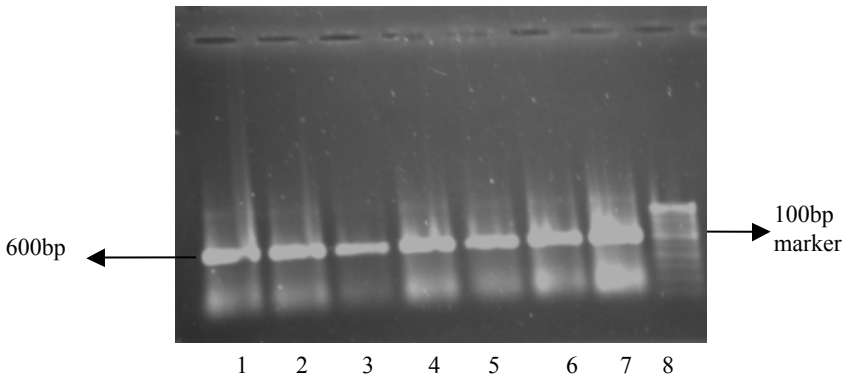


Fig. 19: PCR amplification of *phaC* fragment from various *Bacillus* isolates: Lane 8: 100 bp marker; lanes: 1-6 isolate numbers 5, 6, 13, 21 and 32 (as in Table 12), respectively; Lane 7: *B. megaterium*

1.3 DISCUSSION

Bacteria belonging to the genus *Bacillus* are Gram-positive rods that produce heat resistant endospores during their growth cycle. Morphological groups have been formed based on the shape and position of the endospores and shape of the sporangium or mother cell (Gordon *et al,* 1973). Genus *Bacillus* are widespread and can be isolated from various habitats such as marine and aquatic regions, thermal or Antarctic areas and from soil. They are isolated from acidic and alkaline environments. *Bacillus* species are also found in the inner tissues of various plants such as cotton, sweet corn (Misaghi and Donndelinger, 1990; McInroy and Kloepper, 1995) etc, where they are known to protect the plants from pathogenic fungi and support growth promotion and enhance the plants natural resistance (Emmert and Handelsman, 1999). These endophytic bacteria are known to occur as free-living soil bacteria and this includes *B. cereus* (Pleban *et al*., 1997), *B. megaterium* (McInroy and Kloepper, 1995) and *B. pumilus* (McInroy and Kloepper, 1995; Benhamou *et al*, 1998). Out of 78 strains of bacteria isolated and characterized from the inner tissues of healthy cotton plants (*Gossypium sp*) majority of isolates were identified as *Bacillus amyloliquifaciens*, *Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus* and *Bacillus subtilis* but four strains could not be assigned to any known species of *Bacillus* or the related genera of the aerobic, endospore forming bacteria. Further the authors identified these as the novel species of the genus *Bacillus*, for which the name *Bacillus endophyticus sp* nov was proposed (Reva *et al*, 2002). Phylogenetic analyses indicated that the strain belonged to the genus *Bacillus* and was most closely related to *Bacillus sporothermodurans* with a sequence similarity of 98% (Fig. 20).

In the present study, 38 *Bacillus spp* were isolated from different dry soil samples collected from various parts of the country. The soil samples were heated at 60^0 C to eliminate the non-spore forming bacteria. All the isolates possessed catalase activity. Among the different isolates of *Bacillus, Bacillus sp* 256 showed the highest growth and PHA accumulation. This culture was isolated from soil sample collected from a very hot and dry area in the northern region of the country. The strain was characterized by

morphological, biochemical as well as molecular methods. The distinguishing phenotypic characters for this isolate as identified by morphological and biochemical characters are shown in Table 17. The strain isolated was Gram-positive rods and the cells occurred singly and in short chains (Fig. 16A). Growth on PHA agar slants occasionally produced pale pink colour that was not diffusible or water-soluble. The strain was examined further by16S rRNA to determine its relationship at the genomic level. The sequence was aligned with about 80 published and unpublished *Bacillus* related 16S rRNA gene sequences and was found to match by 99% with that of *Bacillus endophyticus.* This species has been isolated only recently (Reva *et al*, 2002), and hence only the biochemical and morphological charaterization and consultation of earlier identification data may lead to its identification as *B. licheniformis.* Differences between these speices as reported in the literature are shown in Table 17. The phylogenetic tree constructed by Reva *et al*, (2002), indicates that *B. licheniformis* is closer to *B. endophyticus* in the evolutionary position (Fig. 20). Based on these factors the isolated culture has been identified as *B. endophyticus*. This is the first report, which indicates that the strain can exist outside the host plant tissue as a soil bacterium and has the ability to produce higher quantity of PHA. The amount of PHA produced is relatively high (55% of biomass) and it appeared encouraging to study various aspects of PHA production and characterize the PHA synthesis genes using this new strain.

Table 17: Distinguishing characters of *Bacillus sp* 256 isolated from soil compared to known endophytic isolates of *B. licheniformis* and *B. endophyticus*

Characters	*B. licheniformis*	*B. enodphyticus**	Current soil isolate
Position of the spore	Central	Terminal	Terminal
Spore shape	Oval	Ellipsoidal	Ellipsoidal
Cell width >1.0µm	-	+	+
Motility	Motile	Non motile	Non motile
Oxidase	+	+	+
Catalase	+	+	+
Nitrate reduction	+	-	+
Voges-Proskauer	+	-	-
Hydrolysis of Starch	+	-	-
" Gelatin	+	-	-
" Casein	+	-	-
Acid from Arabinose	+	+	+
Mannitol	+	+	+
Lactose	+	-	-
Xylose	+	+	+
Rhamnose	-	+	+
Cellobiose	+	-	+
Glucose	+	+	+
Maltose	+	+	+
Growth in 5% NaCl	+	+	-
Growth at 50^{o} C	+	-	-

* Reva *et al*, 2002

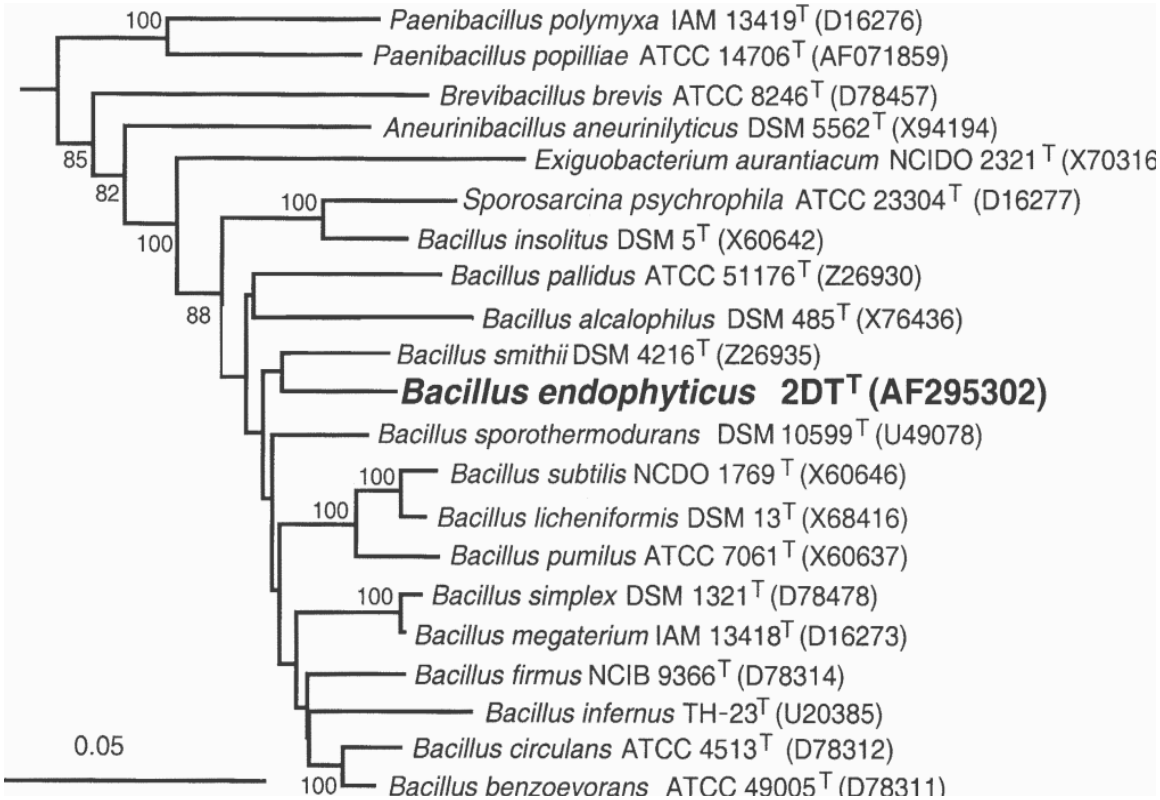


Fig. 20: Phylogenetic tree showing the evolutionary position of *B. endophyticus* in the family of Bacillaceae (Reva *et al*, 2002). This indicates the proximate relationship of the species with that of other Bacilli with special reference to *B. licheniformis*

1.4 CONCLUSIONS

To date, *B. endophyticus* has been isolated from the inner tissues of cotton plants, and this is the first report to show that it exists outside this habitat similar to other endophytic bacteria that have been isolated as free living forms from the soil. Importance of *Bacillus* in food fermentation has been known since long time. The genus is industrially important for the production of extracellular amylases, proteases. The genus also includes several species that are pest control agents. This is the first report to show that *Bacillus endophyticus* can exist in the soil habitat as a free living form and can produce industrially important polymer such as polyhydroxyalkanoate.

2.0 INTRODUCTION

The family of polyhydroxyalkanoates includes a large number of different polyesters formed from a variety of monomeric units. The monomeric groups involved are 3-hydroxy, 4-hydroxy and 5-hydroxy groups, the length of the carbon backbone varies between 4 and 16 carbon atoms with broad range of functional groups. The physical properties of PHAs are highly dependent upon their monomer units; hence biodegradable polymers having a wide range of properties can be produced by incorporating different monomer units. The homopolymer PHB is stiff and brittle and finds limited use. PHAs made of PHB and PHV (poly (3-HB-co-HV) copolymers are more flexible and tougher plastics and can be used in a wide variety of applications.

The commercialization of PHA has been hampered by the high production cost of PHA compared to petrochemical plastics. PHA production from renewable sources such as plant seed oils, industrial waste products like molasses etc as inexpensive carbon sources can bring down the cost of production to certain level. *Bacilli* are able to produce PHA from various simple, complex and cheap carbon substrates such as sucrose, caprolactone, molasses etc. There are *Bacillus spp* that can produce co-polymers of poly-(3 HB-co 3-HV) and some are reported to produce medium chain length PHA (Labuzek and Radecka, 2001; Caballero *et al*, 1995).

Bacteria generally accumulate the PHA inclusion bodies during metabolic stress caused by nutrient limiting conditions in the presence of excess carbon source. The metabolic stress is due to the limitations of certain essential nutrient such as ammonia or nitrogen, iron, magnesium, manganese, oxygen, phosphate, potassium etc. *R. eutropha, A. latus, P. oleovorans* etc are known to produce PHA when the nitrogen is limited or absent in the medium while *Bacillus spp* accumulate intracellular PHA when potassium is limited.

Therefore it is important that the PHA production media have to be designed according to the microorganism, which is used as production host. The media optimization for higher PHA production by traditional method is tedious and time consuming. Hence response surface methodology (RSM) has been used for optimization of different process parameters in a single experiment (Triveni *et al,* 2001). The RSM study can reveal the influence of different factors and their interactions for growth and maximum accumulation of PHA, in a limited number of experimentation.

PHAs are accumulated intracellularly and hence their extraction from the cell is an important step in the economic production of the polymer. PHA is extracted by various methods: solvent extraction, lysis of cells by hypochlorite and solubilization of the polymer by solvent, enzymatic treatment of the cell by proteolytic enzymes or phospholipases etc followed by polymer recovery.

Several methods have been developed for the determination of the monomer composition of scl-PHA based on the analytical methods as Gas Chromatography (GC), Gas Chromatography Mass Spectroscopy (GCMS), Nuclear Magnetic Resonance spectroscopy (NMR) etc. GC analysis of PHA offers quantitative and qualitative information about the polymer. When combined with MS detection it also adds information about the mass and the identity of the monomer involved.

The chapter deals with optimization of media for PHA production by the selected *Bacillus sp*, extraction and characterization of the polymer produced by the bacterium.

2.1 MATERIALS AND METHODS

2.1.1 Production of PHA using shake flasks

2.1.1.1 Microorganism and maintenance

Bacillus sp-256 culture was maintained on nutrient agar (Himedia, Mumbai, India) slant at 4 ^{0}C. The slants were subcultured once a month.

2.1.1.2 Production medium

The following components were used for the preparation of the medium:

Composition	**g/l of distilled water**
Na_2HPO_4 2 H_2O	4.4
KH_2PO_4	1.5
$(NH_4)_2SO_4$	1.5
$MgSO_4$ 7 H_2O	0.2
Sucrose	20.0
(pH 7.0)	

2.1.1.3 Inoculum

Inoculum was prepared by transferring growth of the fresh slant into 10 ml nutrient broth. The inoculated tube was incubated at 30 ^{0}C at 200 rpm for 12-18 h and used at 10 % level. Viable cells in the inoculum were 1 x 10 ^{3}/ml.

2.1.1.4 Optimization of carbon and nitrogen sources for PHA production

Media optimization studies were carried out using various carbon and nitrogen sources to attain maximum PHA accumulation. Carbon sources such as rhamnose, inositol, sorbitol, dextrin, mannitol, glucose, arabinose, fructose and sucrose were used in the media to

impart 8.42 g C/l. Nitrogen sources such as KNO_3, peptone, NH_4NO_3, yeast extract, $(NH_4)_2SO_4$, $(NH_4)_2$ H PO_4, tryptone and $NaNO_3$ were used in the media to impart 0.368 g N/l.

2.1.1.5 Extraction of pongemia (*Pongemia glabra*) oil

100g of pongemia seeds was powdered by grinding and mixed with 100 ml of hexane and refluxed for 2-3h at 45 ^{0}C. Hexane layer was collected and evaporated to dryness. Approximately 20g of oil was collected from 100g of seeds.

2.1.1.6 Saponification of pongemia oil

To 400 ml of methanol, 75g of NaOH was added, boiled and refluxed for 2-3 h, 25 ml of pongemia oil was added to this mixture and further refluxed for 30 minutes. To this equal volume of water and ice were added, mixed with saturated NaCl and the precipitated saponified pongemia oil was collected and dried.

2.1.1.7 PHA production using organic acids and oils

PHA production by *Bacillus* sp 256 was carried out in triplicate in 500 ml Erlenmeyer flasks in liquid medium (100 ml). The medium contained (g/l): Na_2HPO_4 2 H_2O, 4.4; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.5; $MgSO_4$ 7 H_2O, 0.2 and sucrose 20. The flasks were inoculated with 10% (v/v) of inoculum and incubation was performed at 250 rpm and 30 ^{0}C for 48-72 h. Experiments were carried out using various organic acids and plant oils at a concentration of 3 g/l of medium. Organic acids used were propionic acid, acetic acid, succinic acid, malic acid, valeric acid, oleic acid, linoleic acid, palmitic acid and stearic acid; plant oil such as pongemia oil (*Pongemia glabra*) rice bran (*Oryza sativa*) oil castor oil (*Ricinus communis*) were also used for PHA production. The commercial plant oils obtained locally had the following fatty acid composition: Rice (*Oryza sativa*) bran oil mainly contained: Palmitic acid 15%, oleic acid 43%, linoleic acid 39%; Pongemia oil: Oleic acid 71.3% and linoleic acid 10.8%; Castor oil: Ricinoleic acid 90%, linoleic acid

4%, oleic acid 3%, stearic acid 1% and palmitic acid 1%. The organic acids were suspended in 5 ml of sterile water, neutralized, filtered through 0.45µl filter and used. Plant oils were sterilized in 5 ml portion of water prior to addition.

2.1.1.8 PHA production using various economic substrates

PHA production study was conducted using media containing economic substrates such as molasses, corn steep liquor and corn starch (enzymic hydrolysate). Molasses contained 50g% of total sugars. Corn steep liquor obtained from Anil starch products, Ahmedabad, India, contained:- protein-11%, ash-9.6%, reducing sugars 1.6%. The cornstarch was hydrolysed by α-amylase and amyloglucosidase. The overall composition of various media for cultivation of the bacterium is given in Table 18. The flasks were inoculated with 10% (v/v) of inoculum and incubation was performed at 250 rpm and 30 ^{0}C for 48-72 h.

2.1.1.9 Optimization of PHA production by Response surface methodology (RSM)

RSM was carried out for optimization of PHA production (Table 19 & 24). Different concentrations of succinic acid, malic acid and oleic acid were considered for optimization of PHA production. The composition of basal medium is described above, under 2.1.1.2. The protocol used for inoculum preparation of *Bacillus sp* 256 is as detailed above.

2.1.1.9.1 Experimental design

A central composite roratable design (CCRD) with three variables at five levels was used to study the effect of organic acids on the response pattern (Table 19 and 24). CCRD was arranged to fit the regression model using multiple regression program (as detailed under chapter 5, 5.2.4). Six replicates (treatments 15-20) were used for estimation of a pure error of sum of squares.

2.1.1.9.2 Analyses

Estimation of biomass, PHA and identification of the polymers by GC were carried out as per the already described methods (Page 56 and 57)

A second order polynomial equation was used to fit the experimental data presented in Table 24. A non-linear mathematical optimization procedure of the Quattro pro software was used for the optimization of the fitted polynomials for PHA and biomass yields. Response surface plots were generated through which it was possible to visualize the relation ship between various levels of substrates and their interaction on the yield of biomass and yield of polymer.

2.1.1.10 PHA production in fermentor

The fermentative production of PHA by *Bacillus* sp 256 was carried out using sucrose as major carbon source and pongemia oil and saponified pongemia oil as co carbon substrates. Inoculum was prepared in 500 ml capacity Erlenmeyer flasks. Fermentation was carried out in a jar fermentor (Bio flo - 110, New Brunswick Scientific Co. USA, Fig. 22) of 3 l capacity, containing 2 l of mineral medium and 200 ml inoculum as mentioned above (2.1.1.2, 2.1.1.3). Cultivation was carried out at 30 ^{0}C, pH 7.0, 40% dissolved oxygen. Dissolved oxygen concentration was maintained by cascading effect. The experiment was carried out for a total period of 40 h.

2.1.2 Extraction of PHA

PHA extraction was carried out by sodium hypochlorite digestion method (Williams and Willkinson). The method is described in materials and methods (Page 56).

2.1.3 Film casting

PHA film was prepared by solvent casting method (Sevenkova *et al,* 2000). A 2 % solution of PHA in chloroform was poured on to leveled glass plates (30 x 200 cm) and was allowed to dry without any air turbulence at room temperature. After drying, the film was peeled off from the plate (Fig. 23).

Table 18: Composition of various economic media

Ingredients (g/l)	1	2	3	4	Control
K_2HPO_4	1.5	1.5	1.5	1.5	1.5
$Na_2HPO_4\,2H_2O$	2.2	2.2	2.2	2.2	2.2
$MgSO_4\,7H_2O$	0.2	0.2	0.2	0.2	0.2
$(NH_4)_2SO_4$	1.5	1.5	0	0.5	1.5
Sucrose	-	-	20	-	20
Molasses	40	-	-	-	-
Corn starch (hydrolysate)	-	20	-	-	-
Corn steep liquor	-	-	10	-	-

Table 19: Variables and their levels for CCRD

Acids	Symbols	-1.682	-1	0	1	1.682	Mean	Standard deviation
Succinic acid (g/l)	X1	0	0.41	1.0	1.59	2.0	1.0	0.59
Malic acid (g/l)	X2	0	0.41	1.0	1.59	2.0	1.0	0.59
Oleic acid (g/l)	X3	0	0.41	1.0	1.59	2.0	1.0	0.59

2.1.4 Characterization of PHA

Various methods such as FTIR, GC and ^{1}H NMR were used for the characterization of the polymer as described below:

2.1.4.1 Fourier Transform Infrared Spectroscopy

Standard PHA (Sigma Aldrich, USA) and PHA from *Bacillus sp* 256 (5 mg each) were mixed individually with 100 mg of FTIR grade KBr and pelletized. The FTIR spectrum was recorded at 400-4000 cm^{-1} in FTIR Nicolet Magna 5700 spectrophotometer.

2.1.4.2 Gas Chromatography

GC analysis was carried out using lyophilized cells and purified polymer after subjecting them to methanolysis. Calibration was performed using standard P (HB), P (HB-co-HV) containing 5/8-mol % of hydroxyvalerate (Sigma Aldrich, USA) with benzoic acid as internal standard.

a) Sample preparation

Air-dried biomass or purified PHA was weighed into a clean glass tube, to this 1 ml of chloroform, 850µl methanol and 150µl H_2SO_4 were added. The glass tube was sealed and kept for hydrolysis at 100 ^{o}C for 160 minutes. To the hydrolysed material equal volume of water was added, mixed thoroughly and 2µl of the sample was taken from the bottom layer of injection. Benzoic acid was used as internal standard.

b) ***GC conditions*** (Brandl *et al,* 1988)

The methyl esters formed were analysed with a flame ionization detector in a 30 m DB-1 capillary column of 0.25 mm internal diameter and 0.25µm film thickness. The analysis

parameters used were: injector 170 ^{o}C, detector 220 ^{o}C, and temperature programme of 55 ^{o}C for 7 min, ramp of 4 ^{o}C per minute to 100 ^{o}C and 10 ^{o}C per minute rise to 200 ^{o}C and hold for 10 min. Nitrogen (1ml/min) was used as carrier gas. Standardization was performed using standard P(HB-co-HV) obtained from Sigma Aldrich, USA, with benzoic acid as internal standard.

2.1.4.3 NMR analysis

Lyophilized cells (100 mg) were suspended in ten ml of chloroform and extracted overnight at 40 ^{0}C. Cell sediment was separated by centrifugation at 8000 rpm for 20 minutes and PHA was isolated from clear chloroform layer by the addition of 2 volumes of hexane. Precipitated polymer was air-dried at 40 ^{0}C. ^{1}HNMR of the polymer was carried out in deuterated chloroform at 400 MHz on an AMX 400 spectrophotometer. P(HB), P(HB-co-HV) from Sigma Aldrich, USA were used as standards.

2.2 RESULTS

2.2.1 Optimization of PHA production media

Bacillus sp 256 accumulated significant amount of PHA from a variety of carbon sources. The maximum accumulation was found during 48 to 72 h. The organism was able to utilize various carbon sources for growth and production of PHA (Table 20). Sucrose containing medium was found to be suitable for growth and accumulation of PHA by *Bacillus sp* 256.

Among various N_2 sources studied (Table 21), $(NH_4)_2 H PO_4$ was found to be a better nitrogen source for PHA production. The yield of PHA in medium containing peptone was high (70%) but the growth was not optimal. Highest biomass and PHA was obtained in the medium provided with $(NH_4)_2 H PO_4$, as nitrogen source.

PHA production studies using various economic substrates was conducted and it was found that the *Bacillus sp* 256 grew well and produced PHA in media containing molasses, cornstarch and corn steep liquor (Table 22). Maximum production of PHA was obtained in the medium containing hydrolysed cornstarch, while biomass was highest in molasses medium.

Table 20: Utilization of various C-sources by *Bacillus sp* 256

Carbohydrate	Biomass (g/l)	PHA (% of biomass)
Rhamnose	0.9	55.5
Inositol	0.95	31.5
Sorbitol	0.8	37.5
Dextrin	1.1	54.5
Mannitol	1.1	59.0
Glucose	1.4	50.0
Arabinose	1.6	46.8
Fructose	1.55	41.9
Sucrose	1.65	54.5

Table 21: Utilization of nitrogen sources by *Bacillus sp* 256

Source	Biomass (g/l)	PHA%
KNO_3	0.85	35
Peptone	0.40	70
NH_4NO_3	1.25	56
Yeast extract	0.35	57
$(NH_4)_2SO_4$	1.40	64
$(NH_4)_2 H PO_4$	1.65	69
Tryptone	0.60	33
$NaNO_3$	0.85	64

Table 22: Cultivation of *Bacillus sp* 256 on various economic substrates

Substrates	Biomass g/l	PHA % of biomass
Molasses	2.6	31
Corn starch (hydrolysed)	2.4	54
Corn steep liquor	2.4	43
Control	2.0	55

2.2.2 PHA production from organic acids and plant oils

PHA production was carried out using plant oils such as pongemia oil, rice bran oil and castor oil and organic acids (Table 23; Fig. 21 and 24). *Bacillus sp* grew well in plant oil containing media and accumulated up to 70% PHA (Fig. 21). Analysis of the polymer indicated that only PHB was synthesized in medium containing castor oil. Valerate fraction was found in the polymer obtained from pongemia (7 mol%) and rice bran oil (5 mol%) media. These oils were rich in oleic acid. Hence the bacterium was cultivated in medium with oleic acid as co substrate and the results indicated that growth and PHA production and copolymer synthesis was enhanced in the presence of oleic acid compared

to control (Fig. 24). Overall results showed that pongemia oil is suitable for growth and PHA copolymer production. Pongemia oil was selected for fermentor studies.

2.2.3 Fermentative production of PHA from pongemia oil

Fermenter studies were carried out using pongemia oil as the co-carbon substrate. The experiment was conducted in using three different media: a) medium containing sucrose as the sole carbon, b) medium containing sucrose and pongemia oil as co-carbon substrate, c) medium with sucrose and saponified pongemia oil. The experimental samples were collected at different time intervals of 24 h, 48 h and 72 h and analysed for biomass and PHA. Results obtained are shown in Figs 25 and 25A. The cells cultivated in sucrose medium showed good exponential growth up to 20 h during which biomass and PHA obtained were 4.1 g/l and 2.4 g/l, respectively, and accumulation of PHA in the cells was 58% of dry biomass. The polymer obtained was polyhydroxybutyrate, which formed brittle film on solvent casting. Maximum production of PHA varied in sucrose (2.4 g/l at 20 h), sucrose + pongemia oil (1.8 – 2 g/l during 20-40 h) and sucrose + saponified oil (1.8-2.5 g/l during 30-40 h) containing media. Degradation of PHA during later period of fermentation (20-40 h) in the sucrose fed cells was rapid (58-31%) compared to pongemia oil (64-50%) and saponified oil fed (64-58%) cells. PHA extracted from cells cultivated in pongemia oil containing medium formed flexible film after solvent casting (Fig. 23). This was due to presence of P(HB-co-HV) copolymer of 93:7 mol%.

Table 23: Effect of various organic acids and plant oils on PHA production by *Bacillus sp* 256 in shake flask cultures

Co substrates	Biomass (g/l)	PHA (% of biomass)	PHB: PHV(mol%)
Propionic acid	4.7	40	100:0
Acetic acid	3.8	28	100:0
Succinic acid	3.7	32	98:2
Malic acid	3.4	40	98:2
Valeric acid	3.0	30	100:0
Oleic acid	4.6	47	96:4
Linoleic acid	2.2	24	100:0
Palmitic acid	2.8	34	100:0
Stearic acid	3.6	33	100:0
Pongemia oil	4.0	70	93:7
Rice bran oil	4.0	72	95:5
Castor oil	3.5	63	100:0
Control (Sucrose)	3.8	59	100:0

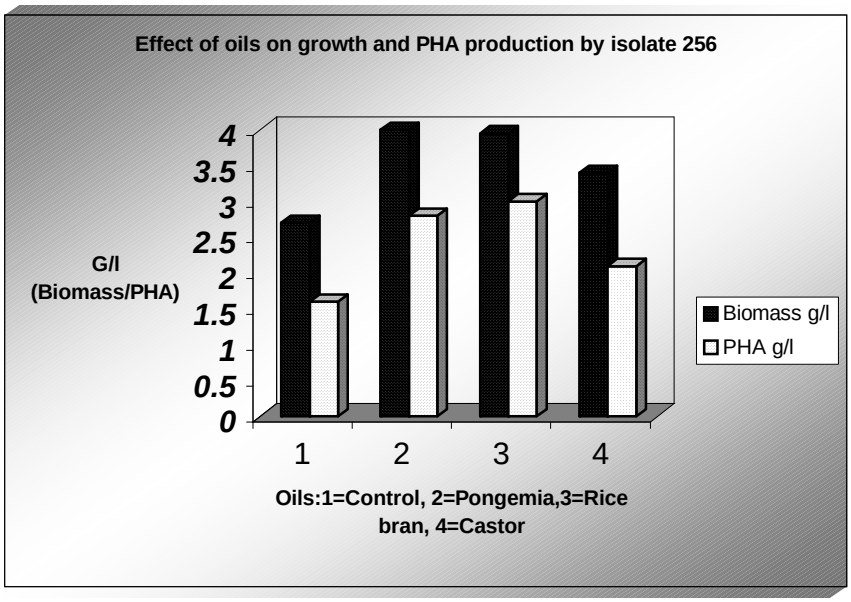


Fig. 21: Effect of oils on PHA production by *Bacillus sp* 256

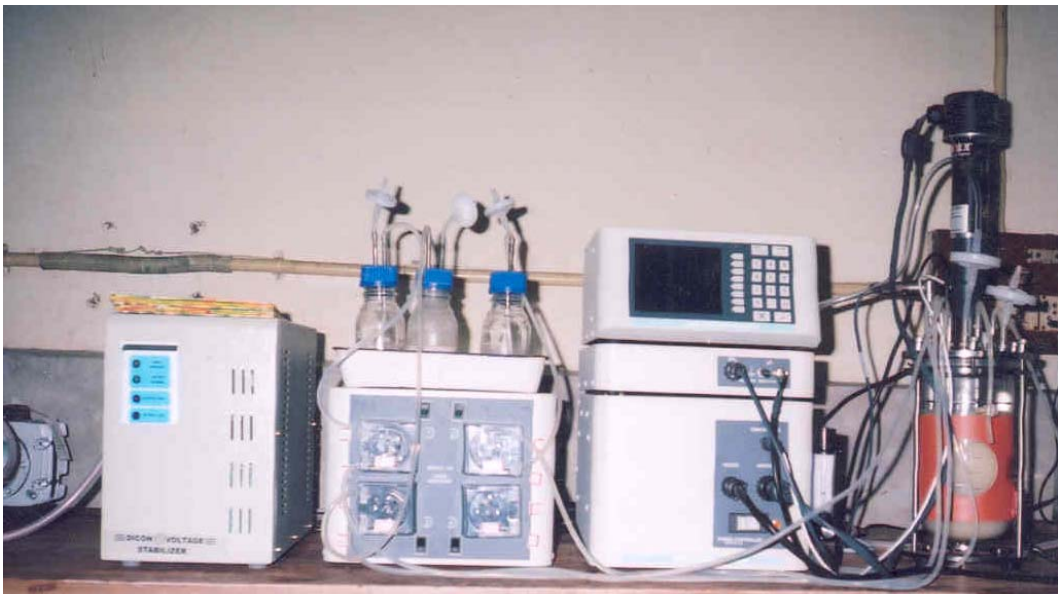


Fig. 22: Fermentor cultivation of *Bacillus sp* 256

Fig. 23: Film (solvent caste) prepared from PHA obtained from *Bacillus sp* 256

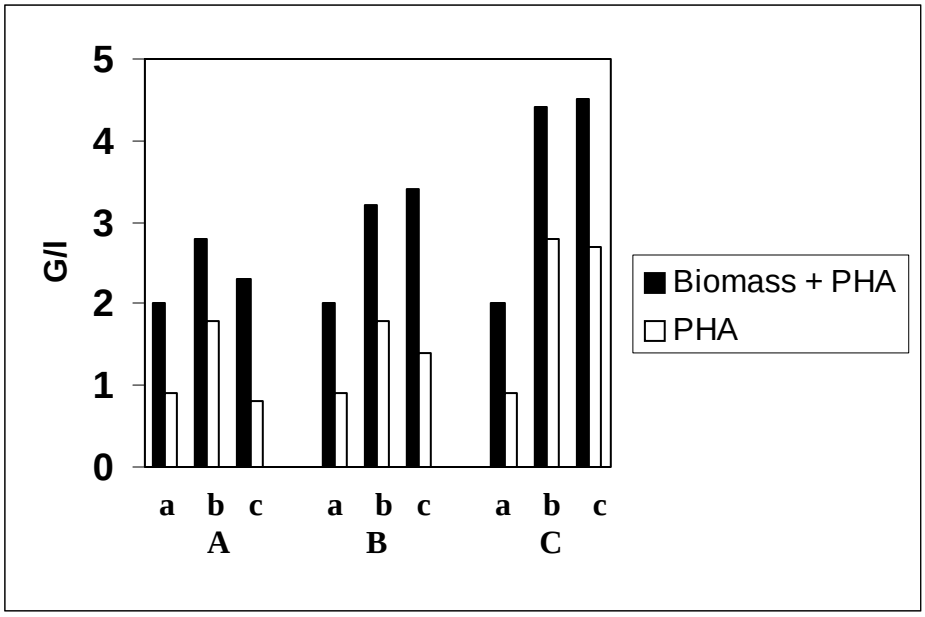


Fig. 24: Effect of carbon substrates and period of fermentation on biomass and PHA production by *Bacillus sp* in shake flask culture.
Culture conditions: 250 rpm and 30 ^{0}C for 72 h. Substrates;
A=Sucrose, B=Sucrose + Oleic acid. C=Sucrose and pongemia oil. Period of fermentation: a=24 h, b=48 h and c=72 h.

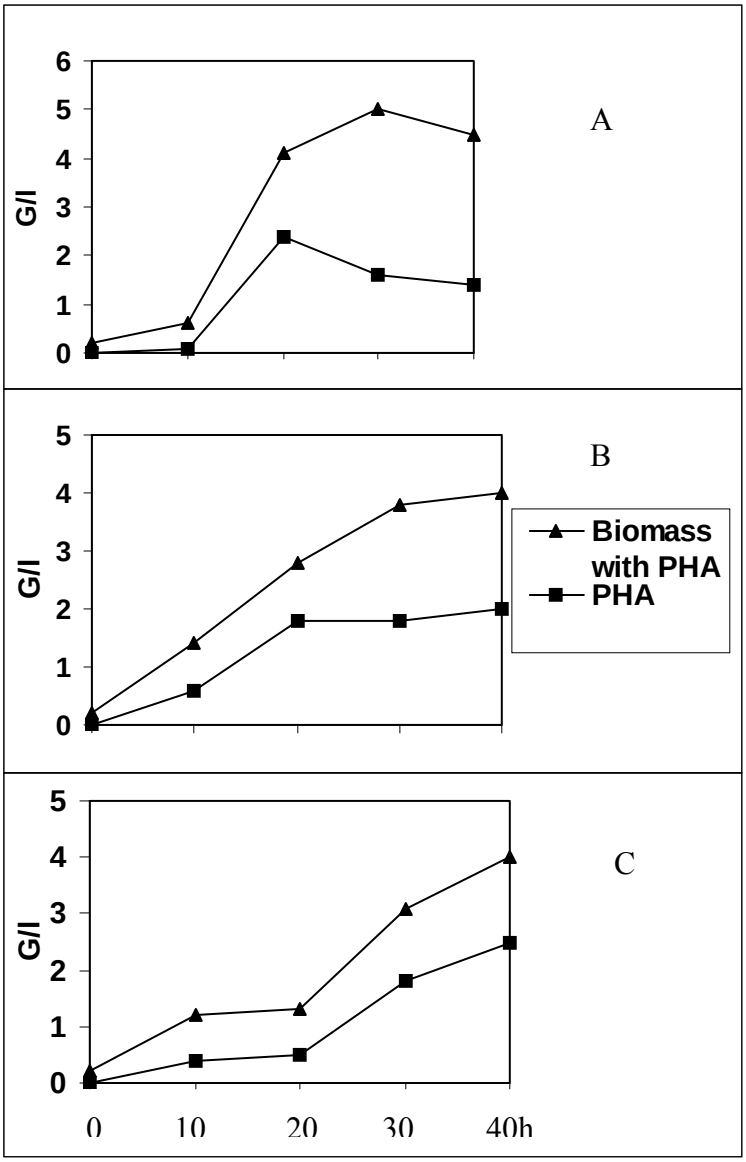


Fig. 25: Effect of carbon sources on biomass and PHA production by *Bacillus sp.* 256 during 40 h cultivation in a fermentor. Carbon sources used: A =Sucrose (25 g/l); B=Sucrose (20 g/l) + Pongemia oil (4.3 g/ l); C=Sucrose (20 g/l) + Saponified pongemia oil (2.5 g/l)

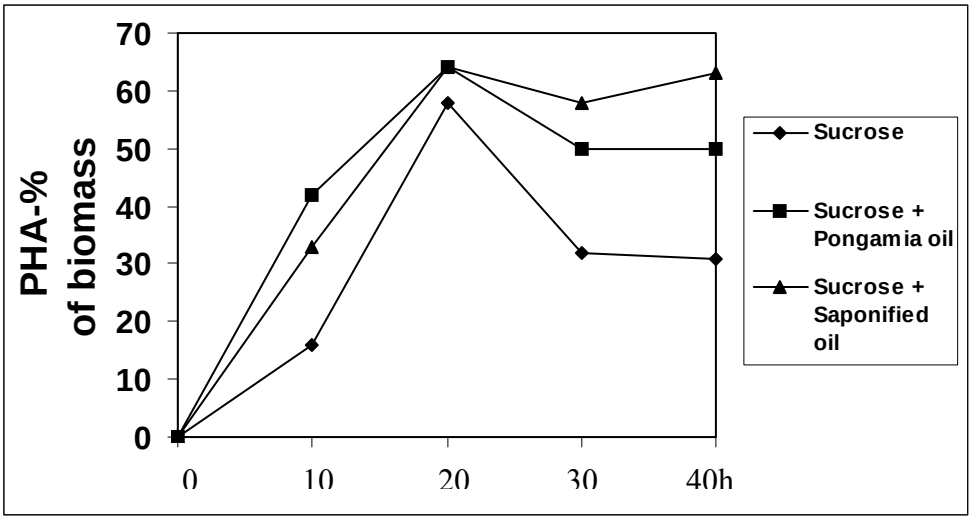


Fig. 25A: PHA (% of biomass) concentration of cells cultivated (corresponds to fig 25) using various carbon sources for 40 h in a fermentor.

2.2.4 Optimization using Response Surface Methodology

RSM was used for optimization of mixed organic acids that can supplemented in the medium for PHA production. The responses measured in the experiments were biomass and PHA yield. The effect of malic, oleic and succinic acids on growth and polymer yields are shown in Table 24. The response surface graphs for biomass and PHA yields showed that succinic acid favored growth of the bacterium and oleic acid enhanced the polymer production (Figs. 26 and 27). However the results on interaction of organic acid for enhanced growth/polymer yields were not very significant.

Table 24: Treatment Schedule for five-factor CCRD and response in terms of biomass and PHA yield

Exp No	Succinic acid (g/l) X1	Malic acid (g/l) X2	Oleic acid (g/l) X3	Biomass (g/l)	PHA yield (g/l)	PHB:HV
1	-1	-1	-1	2.30	1.35	98:2
2	1	-1	-1	2.20	1.50	98:2
3	-1	1	-1	2.20	1.45	98:2
4	1	1	-1	2.25	1.45	97:3
5	-1	-1	1	2.20	1.45	98:2
6	1	-1	1	2.45	1.55	98:2
7	-1	1	1	2.20	1.35	98:2
8	1	1	1	2.55	1.30	97:3
9	-1.682	0	0	2.20	1.40	98:2
10	1.682	0	0	2.33	1.45	96:4
11	0	-1.682	0	2.30	1.45	97:3
12	0	1.682	0	2.25	1.35	97:3
13	0	0	-1.682	2.30	1.60	96:4
14	0	0	1.682	2.38	1.50	97:3
15	0	0	0	2.25	1.42	97:2
16	0	0	0	2.26	1.40	97:3
17	0	0	0	2.26	1.39	97:3
18	0	0	0	2.25	1.40	97:3
19	0	0	0	2.24	1.40	97:3
20	0	0	0	2.25	1.40	97:3

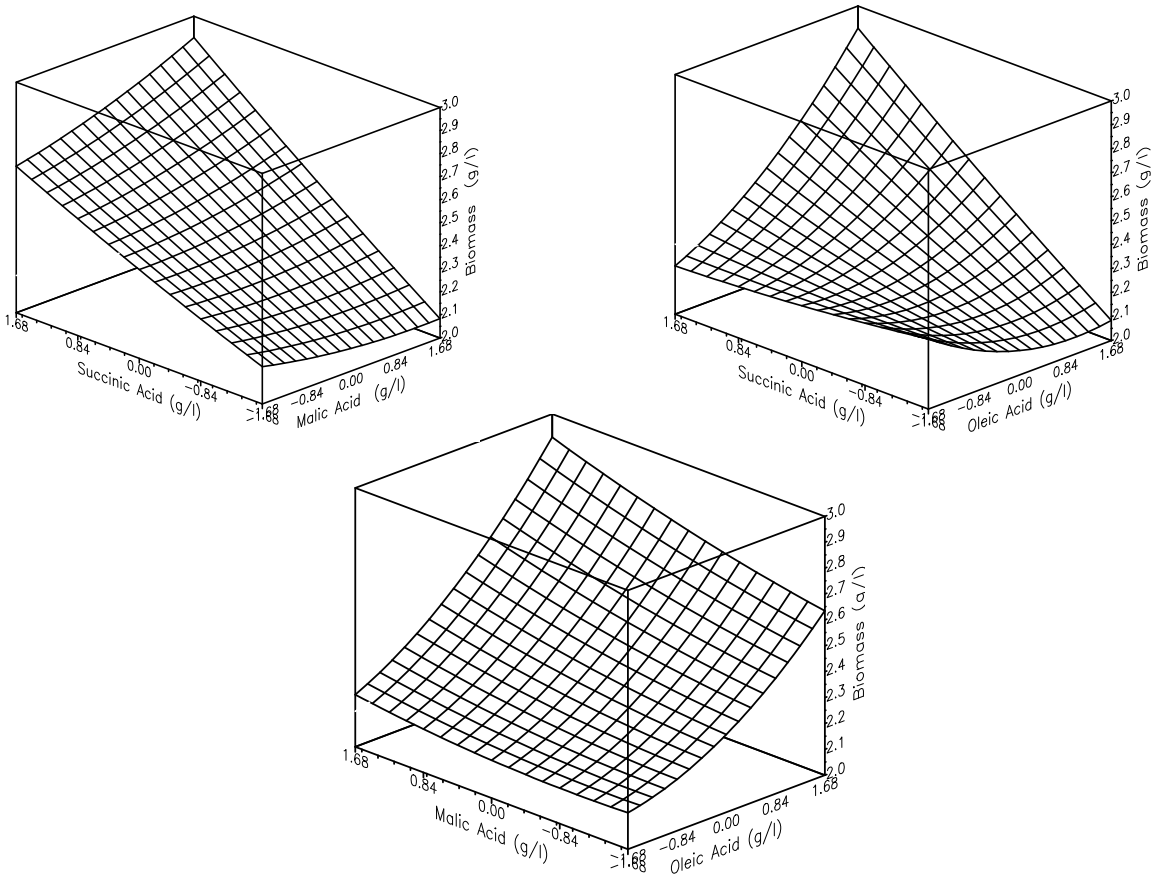


Fig. 26: Effect of interaction of organic acids on biomass yield

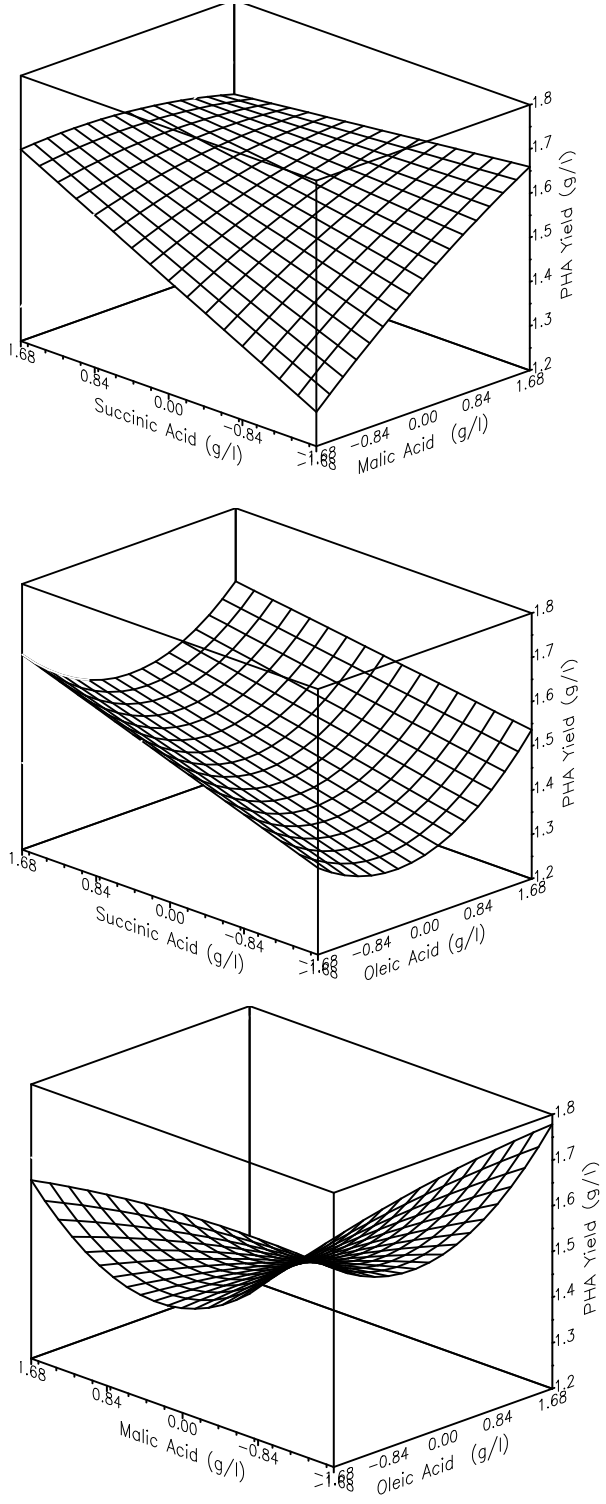


Fig. 27: Effect of interaction of organic acids on PHA yield

2.2.5 Characterization of PHA polymer

The polymer obtained from the *Bacillus sp* 256 was characterized by Fourier transform infrared spectroscopy (FTIR), Gas Chromatography (GC) and Nuclear Magnetic Resonance spectroscopy (NMR) (Figs: 28-30).

FTIR is one of the powerful and rapid tools to obtain information on polymer structure because every chemical compound in the sample makes its own distinct contribution to the absorbance/transmittance spectrum. Based on the following it was confirmed that the polymer synthesized was PHA: The transmittance bands located at 1725cm^{-1}, is attributed to the stretching vibration of the C=O group (ester carbonyl) in the PHA polyester (Fig. 28). Accompanying bands of the C-O-C groups appear in the spectral region from 1150 cm^{-1} to 1300cm^{-1} (1303, 1229, 1196 cm^{-1}). Transmittance regions from 2800 to 3100 cm^{-1} correspond to the stretching vibration of C-H bonds of the methyl (CH_3), and methylene (CH_2) groups. Other characteristic bands present for scl-PHA were 2976, 2933, 1279 (CH3 bend), 1101, 1057 (C-O) 979 and 515.

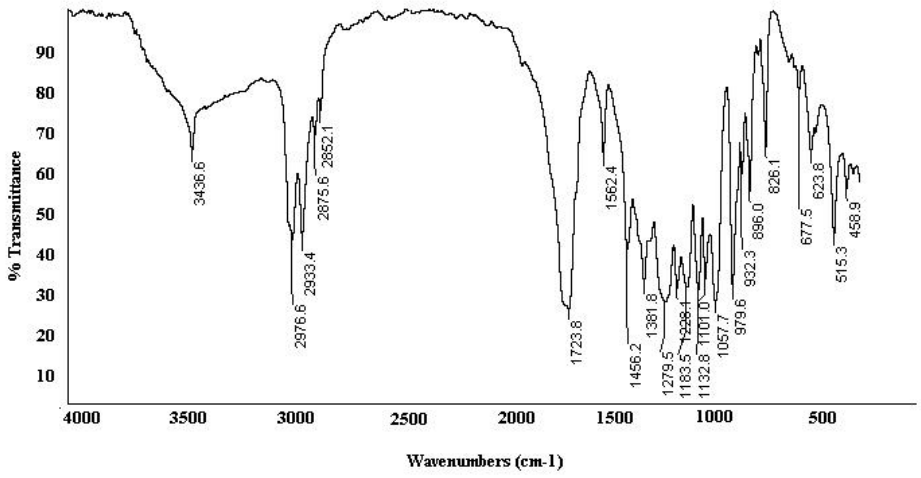


Fig. 28: FTIR spectrum of PHA sample extracted from *Bacillus sp* 256

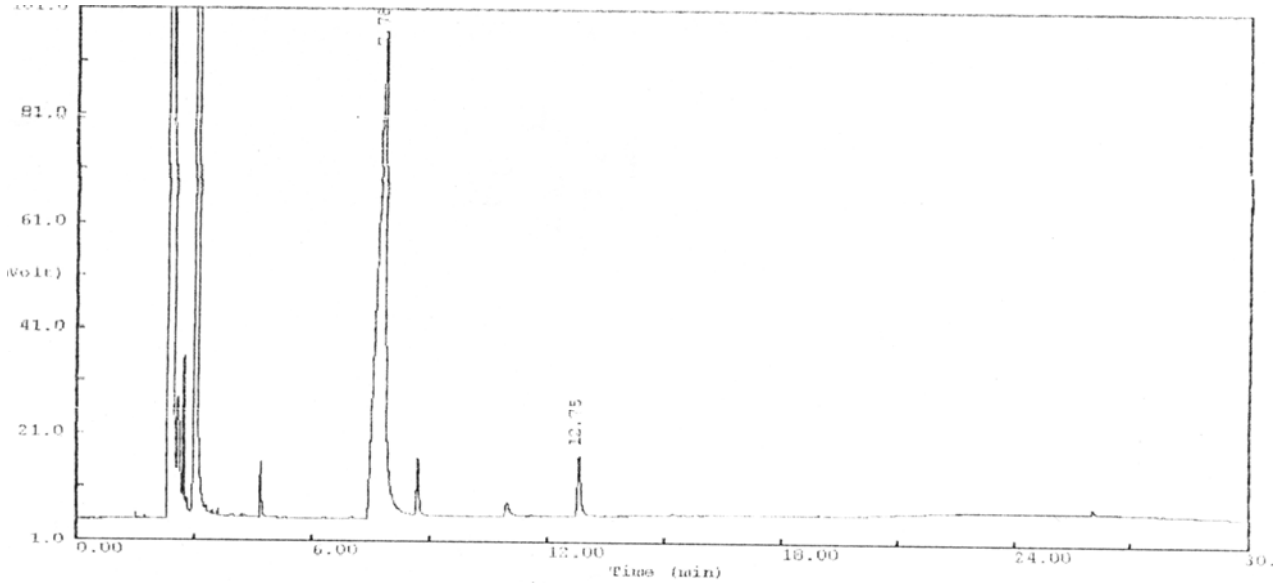


Fig. 29: GC profile of PHA sample obtained by cultivation of *Bacillus sp* 256 on rice bran oil

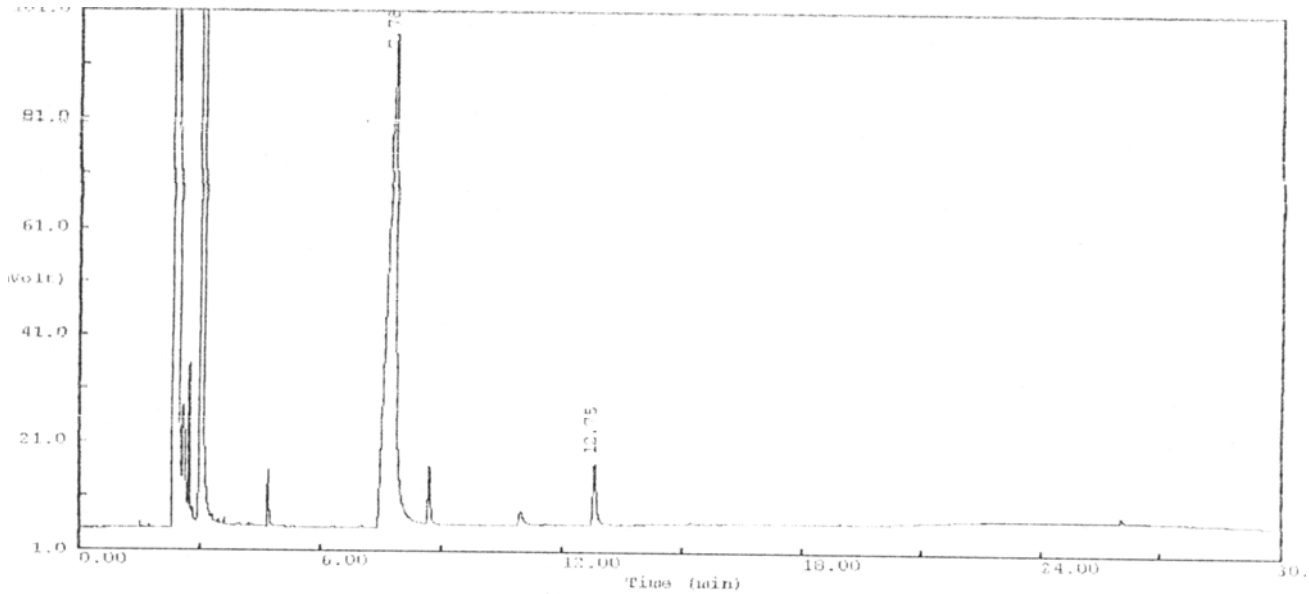


Fig. 30: GC profile of PHA sample obtained by cultivation of *Bacillus* 256 on oleic acid

Gas chromatography is a very efficient method for quantitative estimation as well as characterization of PHA. The butyrate methyl ester eluted at 9.5 min and valerate methyl ester at 12.8 min. Results in figure 29 and 30 show that the PHA obtained after cultivation of *Bacillus sp* 256 on rice bran oil and oleic acid as co substrates was a copolymer of P(HB-co-HV). Mol% of hydroxybutyrate and hydroxyvalerate was 95:5 and 96: 4, respectively, of the total polymer content.

Nuclear Magnetic Spectroscopy (NMR) is a powerful technique used for elucidating the chemical structure of the compounds. NMR has been used to investigate various aspects of PHA like monomer composition, cellular content, conformational analysis, monomer linkage sequence, copolymer analysis and PHA metabolic pathway studies (Jacob *et al*, 1986). The ^{1}H NMR spectrum of PHA (Fig. 31) showed three characteristic groups signals of PHB: a doublet at 1.29 ppm which is characteristic of methyl group, a doublet of a quadruplet at 2.5 ppm which is attributed to methylene group and a multiplet at 5.28 ppm characteristic of a methylene group. A triplet at 0.9 ppm and a methylene resonance at 1.59 and methyne resonance at 5.5 indicated the presence of valerate in the polymer.

Synthesis of PHA and copolymer of P(HB-co-HV) was thus confirmed by FTIR, GC and NMR studies.

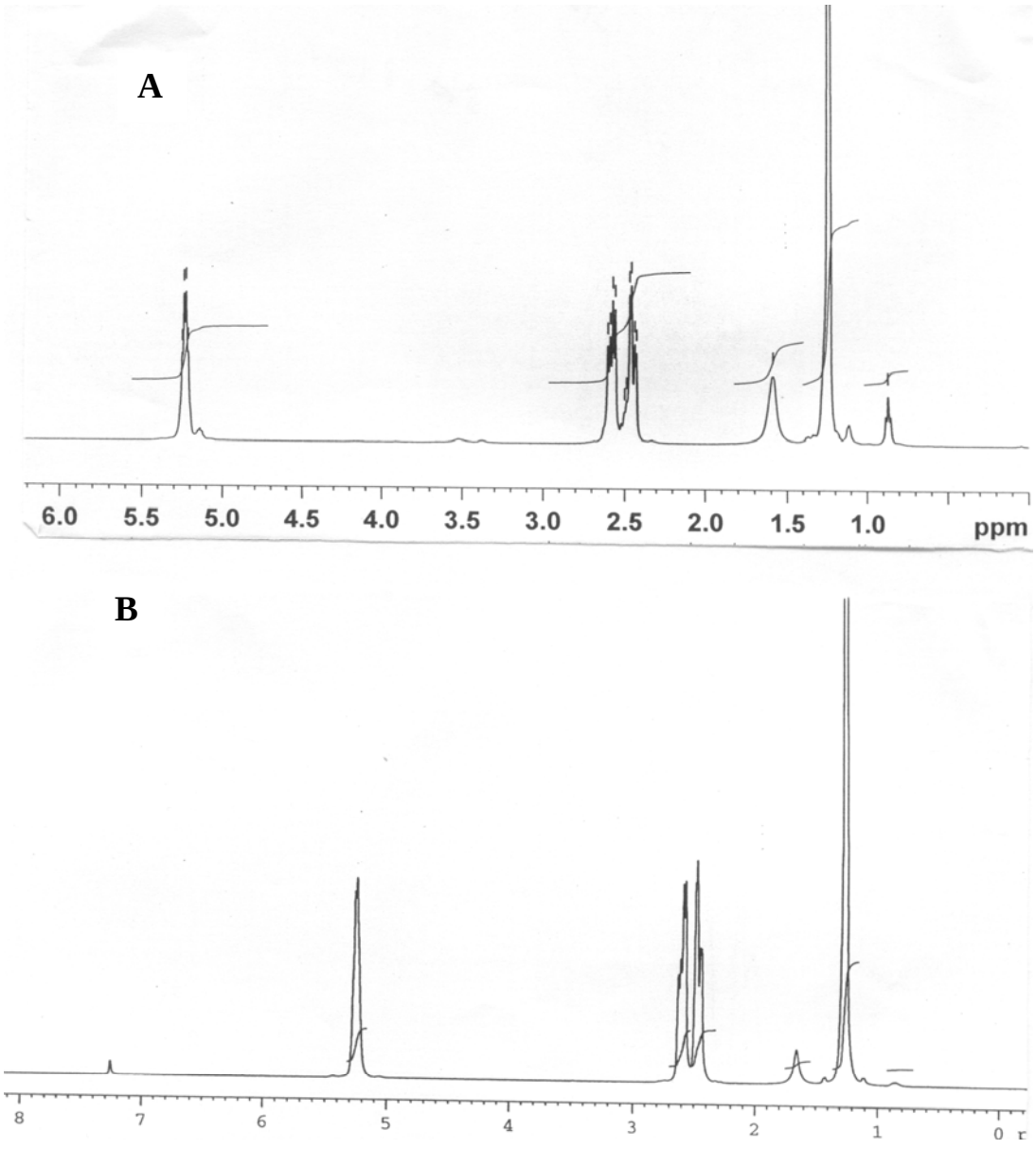


Fig. 31: ^{1} H NMR spectrum of PHA samples

A: Standard PHA copolymer (PHB-co-HV)

B: PHA from *Bacillus sp* 256

2.3 DISCUSSION

Imbalances of nutrients such as depletion of essential nutrients, excess carbon, are the major factors that influence bacterial accumulation of intracellular PHA. Type of limiting nutrient involved may differ from one genus to other genus of bacteria. Several *Bacillus spp* are known to produce PHA under varied growth conditions and they are found capable of producing various PHAs from structurally unrelated carbon sources (Chen *et al,* 1991; Valappil *et al,* 2007[a]). Generally *Bacillus spp* produce PHA due to depletion of potassium and phosphorous in the medium. Excess carbon source is another inducing factor for PHA production. So it is important to design a desirable medium for the optimum PHA production. In the present chapter media optimization studies were carried out using different carbon and nitrogen sources. *Bacillus spp*. are known to produce optimum PHA concentration in sucrose medium (Shamala *et al,* 2003). They are also known to produce PHA from economic substrate like molasses (Wu *et al,* 2001). Similar results were observed in the present study wherein data in Table 20 shows that amongst the carbon substrates tested, glucose and sucrose were found suitable for PHA production. Bacteria can metabolize these two sugars rapidly and utilize the metabolic intermediates as acetyl CoA for PHA synthesis. *Bacillus sp* 256 grew well and accumulated PHA in sugar rich economic substrates such as molasses and corn starch hydrolysate. Molasses is a bi product from sugarcane industry, which contains about 50% of sugars and it is used as an economic substrate for various fermentation processes. Due to the presence of glucose and sucrose, molasses appeared to be well-suited economic substrate for PHA production. Similarly the bacterium produced optimal concentration of PHA when glucose rich cornstarch hydrolysate was used as a carbon source in the medium.

Bacillus sp 256 grew well in organic acid containing media and it produced copolymer of poly-3(HB-co-HV) in media containing succinic, malic and oleic acids. Bacteria utilize various organic acids as substrate for various metabolic activities to derive energy for its growth and survival. Succinic and malic acids can be converted in to

acetyl CoA by reverse reaction including decarboxylation in glycolysis via oxaloacetic acid and phosphoenol pyruvic acid. Succinic acid can also support PHV synthesis by methyl malonyl CoA pathway. Oleic acid can be directly converted in to precursor molecules for PHA biosynthesis by β-oxidation. The formation of PHV may be due to the conversion of some acetyl CoA molecule in to succinyl CoA via TCA cycle in the presnce of excess carbon (sucrose). The succinyl CoA can be converted in to propionyl CoA by methyl malonyl CoA pathway. The intermediate of β-oxidation cycles with five carbon atoms of acyl chain may be converted to 3HV, if the respective enzymes are present (Steinbuchel *et al*, 2003).

Supplementation of plant oils in the medium resulted in accumulation of PHA. Amongst the oils tested, pongemia and rice bran oils supported PHA copolymer synthesis.

Cell activity and function, and overall production rate of PHA is dependent on the type of carbon source/co carbon source used in the medium and its utilization rate. Cost of raw material is considered for economic production of polymer. Due to high prices, fatty acids cannot be employed for economic production for PHA. Amongst various carbon sources, utilization of oils is species specific and for economical production of the polymer it is essential to use non-edible or underutilized plant oils. Plant oils are natural sources of fatty acids, and the use of these renewable energy sources as co-substrates for PHA production can lead to economic production of the polymer with required molar concentrations of the copolymers In the present work, non-edible oils of pongemia *(Pongemia glabra),* and castor *(Ricinus communis*), were used as co carbon substrates in the medium. Rice (*Oryza sativa*) bran oil is used for edible purpose, but rancid oil may find application as co substrate for PHA synthesis. As mentioned above the strain used was able to utilize oleic acid for PHA copolymer production and high titers of this fatty acid was present in pongemia oil (71 %) and rice bran oil (42 %). In fermentor studies, in control medium maximum synthesis of PHA (2.4 g/l) was observed at the end of 20 h

of fermentation and it declined (1.6 g/l) thereafter. Subsequent to this, a concomitant increase in the weight of the residual biomass (Weight of total biomass- weight of PHA) was attained (3.4 g/l) indicating the onset of depolymerization of PHA and its utilization towards cell growth. Compared to this, the degradation of PHA and build up of residual biomass was marginal in the medium containing pongemia oil (2 g/l) and saponified oil (1.3 g/l). Saponified oil resulted in slow but steady rise in PHA concentration. The slow growth could have occurred due to the ready presence of fatty acids compared to that of the oil wherein the oil is utilized subsequent to its enzymatic hydrolysis. It is known that acids affect the gradient of protons through the membrane and the production of energy and the transport system is dependent on this gradient. This finally may lead to decline in microbial growth and activity (Lawford and Rousseau, 1993). Overall the molar concentration of P (HB-co-HV) was maximum in the presence of saponified oil (80:20 mol%) compared to unsaponified oil (93:7). Homopolymer of PHB was obtained from the cells fed with only sucrose. Hydroxyacyl coenzyme A thioesters required for quantitative and qualitative productivity of PHA are supplied by fatty acid biosynthesis and degradation pathway (Eggink *et al,* 1992). The extra metabolic flux of fatty acid degradation appears to have channelised towards enhanced copolymer production in oil fed cells, which was absent in sucrose fed cells.

The formation and breakdown of PHA in the cells appear to be important in defining nutritional status of the microbial cells. In *Bacillus spp.* it serves as an endogenous source of carbon and energy for cell activities and spore formation. The co substrate absorption may lead to availability of energy to the cells which may lead to delayed degradation of PHA polymer. The overall results indicate that plant oils may be better suited for the stabilized production of PHA copolymer by *Bacillus spp*.

2.4 CONCLUSIONS

A PHA producing *Bacillus sp* 256 has been identified and has been found to use a variety of nitrogen and carbon substrates for growth and polymer production. The bacterium was capable of producing PHA copolymers. In addition, the fermentation conditions reported in this chapter using non-edible plant oil such as pongemia oil as co carbon substrate shows that the oleic acid containing co-carbon substrate can lead to synthesis of copolymers. Supplementation of this substrate also prevented degradation of PHA once maximal production was achieved, which indicates that such fermentation conditions can be used for this strain for commercial process. It appeared that the strain utilizes various metabolic pathways and possesses robust genes for PHA biosynthesis, which can be exploited.

3.0 INTRODUCTION

The organization of the genes involved in PHA biosynthesis is varied among the organisms; they are clustered in some bacteria, while they occur independently in some other bacteria. The difference in gene organization leads to different PHA operons. The different modes of arrangement of PHA genes are given Fig: 13. The *phaC* (coding for PHA synthase) is the prime gene in the *pha* operon, which is wide spread in all PHA producing organisms. In *Bacillus megaterium* the size of *phaC* is 1089 base pairs (McCool and Cannon, 1999). The *phaB* gene is present in most of the scl-PHA-producing organisms and in many bacteria *phaB* is associated with PHA synthase. In *Bacillus sp* it forms *phaRBC* operon, flanked by the *phaR* and *PhaC*, (McCool and Cannon, 1999). The gene *phaA* codes for the enzyme β-ketoacyl CoA thiolase, which catalyzes the first step in the PHB synthesis. The *phaA* gene of *Bacillus* is not associated with the PHA operon.

Many prokaryotic and eukaryotic organisms are able to produce low molecular weight PHB molecule that is complexed with other biomolecules. A number of microorganisms and their PHA biosynthesis genes have been characterized in the recent past, which resulted in the development of highly efficient recombinant bacteria. Nucleotide sequence of fifty nine PHA synthase genes have been obtained from fortyfour different bacteria (Rehm, 2003). According to the subunit requirement and substrate specificity PHA synthase have been classified in to class I, II, III and IV. The PHA synthase of *Ralstonia eutropha*, *Pseudomonas aeruginosa*, *Allochromatium vinosm* and *Bacillus megaterium* represent class I, II, III and IV, respectively (Rehm, 2003).

In *Bacillus* the Pha operon consists of a cluster of five genes namely *phaP*, *phaQ*, *phaR*, *phaB*, and *phaC* (McCool and Cannon, 1999). The *phaP* and -*Q* genes are transcribed in one orientation, each from a separate promoter, while the *phaR*, -*B*, and –*C* are present in the opposite strand in different orientation from a separate promoter. PHA

synthase of *Bacillus megaterium* and *Bacillus* INT005 is reported to require *phaR* protein subunit for its activity (McCool and Cannon, 2001; Satoh *et al*, 2002). PHA synthase activity was detected in *E. coli* carrying the *phaR*, *phaB* and *phaC* genes, but not in *E. coli* carrying only the *phaC* gene (Satoh *et al*, 2002).

To elucidate the mechanism of PHA biosynthesis, studies on metabolic pathways for PHA production and molecular analyses of PHA biosynthesis genes in various bacteria have been conducted.

The present chapter deals with isolation and characterization of three important genes of PHA biosynthesis pathway from *Bacillus spp*.

3.1 MATERIALS AND METHODS

3.1.1 Strains, plasmids and their maintenance and cultivation

Bacillus spp were maintained on nutrient agar (Himedia, Mumbai, India) slant at 4^{0} C. *E. coli* strain DH5α was maintained on LB agar medium (Himedia, Mumbai, India). The strains were grown in NB or LB medium overnight at 30^{0} C and 200 rpm for various experiments.

3.1.2 DNA manipulation

Isolation of total genomic DNA from *Bacillus sp*-256 was performed using Nucleospin Extraction kit (Macheri Nagal, Germany). Isolation of plasmid, restriction digestion, transformation of *E. coli* was carried out according to standard procedures (Sambrook and Russell, 2001).

3.1.3 Designing of oligonuleotide primers

A PCR cloning strategy was used to clone the PHA biosynthesis genes from *Bacillus* ***spp.*** Various oligonucleotide primers were designed and synthesized using sequence data available at gene bank (www.ncbi.nlm.nih.gov). The sequences of the primers were modified, wherever it was necessary. The list of primers used in the study is given below (Table 25).

3.1.4 PCR cloning of *phaB* (NADPH dependant Acetoacetyl CoA reductase)

All the PCR reactions were carried out in thermocycler: Gene Amp PCR system 9700 (Perkin-Elmer, USA) and Primus-25 (PeQLab, Germany). The *phaB* gene, which code for the enzyme acetoacetyl CoA reductase, was amplified from the genomic DNA of *Bacillus sp* 256 by PCR using the following parameters. All the components (as described earlier) were mixed in a 25µl reaction mixture using the primers PhaBFX and PhaBR and subjected to PCR.

Table 25: List of primers used for *pha* gene amplification

Primer	Sequence	Target gene	Expected Size (bp)
PhaBF	5'..ATGGTTCAATTAAATGGAAAAGTAGCA..3'	phaB	744
PhaBFX	5'.TAAAGGAAACAGCTAATGGTTCAATTAAATGGAAAAGTAG CA..3'	phaB	759
PhaBR	5'..TTACATRTATAAACCGCCGTTAATG..3'	phaB	
PhaBR1	5'..THGCWGCTGAGTAGTTTGATTTGAC..3'	phaB	450
PhaCFX	5'..TAAAGGAAACAGCTAATGACTACATTCGCAACAAGAAT..3'	phaC	
PhaCR	5'..TTAHTTAGARCCYTYATCWA..3'	phaC	1100
PhaCNF	5'..TAGTTTAGTGGAATATCTAGT..3'	phaC	
PhaCNR	5'..ATCCACTGTCTGTATGATTC..3'	phaC	621
PhaAFX	5'..AGACGTCCCCGGGGAATTCTAAAGAAAACAGCTAATGAG AGAAGCTGTCATTGTT..3'	phaA	
PhaAR	5'..CAGCGTGGTACCCTCGAGTTAAAGTAATTCAAATACT..3'	PhaA	1200
PhaAR1	5'..AGC TGT TAC AGA ACC GCG AAC GT..3'	phaA	744

PCR conditions:
Initial denaturation: 94 ^{0}C for 1 min

Denaturation:	94 ^{0}C for 1 min	35 cycles
Annealing:	58 ^{0}C for 1 min	
Extension:	72 ^{0}C for 1min	
Final extension:	72 ^{0}C for 10 min	

A 10 µl aliquot of the PCR product was analyzed by agarose gel (1.0%) electrophoresis as described earlier. The size of the *phaB* gene amplicon was checked by comparing with a 100bp DNA ladder (Bangalore Genei, India). The authenticity of the amplicon was again checked by nested PCR using the diluted *phaB* amplicon as the template. The PCR product was purified using the method described earlier and stored at –20^{0}C.

3.1.4.1 Cloning of *phaB* in to T-tailed vector

The *phaB* PCR product was purified using GenElute PCR Clean - Up Kit (Sigma, USA). The purified PCR product was taken for A- tailing. The method of A-tailing is described in materials and methods (page 60). The PCR product was incubated with *Taq* polymerase, *Taq* assay buffer and dATP solution at 70 ^{0}C. To remove residual dATP and *Taq* buffer the A-tailed mixture was purified using PCR Clean - Up Kit. The A- tailed PCR product was ligated to T-tailed vector pTZ57R/T.

3.1.4.2 Ligation of A-tailed *phaB* PCR product to T-tailed vector

The A-tailed purified PCR product for *phaB* gene was T/A ligated to pTZ57R/T vector using InsT/A Clone PCR Product Cloning Kit (MBI Fermentas, Lithuania). The A-tailed purified PCR product was T/A cloned by ligating to pTZ57R/T vector using InsT/A Clone PCR Product Cloning Kit (MBI Fermentas, Lithuania) and transforming competent cells of *Escherichia coli* strain DH5α.

Methodology

The following components were pipetted into a thin-walled 0.2 ml PCR reaction tube:

Plasmid vector pTZ57R/T DNA	2.0 µl
Purified PCR fragment	10.0 µl
10X Ligase Buffer	3.0 µl
PEG 4000 solution	3.0 µl
T4 DNA Ligase, 5U/µl	1.0 µl
Deionized water (to make up to 30.0 µl)	11.0 µl

The reaction components were mixed by brief spin. The samples were incubated at 22 ^{0}C for overnight. Heating the reaction mixture at 65 ^{0}C for 10 min inactivated the enzyme.

Solutions

10 x Ligation Buffer*

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

PEG 4000*

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

*T4 DNA Ligase, 5U/µl**

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

* Supplied with the kit

3.1.4.3 Transformation of *E. coli* using the ligation reaction mix

Solutions & Media

Luria-Bertani broth (LB)	(per liter)
Bacto-tryptone	10.0 g
Bacto-Yeast extract	5.0 g
Sodium chloride	10.0 g

The pH was adjusted to 7.0 with 2N NaOH and the total volume was made up to 1 liter with deionized water.

SOB (per liter)

Bacto-tryptone	20.00 g
Bacto-Yeast extract	5.00 g
Sodium chloride	0.60 g
Potassium chloride	0.19 g
Magnesium sulphate	10.0 mM (added from 1.0 M stock)
Magnesium chloride	10.0 mM (added from 1.0 M stock)

The first four components and the magnesium salt were autoclaved separately and then mixed to constitute the SOB medium.

SOC medium

To 1.0 ml of the basal SOB medium, 7 µl of filter-sterilized (Millipore, 0.4 µm) glucose solution (50%w/v) was added.

0.1 M $CaCl_2$ stock solution

1.47 g of $CaCl_2$ was dissolved in 100 ml of deionized water. The solution was sterilized by filtration and stored as 20 ml aliquots at -20^0C.

Ampicillin stock solution

100 mg ampicillin (Himedia, Mumbai, India) was dissolved in 1.0 ml of deionized water. The solution was sterilized by filtration and stored at -20^0C and used at a working concentration of 100 µg ml^{-1}

Chloramphenicol stock solution

100 mg of chloramphenicol (Himedia, Mumbai, India) was dissolved in 200µl distilled ethanol and diluted to 1 ml using sterile water. The solution was sterilized by filtration and stored at -20^0C and used at a working concentration of 10 µg ml^{-1}

0.1 M IPTG stock solution

0.12 g of IPTG was dissolved in 5.0 ml of deionized water. The solution was filter-sterilized and stored as aliquots at -20^0C.

X-Gal stock solution

100 mg of X-Gal was dissolved in 2.0 ml of N, N'-dimethylformamide (DMF). The solution was stored in micro centrifuge tube, wrapped in aluminum foil at -20^0C.

3.1.4.3.1 Preparation of competent cells using $CaCl_2$

A single colony of *E. coli* (DH5α strain/JC7623) from a plate, freshly grown for 16-20 h at 37^0C was picked up and transferred into 50 ml sterile LB (composition of which is given below) broth in a 250 ml conical flask. The culture was incubated at 37^0C at 200 rpm. The OD_{600} of the culture was determined periodically to monitor cell growth. When the OD_{600} reached 0.40-0.50, the cells were transferred aseptically to 50 ml sterile polypropylene tube. The culture was cooled by storing the tube on ice for 10 minutes. The cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4^0C. The medium was decanted from the cell pellet. The cell pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice. The cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4^0C. The fluid from the cell pellet was decanted and the tubes were kept in an inverted position for 1 minute to allow the last traces of fluid to drain away. The cell pellet was resuspended in 2.0 ml of ice-cold 0.1 M $CaCl_2$ and cells were stored at 4^0C overnight.

3.1.4.3.2 Transformation of competent cells

About 200 µl suspensions of competent cells were added to sterile micro-centrifuge tubes. Plasmid DNA (~50 ηg) or 2 to 5 µl of ligation mixture was added to each tube. The contents of the tubes were mixed by swirling gently and the tubes were stored on ice for 30 minutes. Control samples used were: (a) competent cells that received plasmid DNA and (b) competent cells that received no plasmid DNA. The tubes were transferred to water bath set at 42^0C for 90 seconds to subject the cells to heat shock. The tubes were rapidly transferred to ice and the cells were allowed to chill for 1-2 minutes. 800 µl of SOC medium (composition of which is given below) was added to each tube and the cultures were incubated for 45 minutes at 37^0C in a shaker incubator at 150 rpm.

31.4.3.3 Selection of transformants

The transformants were selected on ampicillin containing LB agar plates. The plasmids were isolated from the transformed *E. coli* by alkali lysis method **(**Birnboim and Doly, 1979**)**

3.1.4.4 Isolation of plasmid DNA from the transformed colonies (Birnboim and Doly, 1979**)**

Solutions and Reagents

Luria-Bertani broth (LB)	(per liter)
Bacto-tryptone	10.0 g
Bacto-Yeast extract	5.0 g
Sodium chloride	10.0 g

Solution I

50 mM glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Solution II

0.2 N NaOH (freshly prepared from 10 N NaOH)

1.0% SDS. Prepared freshly before use.

Solution III

5.0 M Potassium acetate	60.0ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

The resulting solution is 3.0 M and 5.0 M with respect to potassium and acetate, respectively.

Methodology

Single colony of appropriate strain was inoculated into 2 ml of LB broth containing required antibiotic and grown overnight in a shaker incubator at 37^0C and 180 rpm. 1.5 ml of the overnight culture was transferred to a 1.5 ml micro centrifuge tube and the cells were harvested by centrifugation at 10,000 rpm for 2 min. After discarding the

supernatant, 100 µl of solution I was added and vortexed vigorously to obtain a homogenous suspension. The samples were kept on ice for 5 min. About 200 µl of freshly prepared alkaline solution (solution II) was added to the tube and mixed gently by inverting the tube several times until the lysed cell suspension became clear. The samples were kept on ice for 5 min, 150 µl of ice-cold potassium acetate solution (solution III) was added, and tubes were inverted gently. The tubes were centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a fresh tube and equal volume of phenol-chloroform was added and vortexed thoroughly. Centrifugation at 10,000 rpm for 10 min separated the two phases. The upper aqueous phase was transferred to a fresh tube and equal volume of chloroform was added. The tubes were centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was transferred to a fresh tube and 2 volumes of absolute ethanol were added. The tubes were kept at -20^{0}C for 1 hr to overnight for precipitation. The tubes were centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. The pellet was washed with 300 µl of 70% ethanol and the air-dried pellet was dissolved in 20 µl of TE buffer. Agarose gel (0.8%) electrophoresis of the samples was carried out along with control plasmid.

The isolated plasmids were run in an agarose gel to screen and select the recombinant plasmid.

3.1.4.4.1 Agarose gel Electrophoresis

Materials and solutions

1. Agarose (SRL, Mumbai, India).
2. TAE 50 X buffer: (100ml.)

   24.2 g Tris base, 5.71 ml of glacial acetic acid and 10 ml of 0.5 M EDTA (pH 8.0) were added to 80 ml of distilled water. The pH was adjusted to 7.2 and the final volume was made up to 100 ml with distilled water. The buffer was sterilized by autoclaving and stored at room temperature.
3. 100bp DNA ladder (Bangalore Genei).
4. Gel casting boat
5. Mini gel apparatus and power supply (Bangalore Genie, India).

6. Ethidium bromide stock solution (10 mg ml^{-1}):

   10 mg of ethidium bromide (Sigma, MO, USA) was dissolved in 1 ml of distilled water. The solution was stored in a micro centrifuge tube wrapped in aluminum foil at 4^0C.

Methodology

The boat was sealed with an adhesive tape and the comb held in place for the formation of wells. 1.2 g of agarose was added to 100 ml of 1 X TAE buffer. The mixture was heated for the solubilization of agarose. The solution was cooled to 50^0C and poured into the sealed boat. The gel was allowed to polymerize. The comb and the adhesive tape were removed and the gel was placed in the electrophoresis tank with sufficient volume of 1 X TAE buffer to cover the surface of the gel. The samples and the standard DNA size marker were loaded in the wells. Electrophoresis was carried out at 50 volts till the dye reached 75% of the total gel area. The gel was removed from the tank and stained by soaking in a solution of 0.5 μg ml^{-1} ethidium bromide for 30 min at room temperature. The gel was destained in distilled water for 10 min, examined on a UV transilluminator and documented using Gel Documentation system (Herolab, Germany).

3.1.4.5 Sequencing of the clones

DNA sequencing was carried out using M13F and M13R primers by dideoxy chain termination method (Sanger *et al*, 1977). The reaction was carried out in an automatic DNA sequencer (ABI prism, Applied Biosystems, USA) with fluorescent dideoxy chain terminators at the Dept. of Biochemistry, University of Delhi, South Campus, and New Delhi.

3.1.5 PCR cloning of *phaC* gene (PHA synthase)

The phaC gene was amplified from the genomic DNA of *Bacillus sp* by PCR method. The primer set PhaCFX and PhaCR were used to amplify the full-length gene of PHA synthase. The PCR was carried out by following PCR parameters as: initial denaturation

at 94 ^{0}C for 2 min, annealing at 59 ^{0}C for 2 min and the extension temperature at 72 ^{0}C for 2 min and a final extension step at 70 ^{0}C for 10 min. The PCR amplifications were performed using XT-5 PCR system (Bangalore Genei). The PCR reactions were conducted in 25 µl volumes containing *Bacillus sp* genomic DNA, 10 mM of dNTP mix, 1µl each of PhaCFX and PhaCR primers and 1X XT-polymerase buffer 5A (100 mM TAPS (pH 8.0), 500 mM KCl, 17.5 mM $MgCl_2$, 0.01% gelatin).

The PCR amplicon was purified using gene elute PCR clean up kit and A-tailed as described earlier. The A-tailed purified PCR product for PHA synthase gene was T/A cloned by ligating to pTZ57R/T vector using InsT/A Clone PCR Product Cloning Kit (MBI Fermentas, Lithuania) and transforming competent cells of *Escherichia coli* strain DH5α. The recombinant plasmid was screened and checked by PCR and insert release using *BamHI* and *EcoRI* (MBI Fermantas, Lithuania). The insert release was conducted as follows.

The following constituents were added in a micro centrifuge tube in the order mentioned:

Constituents	**Volume (µl)**
Nuclease-free water	33.0
TY^+ tango 10 x buffer	10.0
Plasmid DNA	5.0
BamHI	1.0
E.*coRI*	1.0
Final volume	50.0

The contents of the tube were mixed gently by pipetting and the tube was centrifuged briefly at 10, 000 x g to collect the contents at the bottom of the tube. The reaction was carried out at 37 ^{0}C for 4 - 8 h. The samples were analyzed by agarose gel electrophoresis along with 100bp DNA ladder.

3.1.5.1 Sequencing of the *pTZC* construct

The phaC gene insert of pTZC was completely sequenced using M13F and M13R primers by dideoxy chain termination method (Sanger *et al*., 1977). The reaction was carried out in an automatic DNA sequencer (ABI prism, Applied Biosystems, USA) with fluorescent dideoxy chain terminators at the Dept. of Biochemistry, University of Delhi, South Campus, New Delhi, India.

3.1.6 PCR cloning of *phaA* gene (β-ketothiolase)

The ketothiolase gene was amplified from the genomic DNA of *Bacillus sp* 256. The PCR reaction was carried out by combining the following reaction components in 25µl reaction volume:

Components	Volume (µl)	Final concentration
Nuclease-free water	18.7	
10X XT-polymerase 5A buffer	2.5	1 X
dNTP mix (10 mM)	0.5	0.2 mM
XT *Taq* polymerase (3U/ µl)	0.3	0.03U/µl
Primer *PhaA*FX (Forward)	1.0	0.2 µM
Primer *PhaA*R (Reverse)	1.0	0.2 µM
Genomic DNA Template	1.0	
Total reaction volume	25.0	

10X XT-polymerase 5A buffer (Bangalore Genei) contained 100 mM TAPS (pH 8.0), 500 mM KCl, 17.5 mM $MgCl_2$, 0.01% gelatin. The contents of the tube was mixed and the reaction was carried out in a thermocycler GeneAmp PCR System 9700 (Perkin-Elmer, USA) and the reaction parameters were as follows:

Initial denaturation:	94 ^{0}C for 1 min	
Denaturation:	94 ^{0}C for 30 sec	35 cycles
Annealing:	58 ^{0}C for 1 min	
Extension:	72 ^{0}C for 2 min	
Final extension:	72 ^{0}C for 10 min	

The PCR product was purified and the amplicon size was determined in agarose gel using standard DNA marker. The purified PCR amplicon was cloned in sequencing vector pTZ57R/T (MBI Fermentas, Lithuania). The presence of insert in the plasmid was confirmed by PCR and restriction digestion and the *phaA* gene insert was sequenced completely at Dept. of Biochemistry, University of Delhi, South Campus, New Delhi, India.

3.1.6.1 Analysis of nucleotide sequences

The nucleotide sequences of the cloned genes were analysed using various online programmes, such as nucleotide BLAST, Dialign, Genomic BLAST, Clone manager etc.

3.2 RESULTS

PCR cloning strategy was used to amplify the PHA biosynthesis genes such as *phaA* (β ketothiolase), *phaB* (Acetoacetyl CoA reductase) and *phaC* (PHA synthase), from the genomic DNA of *Bacillus sp* and the results are presented below.

3.2.1 Isolation, cloning and characterization of *phaB* gene

PCR was carried out to amplify the *phaB* gene from *Bacillus sp* 256. The PCR amplicon was 744 bp in size as expected. The authenticity of the PCR product was again checked by nested PCR, which resulted in the amplification of the 450 bp long internal fragment of *phaB* gene. The amplicon and the nested PCR products were run in an agarose gel along with the standard DNA marker (Fig. 32), which showed the expected size of both the fragments. The *phaB* gene was cloned in sequencing vector pTZ57R and the recombinant *pTZB* plasmid was selected from an agarose gel (Fig. 33). The presence of the *phaB* gene in the *pTZB* construct was confirmed by insert release by restriction digestion and the insert was 800 bp as expected. The *phaB* gene insert was sequenced completely and the sequence data was subjected to BLAST analysis (Table 26). The BLAST results of the sequence showed high homology with acetoacetyl CoA reductase gene of *Bacillus sp*. The sequence was similar (90%) to *phaB* gene of *B. megaterium*, *B. cereus* (100%) and *B. thuringiensis* (100%). Based on the *phaB* gene sequence restriction map of *Bacillus sp* 256 was deduced using clone manager program (Fig 34). The map was compared with restriction maps of other *Bacillus phaB* gene reported. The restriction pattern showed that *Bacillus phaB* gene is polymorphic in nature. In *Bacillus sp* 256 there are *Hind III* (304^{th} position) and *Hinc II* (105), which were absent in other *Bacillus phaB* genes. Other major unique sites that were present among different *Bacillus spp* were *EcoRV* (*B. antharacis*, 216), *PstI* (*B. megaterium*, 132), and *TaqI* (*B. megaterium*, 224). A *PvuII* site at 130 was conserved in *B. cereus* and *Bacillus* INT005. The *phaB* gene of *Bacillus sp* 256 contained 41 % GC and 59 % AT sequences.

The *phaB* sequence of *Bacillus sp* 256 showed the presence of a 744 bp long complete open reading frame (ORF) (Table 27). The deduced amino acid sequence of the DNA was a continuous stretch of 247 amino acid sequences with an average molecular weight of ~27kDa.

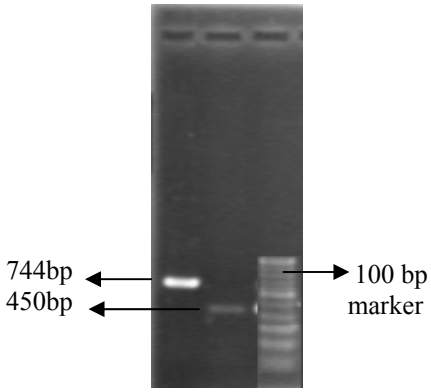


Fig. 32: PCR amplicon of *phaB* gene

Lane 1 744 bp *phaB* gene

Lane 2 450 bp

Lane 3 100 bp DNA ladder

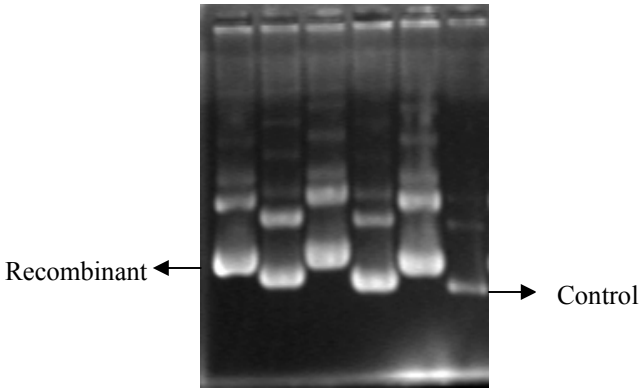


Fig. 33: Cloning of *phaB* in pTZ57R/T

Lane 1,3 & 4 pTZB

Lane 6 pTZ57R

Lane 2 & 5 self ligation

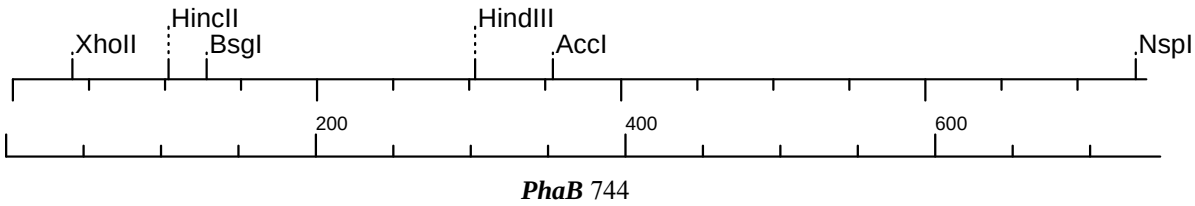


Fig. 34: Restriction map of *Bacillus sp 256 phaB gene*

Table 26: BLAST result of *phaB* sequence of *Bacillus sp* 256

NCBI results of BLAST

Accession	Description	Max score	Tot score	Query coverage	E value	Max identity
AF109909.2	*Bacillus megaterium* polyhydroxyalkanoate gene cluster, complete sequence	147	203	70%	7e-32	90%
AE017355.1	*Bacillus thuringiensis* serovar konkukian str. 97-27, complete genome	103	351	41%	9e-19	100%
CP000001.1	*Bacillus cereus* E33L, complete genome	103	305	34%	9e-19	100%
AE017194.1	*Bacillus cereus* ATCC 10987, complete genome	103	261	31%	9e-19	97%
EF037511.1	Synthetic construct *Bacillus anthracis* clone FLH241992.01L BA1330 gene, complete sequence	95.6	211	34%	2e-16	97%
CP000485.1	*Bacillus thuringiensis* str. Al Hakam, complete genome	95.6	299	41%	2e-16	100%
DQ486135.1	Bacillus cereus strain SPV PHA biosynthetic gene cluster, complete sequence	95.6	161	28%	2e-16	95%
AE017334.2	*Bacillus anthracis* str. 'Ames Ancestor', complete genome	95.6	343	41%	2e-16	100%
AE017225.1	*Bacillus anthracis* str. Sterne, complete genome	95.6	343	41%	2e-16	100%
DQ000291.1	*Bacillus thuringiensis* strain R1 PhaP (phaP), PhaQ (phaQ), PhaR (phaR), PhaB (phaB), and PhaC (phaC) genes, complete cds; and	95.6	215	34%	2e-16	97%
AB077026.1	*Bacillus sp*. INT005 phaR, phaB, phaC genes for PHA synthase subunit PhaR, 3-keto-acyl-CoA reductase, PHA synthase, complete	95.6	161	28%	2e-16	95%
AE016879.1	*Bacillus anthracis* str. Ames, complete genome	95.6	343	41%	2e-16	100%
AY331151.1	*Bacillus thuringiensis* PhaP (phaP), PhaQ (phaQ), PhaR (phaR), PhaB (phaB), and PhaC (phaC) genes, complete cds	79.8	153	29%	1e-11	92%
AE016877.1	*Bacillus cereus* ATCC 14579, complete genome	75.8	231	23%	2e-10	100%

Table 27: *PhaB* gene (Acetoacetyl CoA reductase) sequence from *Bacillus sp* 256

```
 1  atggttcaat taaatggaaa agtagcagtc gtaacaggtg gatctaaagg gatcggagca gctatttcaa aggaattagc gaaaaacgga
     M  V  Q   L  N  G   K  V  A  V   V  T  G   G  S  K   G  I  G  A   A  I  S   K  E  L   A  K  N  G

91  gtgaaggttg ttgtcaacca taacagcaac aaagaaagtg cagaagagat tgtaaagcaa attgaagcag aaggtggagc agcggttgct
     V  K  V   V  V  N   H  N  S  N   K  E  S   A  E  E   I  V  K  Q   I  E  A   E  G  G   A  A  V  A

181 attggagccg atgtttctta tagtgaacaa gctaaacgcc ttattgaaga aacgaaagaa gcatttggac agcttgatat tttagtaaac
     I  G  A   D  V  S   Y  S  E  Q   A  K  R   L  I  E   E  T  K  E   A  F  G   Q  L  D   I  L  V  N

271  aatgcgggca ttacacgcga tagaacgttt aagaagcttg gggaagagga ttggagaaaa gttattgatg tgaacttaaa tagcgtctac
      N  A  G   I  T  R   D  R  T  F   K  K  L   G  E  E   D  W  R  K   V  I  D   V  N  L   N  S  V  Y

361  aacactactt ccgcagcact tacatacctt ttagaatcag aaggtgggcg agtaattaac atttcttcca ttattggaca agcgggagga
      N  T  T   S  A  A   L  T  Y  L   L  E  S   E  G  G   R  V  I  N   I  S  S   I  I  G   Q  A  G  G

451  tttggtcaaa caaactatgc tgctgcgaaa gctggtttgt tagggtttac aaaatctcta gctttagagc ttgcacgcac aggcgtaaca
      F  G  Q   T  N  Y   A  A  A  K   A  G  L   L  G  F   T  K  S  L   A  L  E   L  A  R   T  G  V  T

541  gtaaactcaa tttgtccagg attcattgag acagaaatgg taatggctat gcctgaaaat gtacgtgaac aagttatttc aaaaatccct
      V  N  S   I  C  P   G  F  I  E   T  E  M   V  M  A   M  P  E  N   V  R  E   Q  V  I   S  K  I  P

631  gcgcgtcgtc tcggtcattc agaagaaatt gctcgtggcg ttttatactt atgccaagat ggagcttata ttacaggtca agagctaagc
      A  R  R   L  G  H   S  E  E  I   A  R  G   V  L  Y   L  C  Q  D   G  A  Y   I  T  G   Q  E  L  S

721  attaacggcg gtttatacat gtaa
      I  N  G   G  L  Y   M  -
```

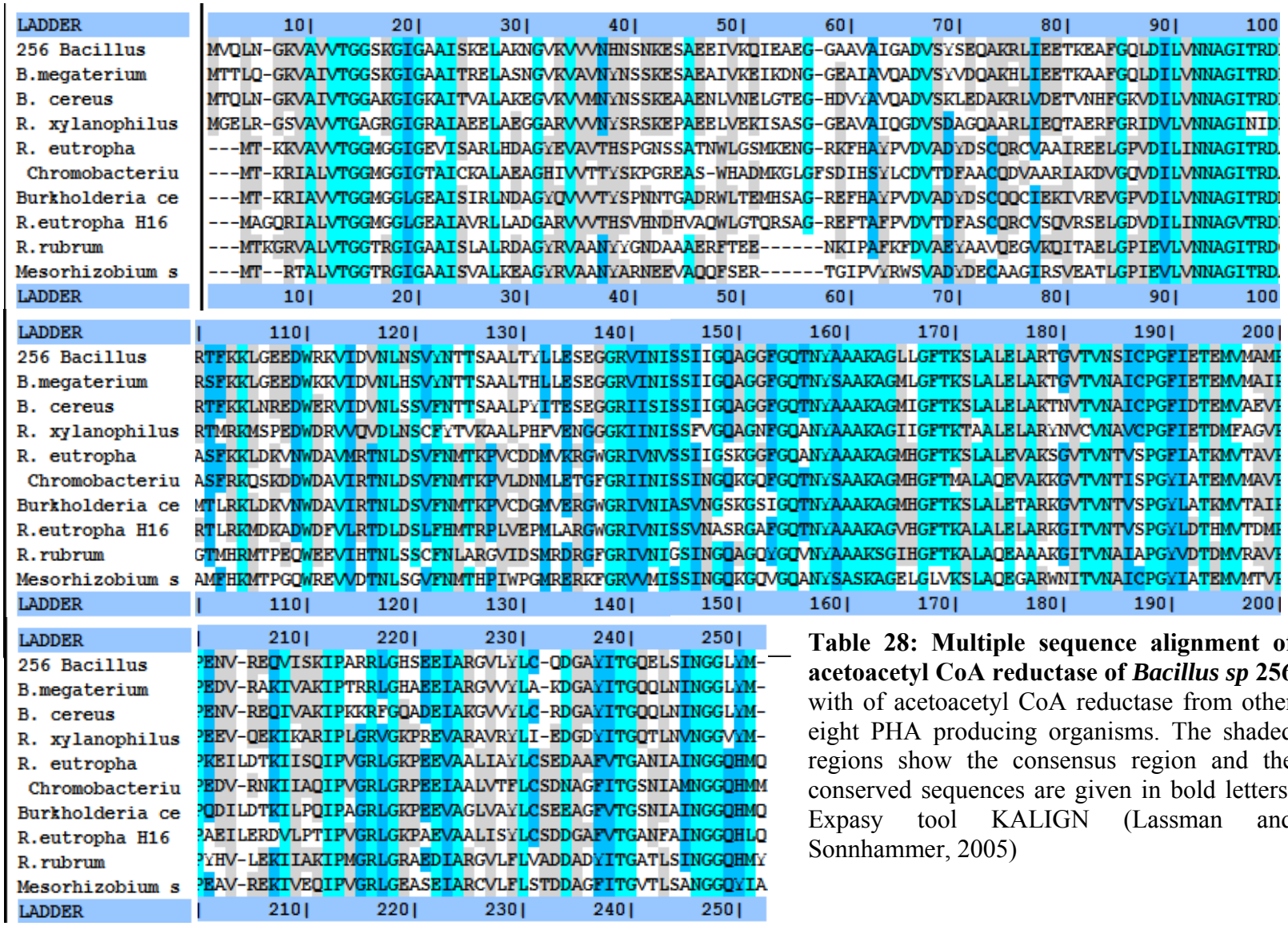
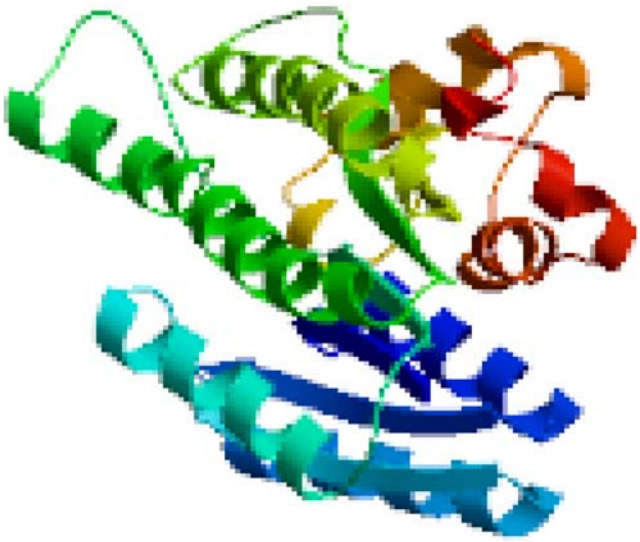


Table 28: Multiple sequence alignment of acetoacetyl CoA reductase of *Bacillus sp* 256 with of acetoacetyl CoA reductase from other eight PHA producing organisms. The shaded regions show the consensus region and the conserved sequences are given in bold letters. Expasy tool KALIGN (Lassman and Sonnhammer, 2005)

d

A



B

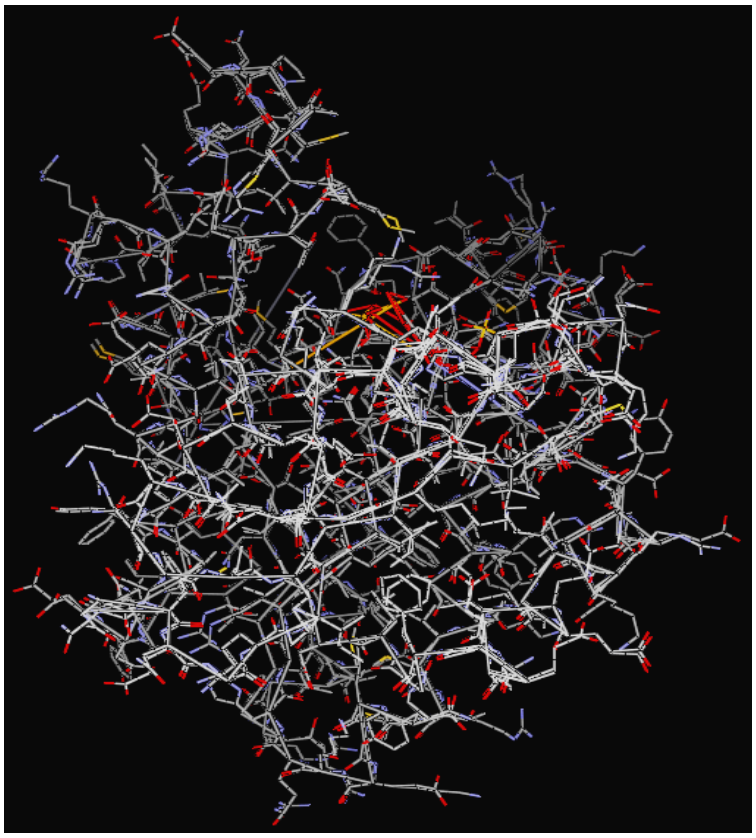


Fig 35: The proposed three-dimensional structure of NADPH dependant acetoacetyl CoA reductase protein from *Bacillus sp* 256 (Swiss-Port model)

A: 3D structure, ribbon model showing all the secondary structure elements
B: 3D structure, stick model

The BLAST search showed that the sequence is putative sequence of *Bacillus phaB* gene. This gene was further compared with *phaB* genes from other major scl-PHA-producing microorganisms by multiple sequence alignment using deduced amino acid sequences by KALIGN multiple sequence alignment program, Expasy tool (Table 28). The amino acid sequences were conserved among all the 10 microorganisms examined. More consensus domains were found towards the C terminal portion of the protein. The region 91 to 100 showed highly conserved domain in all the organisms. Overall there was more than 50% of sequence similarity among the organisms.

The three dimensional structure of the acetoacetyl CoA reductase protein from deduced amino acid sequence was elucidated using SWISS-PORT protein modeling tool The sequence showed putative hits with 432 pdb (Protein Data Bank) protein templates. Sequence showed highest homology with leodA pdb (a plant protein) with 44% identity. The sequence was aligned with this template using anchor residues as Leu 225 and Cys 228 with minimum energy. The proposed 3D structure of the protein suggested that the polypeptide could fold in to eight helices and five plated sheet elements connected with by various loops (Fig. 35). The stick model suggested that the protein folded in to its 3D structure by aligning the hydrophobic amino acids in to the core portion of the enzyme.

3.2.2 Isolation, cloning and characterization of *phaC* gene

The gene coding for PHA synthase (*phaC*) was amplified from the genomic DNA of *Bacillus sp* by PCR method. The PCR product showed the expected size of 1086 bp in an agarose gel with standard DNA marker (Fig. 36). The PCR product was checked by nested PCR, which resulted in the amplification of 600bp long *phaC* fragment. The *phaC* PCR amplicon was cloned in t-tailed vector. The recombinant plasmids (pTZC) were selected based on their movement in agarose gel (Fig. 37).

The *phaC* insert was sequenced completely and the sequence data was subjected for BLAST analysis (Table 29). The *phaC* gene was homologus in sequence with that of *Bacillus sp*. INT005 with highest score and identity (99%) followed by *B. cereus* strain SPV (99%), *B. cereus* ATCC 10987 (99%) and *B. anthracis* str. 'Ames Ancestor', (96%).

The restriction map of the *phaC* gene sequence showed that there is a *HindIII* site at 166th position (Fig. 38), which is conserved in *B. cereus*. The deduced restriction map of *phaC* gene showed that the gene also possesses polymorphism in *Bacillus*. The *phaC* from *Bacillus sp* showed that there was a *HindIII* site at 167th position and an XbaI site at 592 (Fig 38). In *B. megaterium* the *HindIII* site is present at 788 while in *B. cereus* and *B. antharacis* it is present at 166. The other major unique sites present in *phaC* gene were NdeI, 90 (*B. megaterium,* SmaI, 779 (*B. antharacis*) and XbaI, 591 (*Bacillus INT005*). The *phaC* gene from *Bacillus sp* consisted of 38 % GC and 62 % AT sequences.

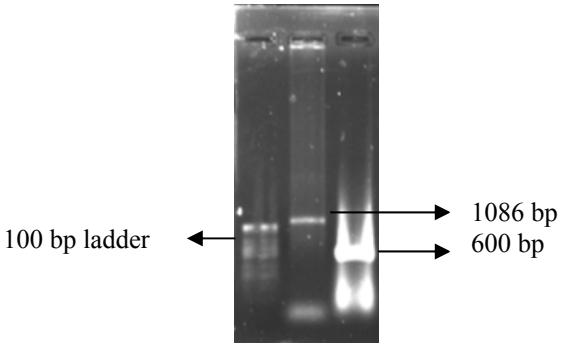


Fig 36: PCR amplicon of *phaC* gene

Lane 1: 100bp DNA ladder

Lane 2: 1086 bp *phaC* gene

Lane 3: 600 bp phaC fragment

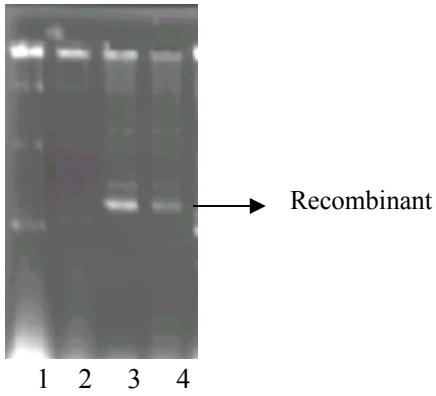


Fig 37: Cloning of *phaC* in pTZ57R/T

Lane 1 control pTZ57R

Lane 3 & 4 pTZC

Lane2 self ligation

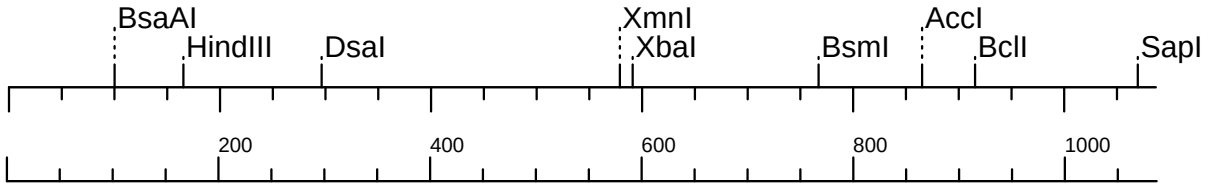


Fig. 38: Restriction map of *phaC* from *Bacillus sp*

Table 29: BLAST results of *phaC* gene sequences

NCBI results of BLAST

Accession	Description	Max score	Tot score	Query coverage	E value	Max ident
AB077026.1	*Bacillus sp.* INT005 phaR, phaB, phaC genes for PHA synthase subunit PhaR, 3-keto-acyl-CoA reductase, PHA synthase, complete cds	2095	2095	99%	0.0	99%
DQ486135.1	*Bacillus cereus* strain SPV PHA biosynthetic gene cluster, complete sequence	2062	2062	99%	0.0	99%
AE017194.1	*Bacillus cereus* ATCC 10987, complete genome	2056	2056	99%	0.0	99%
CP000001.1	*Bacillus cereus* E33L, complete genome	1816	1816	99%	0.0	96%
AE017355.1	*Bacillus thuringiensis* serovar konkukian str. 97-27, complete genome	1804	1804	99%	0.0	96%
AE017334.2	*Bacillus anthracis* str. 'Ames Ancestor', complete genome	1804	1804	99%	0.0	96%
AE017225.1	*Bacillus anthracis* str. Sterne, complete genome	1804	1804	99%	0.0	96%
AE016879.1	*Bacillus anthracis* str. Ames, complete genome	1804	1804	99%	0.0	96%
CP000485.1	*Bacillus thuringiensis* str. Al Hakam, complete genome	1788	1788	99%	0.0	95%
DQ000291.1	*Bacillus thuringiensis* strain R1 PhaP (phaP), PhaQ (phaQ), PhaR (phaR), PhaB (phaB), and PhaC (phaC) genes, complete cds; and oxidoreductase gene, partial cds	1788	1788	99%	0.0	95%
AY331151.1	*Bacillus thuringiensis* PhaP (phaP), PhaQ (phaQ), PhaR (phaR), PhaB (phaB), and PhaC (phaC) genes, complete cds	1776	1776	99%	0.0	95%
AE016877.1	*Bacillus cereus* ATCC 14579, complete genome	1752	1752	99%	0.0	95%
AY907827.1	*Bacillus cereus* strain CFR04 PhaC (phaC) gene, partial cds	682	682	38%	0.0	95%
AF109909.2	*Bacillus megaterium* polyhydroxyalkanoate gene cluster, complete sequence	79.8	121	17%	2e-11	90%

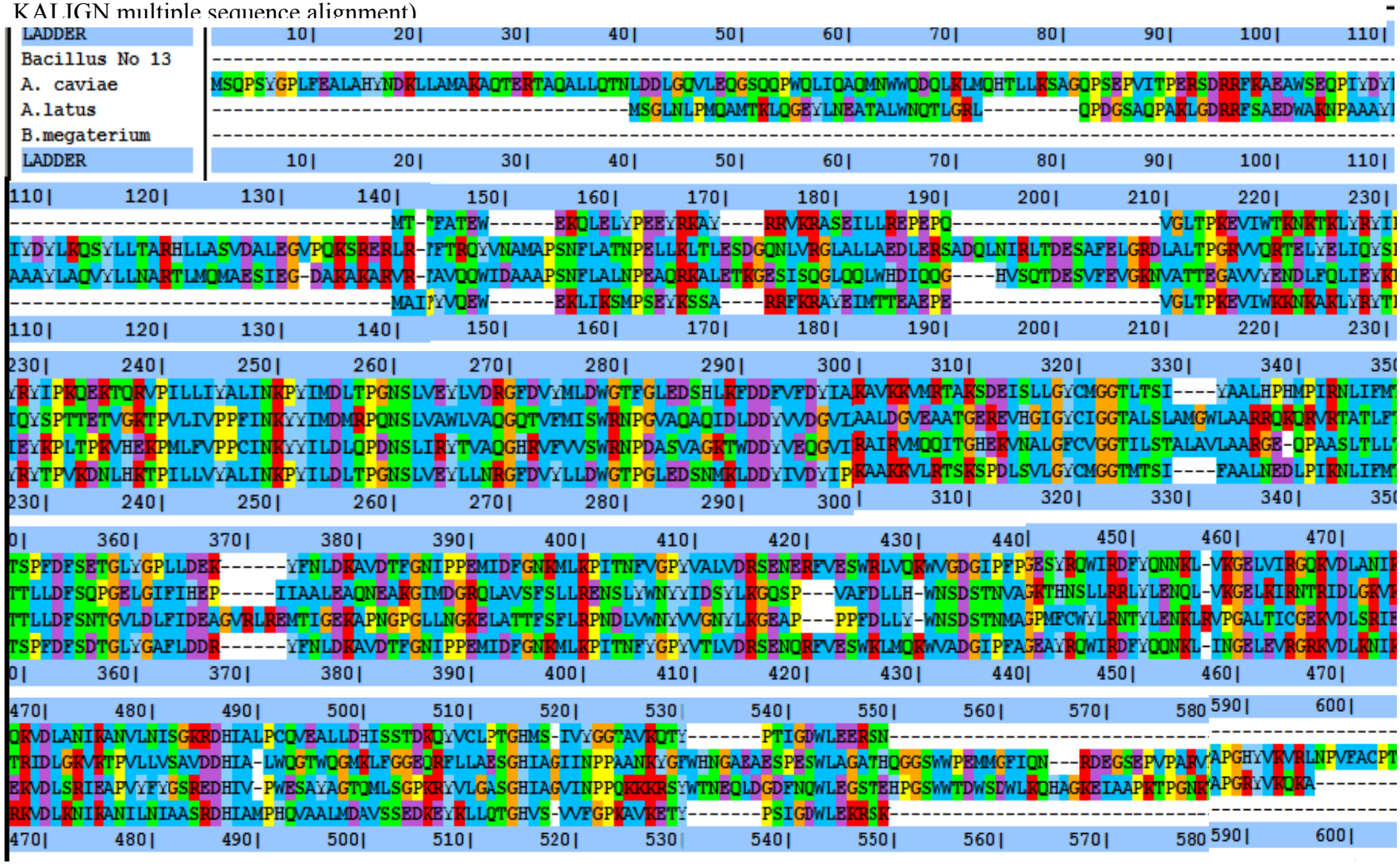
Table 30: *PhaC* gene (PHA synthase) sequence from *Bacillus sp*

```
  1  atgactacat tcgcaacaga atgggaaaag caattagagc tatacccaga agagtaccga aaagcatatc gccgagtgaa aagggcgagt
        M  T  T   F  A  T   E  W  E  K   Q  L  E   L  Y  P   E  E  Y  R   K  A  Y   R  R  V   K  R  A  S

 91  gaaatttttat tacgtgaacc agagccacaa gtaggattaa cgccgaaaga ggttatttgg acgaagaata agacgaagct ttatcgctac
      E  I  L   L  R  E   P  E  P  Q   V  G  L   T  P  K   E  V  I  W   T  K  N   K  T  K   L  Y  R  Y

181  attccaaaac aagaaaaaac acaaagagtt ccaattctat taatatatgc tcttattaat aaaccatata ttatggattt aactcctgga
      I  P  K   Q  E  K   T  Q  R  V   P  I  L   L  I  Y   A  L  I  N   K  P  Y   I  M  D   L  T  P  G

271  aatagtttag tggaatatct agtggaccgt ggttttgatg tgtatatgct tgattggggc acatttggtt tagaagatag tcatttgaaa
      N  S  L   V  E  Y   L  V  D  R   G  F  D   V  Y  M   L  D  W  G   T  F  G   L  E  D   S  H  L  K

361  tttgatgatt ttgtgtttga ttatattgca aaagcagtga aaaaagtaat gcgaactgca aaatcggacg agatttcttt acttggttat
      F  D  D   F  V  F   D  Y  I  A   K  A  V   K  K  V   M  R  T  A   K  S  D   E  I  S   L  L  G  Y

451  tgcatggggg gaacgctaac ttctatttat gcggcacttc atccacatat gccaattcgt aacctaatct ttatgacaag tccttttgat
      C  M  G   G  T  L   T  S  I  Y   A  A  L   H  P  H   M  P  I  R   N  L  I   F  M  T   S  P  F  D

541  ttctctgaaa caggattata tggtccttta ttagatgaga aatacttcaa tctagataaa gcggttgata catttggaaa tattccgcca
      F  S  E   T  G  L   Y  G  P  L   L  D  E   K  Y  F   N  L  D  K   A  V  D   T  F  G   N  I  P  P

631  gaaatgattg atttcggaaa caaaatgtta aaaccaatta cgaactttgt tggtccatat gttgctttag tagatcgttc agagaatgag
      E  M  I   D  F  G   N  K  M  L   K  P  I   T  N  F   V  G  P  Y   V  A  L   V  D  R   S  E  N  E


721  cgcttcgttg aaagctggag gttagttcaa aagtgggttg gcgatggcat tccgttccca ggtgaatcat acagacagtg gattcgtgat
      R  F  V   E  S  W   R  L  V  Q   K  W  V   G  D  G   I  P  F  P   G  E  S   Y  R  Q   W  I  R  D
```

(contd…)

```
811  ttttatcaaa acaataaatt ggttaagggt gaactcgtta ttcgcggaca aaaggtagac cttgcaaata ttaaggcgaa tgtcttaaat
      F  Y  Q   N  N  K   L  V  K  G   E  L  V   I  R  G   Q  K  V  D   L  A  N   I  K  A   N  V  L  N

901  atttcaggga aacgtgatca tatcgccctg ccatgccaag tagaagcgtt gctagatcat atttctagca cagataaaca atatgtatgt
      I  S  G   K  R  D   H  I  A  L   P  C  Q   V  E  A   L  L  D  H   I  S  S   T  D  K   Q  Y  V  C

991  ttaccaacgg gacatatgtc gattgtttac ggtggaacag cggtaaaaca aacgtatccg acgattggag actggcttga agagcgttct
      L  P  T   G  H  M   S  I  V  Y   G  G  T   A  V  K   Q  T  Y  P   T  I  G   D  W  L   E  E  R  S

1081  aattaa
       N  -
```

Table 31: Multiple sequence alignment of PHA synthase of *Bacillus sp* with PHA synthase from other three PHA producing organisms. The shaded regions show the consensus region and the conserved sequences are given in bold letters. (Expasy tool KALIGN multiple sequence alignment)



The analysis of *phaC* sequence showed that the insert was a complete ORF of 1086 bp size (Table 30). The deduced amino acid sequence of the *phaC* showed that the DNA fragment codes for a continuous stretch of 361 amino acid chains with an approximate molecular weight of 42kDa. The BLAST search showed that the sequence is putative sequence of *Bacillus phaC* gene. The deduced amino acid of *phaC* gene was subjected to multiple sequence alignment with that of *A. latus*, *A. caviae* and *B. megaterium* by KALIGN multiple sequence alignment program, Expasy tool (Table 31). The amino acid sequences showed differences between these three groups. The *Bacillus sp*13 PHA synthase was similar to *B. megaterium* (90%) and C terminal end showed much consensus domains.

3.2.3 Isolation, cloning and characterization of *phaA* gene

The *phaA* gene codes for the enzyme β-ketothiolase, which catalyses the first step in scl-PHA biosynthesis. In *Bacillus* this gene is not associated with the pha operon. The *phaA* gene was isolated by PCR technique from the genomic DNA of *Bacillus sp* 256. The PCR amplicon of *phaA* gene showed expected size in an agarose gel (Fig. 39). The PCR product was purified, A-tailed and ligated in to pTZ57R/T vector. The recombinant pTZA plasmid was selected from an agarose gel (Fig. 40). The *phaA* insert in the pTZA plasmid was sequenced completely and the sequence was analysed using online software. Restriction map of *phaA* gene (Fig 41) showed that similar to other *Bacillus spp phaA* gene the *phaA* of *Bacillus sp* 256 contained *PstI* site at 543, *PvuII* site at 721 and *HaeII* at 861 irrespective of their position. The *phaA* gene of *Bacillus sp* 256 contained 44 % GC and 56 % AT sequences.

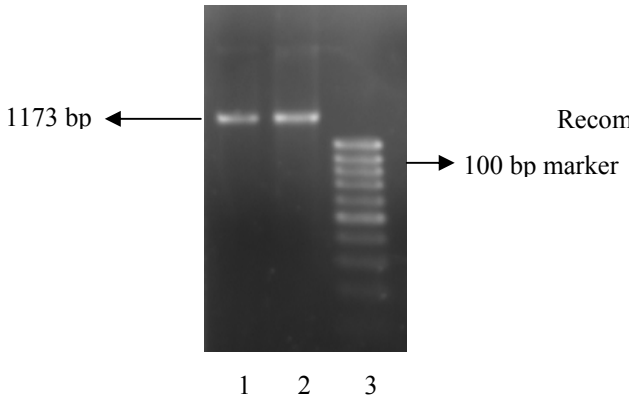


Fig 39: PCR amplicon of *phaA* gene

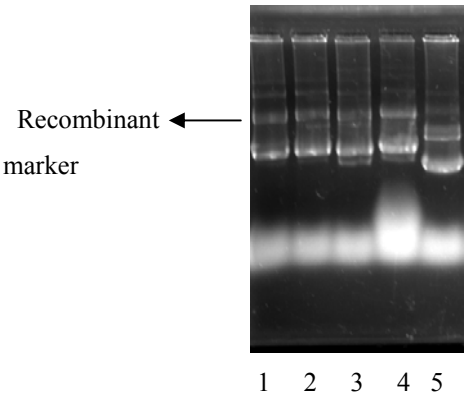


Fig 40: Cloning of *phaA* in pTZ57R/T

Lane 1&2 1173 bp *phaA* gene

Lane 3 100bp DNA ladder

Lane 1, 2, 3 & 4 pTZA clone

Lane 5 control pTZ57R

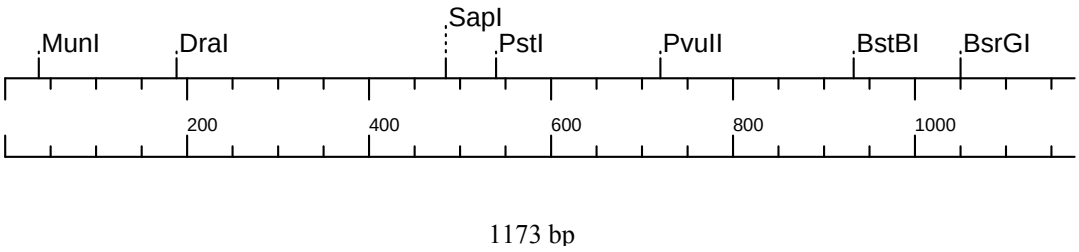


Fig. 41: Restriction map of *Bacillus sp* 256 *phaA* gene

Table 32: *phaA* BLAST result

NCBI results of BLAST

Accession	Description	Max score	Tot score	Query coverage	E value	Max identity
EF038547.1	Synthetic construct *Bacillus anthracis* clone FLH248243.01L BA5248 gene, complete sequence	2183	2183	99%	0.0	98%
AE017334.2	*Bacillus anthracis* str. 'Ames Ancestor', complete genome	2183	2227	99%	0.0	98%
AE017225.1	*Bacillus anthracis* str. Sterne, complete genome	2183	2227	99%	0.0	98%
AE016879.1	*Bacillus anthracis* str. Ames, complete genome	2183	2227	99%	0.0	98%
CP000485.1	*Bacillus thuringiensis* str. Al Hakam, complete genome	2103	2103	99%	0.0	97%
CP000001.1	*Bacillus cereus* E33L, complete genome	2087	2087	100%	0.0	97%
AE017194.1	*Bacillus cereus* ATCC 10987, complete genome	2056	2056	99%	0.0	97%
AE017355.1	*Bacillus thuringiensis* serovar konkukian str. 97-27, complete genome	2040	2040	99%	0.0	97%
AE016877.1	*Bacillus cereus* ATCC 14579, complete genome	2032	2032	100%	0.0	96%
Z99120.2	*Bacillus subtilis* complete genome (section 17 of 21): from 3213330 to 3414388	105	105	15%	4e-19	82%
BA000004.3	*Bacillus halodurans* C-125 DNA, complete genome	75.8	171	21%	3e-10	89%
AP008934.1	*Staphylococcus* saprophyticus subsp. saprophyticus ATCC 15305 DNA, complete genome	60.0	106	5%	2e-05	100%
CP000141.1	*Carboxydothermus* hydrogenoformans Z-2901, complete genome	58.0	58.0	4%	8e-05	88%
CP000002.2	*Bacillus licheniformis* ATCC 14580, complete genome	58.0	104	13%	8e-05	85%
AE017333.1	*Bacillus licheniformis* DSM 13, complete genome	58.0	104	13%	8e-05	85%
CR954246.1	*Pseudoalteromonas* haloplanktis str. TAC125 chromosome I, complete sequence	52.0	98.1	5%	0.005	91%

Table 33: *PhaA* gene (β-ketothiolase) from *Bacillus sp* 256

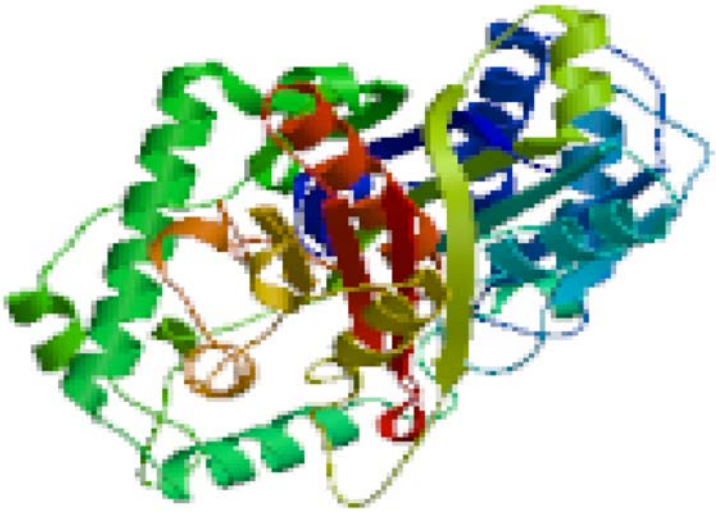
```
1   atgagagaag ctgtcattgt tgcgggagca agaacaccaa ttggaaaagc aaagaggggt tcattaaaaa cagttcgtcc tgacgatcta
      M  R  E   A  V  I   V  A  G  A   R  T  P   I  G  K   A  K  R  G   S  L  K   T  V  R   P  D  D  L

 91   ggggcgttag tagtaaagga aacgttaaag cgtgcgaatt atgaaggacc aatcgatgat ttaattttcg gttgtgcgat gccagaagca
       G  A  L   V  V  K   E  T  L  K   R  A  N   Y  E  G   P  I  D  D   L  I  F   G  C  A   M  P  E  A

181  gagcaaggtt taaatatggc tcgtaatatc ggcggattag caggactttc ttacgatgtt ccagctatta caattaaccg ttactgttct
       E  Q  G   L  N  M   A  R  N  I   G  G  L   A  G  L   S  Y  D  V   P  A  I   T  I  N   R  Y  C  S

271  tcaggtttac aaagtatcgc ttacggagca gagcgcatta tgcttggtca ctcggaagcg gtattatcag gcggagcggg atcaatgagt
       S  G  L   Q  S  I   A  Y  G  A   E  R  I   M  L  G   H  S  E  A   V  L  S   G  G  A   G  S  M  S

361  ttagttccga tgatgggaca cgtcgttcgt ccgaatagtc gccttgtaga agcggctcca gaatattata tgggtatggg acatacagca
       L  V  P   M  M  G   H  V  V  R   P  N  S   R  L  V   E  A  A  P   E  Y  Y   M  G  M   G  H  T  A

451  gagcaagttg ctgtgaaata tggaatttct cgtgaagagc aagatgcatt tgcagtaaga agtcatcaac gcgctgcgaa agcattagct
       E  Q  V   A  V  K   Y  G  I  S   R  E  E   Q  D  A   F  A  V  R   S  H  Q   R  A  A   K  A  L  A

541  gcagggaact ttgctgatga aacagtatct gtagatgtaa cgttacgtac tgttggagca aataacaaac tgcaagaaga aacaatcact
       A  G  N   F  A  D   E  T  V  S   V  D  V   T  L  R   T  V  G  A   N  N  K   L  Q  E   E  T  I  T

631  ttcacgcaag acgaaggtgt aagagctgaa acgacgctag atattttagg taaattacgt ccagcattta acgttcgcgg ttctgtaaca
       F  T  Q   D  E  G   V  R  A  E   T  T  L   D  I  L   G  K  L  R   P  A  F   N  V  R   G  S  V  T
```

(contd..)

```
 721  gctggtaact cttcacaaat gagtgacggc gcagcatctg tactattaat ggatcgtgaa aaagcagtga gcgatggcat gaaaccactt
       A  G  N   S  S  Q   M  S  D  G   A  A  S   V  L  L   M  D  R  E   K  A  V   S  D  G   M  K  P  L

 811  gcgaaattcc gttcatttgc agtagctggc gtaccaccag aagtaatggg aattggccca atcgctgcaa ttccaaaagc gttaaaacta
       A  K  F   R  S  F   A  V  A  G   V  P  P   E  V  M   G  I  G  P   I  A  A   I  P  K   A  L  K  L

 901  gctggcttag agctatctga tattggctta ttcgaactaa atgaagcatt cgcttctcaa tcgatccaag ttattcgtga acttggttta
       A  G  L   E  L  S   D  I  G  L   F  E  L   N  E  A   F  A  S  Q   S  I  Q   V  I  R   E  L  G  L

 991  gatgaagaaa aagtaaacgt aaatggcggt gcaatcgcac ttggacatcc acttggctgt acaggagcaa aactaacact atctcttatt
       D  E  E   K  V  N   V  N  G  G   A  I  A   L  G  H   P  L  G  C   T  G  A   K  L  T   L  S  L  I

1081  cacgaaatga aacgccgcaa cgaacaattc ggtatcgtaa caatgtgtat cggcggcgga atgggagcag cgggagtatt tgaattactt
       H  E  M   K  R  R   N  E  Q  F   G  I  V   T  M  C   I  G  G  G   M  G  A   A  G  V   F  E  L  L

1171  taa
       -
```

Table 34: Multiple sequence alignment of β-ketothiolase of *Bacillus sp* 256 with ketothiolase from other eight PHA producing organisms. The shaded regions show the consensus region and the conserved sequences are given in bold letters. (Expasy tool KALIGN multiple sequence alignment)

A



B

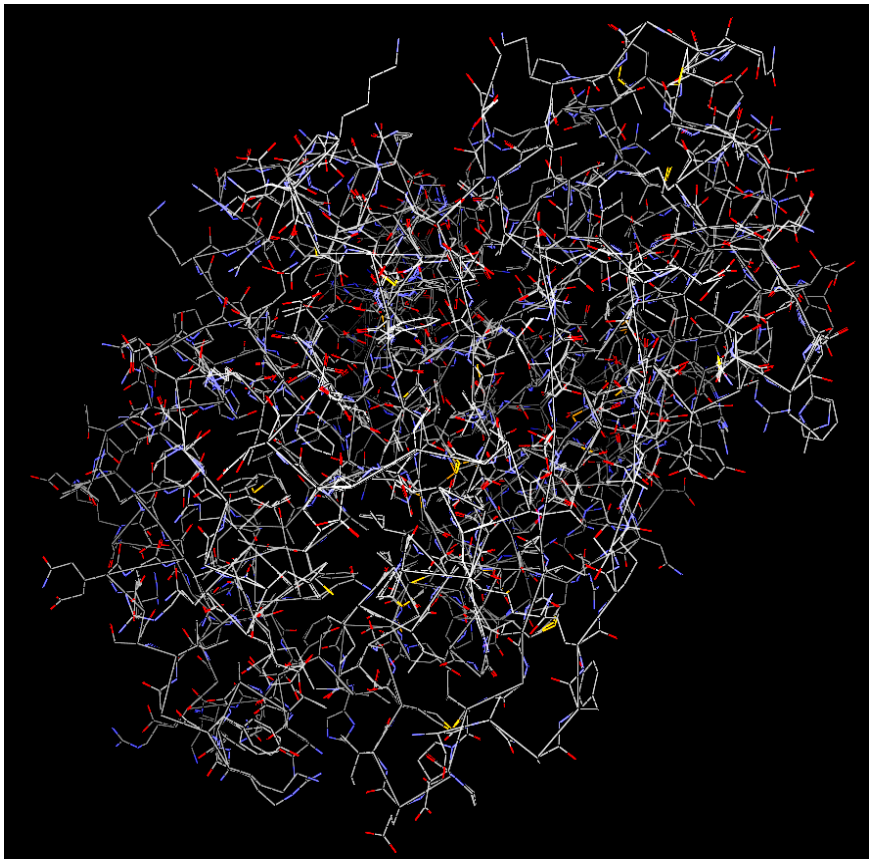


Fig 42: The proposed three-dimensional structure of β-ketothiolase protein from *Bacillus sp* 256 (Swiss-Port model)

A: 3D structure, ribbon model showing all the secondary structure elements
B: 3D structure, stick model

The result of the BLAST analysis confirmed the authenticity of the DNA insert (Table 32). The BLAST analysis showed that the *phaA* sequence was identical to 3- keto acyl CoA thiolase (β-ketothiolase) of many *Bacillus* genome reported. The sequence showed much homology with 3-ketoacyl CoA thiolase of *B. anthracis* str. 'Ames Ancestor' with highest score and similarity (98%) followed by *B. anthracis* str. Sterne (98%), *B. thuringiensis* str. Al Hakam (97%), *B. cereus* E33L (97%) etc. The nucleotide sequence of the *phaA* gene was a complete ORF of 1173 bp long and the deduced amino acid sequence (390 amino acids) revealed that the gene was able to code for a protein with a molecular weight of ~45 kDa (Table 33). The BLAST search showed that the sequence is putative sequence of *Bacillus phaA* gene. This gene was further compared with *phaA* genes from other major scl-PHA-producing microorganisms including *A. hydrophilla*, by multiple sequence alignment using deduced amino acid sequences.

The multiple sequence alignment of deduced amino acid sequences with ketothiolase sequences from some other PHA producing bacteria showed a difference in protein sequences. The sequence homology and conserved regions were very less (Table 34). The three dimensional structure of the β-ketothiolase protein was elucidated from deduced amino acid sequence using SWISS-PORT protein modeling tool (Fig.42). Sequence showed putative hits with 76 protein templates in the pdb library. The highest homology was found with the template 2c7yB pdb with 47% identity. The sequence aligned with the template using the anchor residues Glu 45 and Ile 48 at minimum energy. The model suggested that the polypeptide could be folded in to nine helices and seven plated sheets (Fig 42A). The stick model showed that the protein folded in to its 3D structure by aligning the hydrophobic amino acids in to the core portion of the protein.

Genomic BLAST analysis *Bacillus* with ketothiolase sequence (*bktB*) of *R. eutropha* showed that three to four similar genes are present in the genome with a descending order of similarity; we designated them as *phaA1 phaA2*, *phaA3* and *phaA4*. The *phaA* gene cloned from the *Bacillus sp* 256 resembled *phaA2* of *Bacillus spp*. Phylogenetic analysis of the sequences of *PhaA* from *Bacillus sp* 256, as compared with other *Bacillus spp* and β-ketothiolase enzymes are shown in Fig. 43. It was also compared with the two

ketothiolases of *R. eutropha* (*phaA* and *bktB*) and it was found that the *Bacillus sp* 256 *phaA* sequence matched with both the genes of *R. eutropha* (43% to *phaA* and 44% to *bktB*).

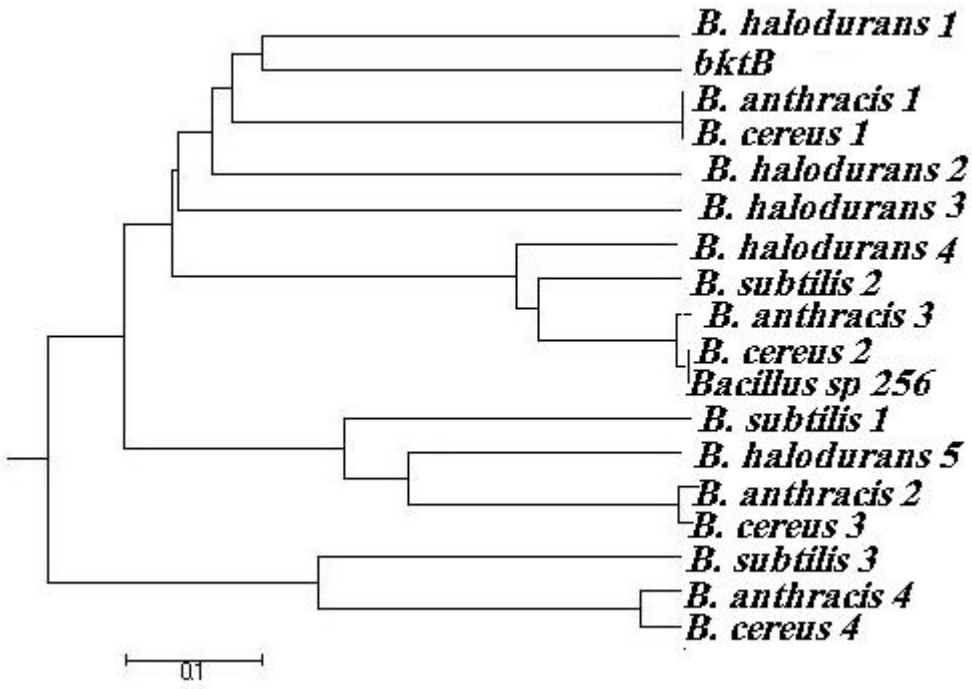


Fig. 43: Phylogenetic analysis of the sequences of *PhaA* from *Bacillus sp* 256, as compared with other *Bacillus spp* and β-ketothiolase enzymes. The enzyme is also compared with that of *bktB* from *Ralstonia eutropha.* Numbers 1-5 indicates descending order of similarity amongst the species compared to *bktB* of *Ralstonia eutropha.*

3.3 DISCUSSION

In recent years, a combination of genetic engineering and molecular microbiology techniques has been applied to enhance PHA production in microorganisms. Natural producers, such as *R. eutropha*, are well adapted to PHA accumulation up to 90% of its dry weight. But most natural producers, including *R. eutropha*, take a long time to grow during fermentation and extraction of polymers from their cells is also difficult due to their rigid cell wall. In order to circumvent this problem recombinant microorganisms were developed by cloning the PHA biosynthesis genes from various natural PHA producing microorganisms, which was followed by the characterization of PHA operon from *R. eutropha* (Schubert *et al,* 1988; Slater *et al*, 1988; Peoples and Sinskey, 1989).

In scl-PHA producing organisms, three genes are responsible for the PHA production in a biosynthetic pathway. The three-step enzymatic reaction of the pathway starts with the condensation of two molecules of acetyl CoA molecules in to acetoacetyl CoA. This initial step is catalyzed by the enzyme β-ketothiolase coded by *phaA* gene. In the next consecutive steps the acetoacetyl CoA is reduced to 3-hydroxybutyryl CoA and this is polymerized to the growing PHA chain, by acetoacetyl CoA reductase (*phaB*) and PHA synthase (*phaC*) enzymes, respectively.

PHA biosynthesis genes were cloned and characterized from *Bacillus sp*. The *phaB* gene, which codes for NADPH dependant acetoacetyl CoA reductase was cloned from *Bacillus sp* 256 and sequenced completely. The *phaB* sequence was a complete ORF for 247 amino acids and the sequence was similar to other *Bacilli*, especially to that of *B. megaterium*. The multiple sequence alignment of the deduced amino acid sequence showed that the *Bacillus* acetoacetyl CoA reductase also resembles with the *phaB* proteins of other PHA producing organisms.

The *phaB* gene is widely present in all scl-PHA producing organisms; acetoacetyl CoA reductase act on specific substrates obtained from PHA biosynthesis pathway or from β-oxidation of fatty acids. During PHA biosynthesis the acetoacetyl CoA is formed from two acetyl CoA molecules by the action of the enzyme β-ketothiolase. Through β-oxidation also acetoacetyl CoA is formed at the end of the oxidation where β- ketoacyl CoA containing three acetyl CoA residues accumulate, which is split into acetoacetyl CoA and acetyl CoA by the enzyme thiolase. This molecule is cleaved into acetyl CoA and acetoacetyl CoA by thiolase enzyme. NADPH dependant acetoacetyl CoA reductase catalytically reduces acetoacetyl coA into 3-hydroxybutyryl CoA by transferring two molecules of hydrogen in to the third carbon atom of acetoacetyl CoA with the help of NADPH molecule. The activity of this enzyme is regulated by the concentration of coenzyme NADPH molecule in the cell. The source of NADPH in the cell is derived from pentose phosphate pathway or sometimes from citric acid cycle. In pentose phosphate pathway two NADPH are formed in the first two-enzymatic steps catalyzed by the enzymes glucose 6-phophate dehydrogenase and 6-phosphogluconate decarboxylase. The phaB enzyme also reduces 3-ketovaleryl CoA into 3-hydroxyvaleryl CoA for PHV production.

The *phaC* gene from *Bacillus sp* was cloned and sequenced completely. The nucleotide sequence was a 1086 bp long complete ORF coding for a polypeptide of 361 amino acids. The *phaC* gene was similar to that of *B. cereus* and *B. megaterium*. The multiple sequence alignment of deduced amino acid sequence showed that the PHA synthase of *Bacillus* is different. The PHA synthase is the key enzyme in the PHA synthesis in microorganisms and *Bacillus* PHA synthase was described as unique one from other PHA synthases reported. The PHA synthase of *Bacillus* polymerizes 3-hydroxybutyryl CoA or 3-hydroxyvaleryl CoA, derived by the action of acetoacetyl CoA reductase enzyme in the PHA biosynthesis pathway, in to the growing chain of PHA. Substrate specificity of class II PHA synthase differs, which can polymerize monomers of 6-14 carbon length. The PHA synthases of *Bacillus* resembles that of *A. vinosum* (class

III) wherein PHA synthase consists of two different subunits and are associated in the operon. Bacillus PHA synthase differs from that of *A. vinosum* in certain aspects such as

the molecular weight of *phaR* is just half of *phaE*, total molecular weight of PHA synthase is 80 kDa but that of *Bacillus* is 60kDa. In *Bacillus phaB* is located between *phaR* and *phaC* while in *A. vinosum phaE* and *phaE* are clustered together. The active PHA synthase of *Bacillus* is a tetramer and it requires *phaR* subunit protein for its catalytic activity.

The *phaA* gene that codes for β-ketothiolase was cloned from *Bacillus sp* 256 and sequenced completely. The sequence data showed that the DNA fragment was 1173 bp in size and was a complete ORF with 390 amino acids. Two β-ketothiolases are known to be present in *R. eutropha* and they are supposed to possess different substrate specificities and one of them is responsible for poly 3-HV synthesis. It is known that the *Bacilli* are capable of synthesizing PHV, which is formed intracellularly by condensing acetyl CoA and propionyl CoA. So it is predictable that *Bacillus sp* also posses a ketothiolase, which has broad substrate specificity.

3.4 CONCLUSIONS

PHAs are formed intracellularly by cascade of enzymatic reactions using cellular metabolites. Scl and mcl PHA are formed through different pathways involving separate precursors. Based on the arrangement of genes and the subunit requirement of PHA synthase, pha operons are grouped in to four categories: class I II, III & IV. Class I operon, represented by *R. eutropha*, is characterized extensively and has been used to construct recombinant organisms for commercial exploitation. The class II and III are represented by *Pseudomonas sp* and *A. vinosum,* respectively. *Bacillus* is placed under class IV group wherein pha operon consists of five genes namely *phaP*, *phaQ*, *phaR*, *phaB*, and *phaC*. The *phaA* gene, which codes for β- ketothiolase is present independently in the genome.

In scl-PHA producing bacteria the pathway mainly consist of three enzymes such as β-ketothiolase, NADPH dependant acetoacetyl CoA reductase and PHA synthase, coded by the genes *phaA*, *phaB* and *phaC,* respectively. These genes are widely present in all scl-PHA producing family. In the present chapter *phaA*, *phaB* and *phaC* were cloned characterized from *Bacillus spp*. The genes were amplified from the genomic DNA of the *Bacillus sp* by PCR using gene specific primers. The *phaB* gene cloned and sequenced from *Bacillus sp* 256 consisted of 744bp. The sequence was a complete ORF with a polypeptide of 247 amino acids. The *phaB* sequence was similar to acetoacetyl CoA reductase of other *Bacilli*. The gene was similar to that of *B. megaterium*. The cloned acetoacetyl CoA reductase was also similar to other reductases of different scl-PHA producing bacteria, as evidensed by multiple sequence alignment of the deduced amino acid sequences. *PhaB* gene is widely present in microorganisms and its protein catalytically reduces acetoacetyl CoA with the help of one NADPH molecule. The enzyme can also reduce 3-hydroxyvaleryl CoA in to ketovaleryl CoA during PHV synthesis. The activity of this enzyme is highly dependent on the availability of NADPH molecules, which is considered as one of the limiting factors of PHA accumulation in scl-PHA producing organisms.

The gene coding for PHA synthase (*phaC* gene) was amplified from *Bacillus sp*. The gene was cloned and sequenced completely. The gene was 1086 bp long and was a complete ORF coding for 361 amino acids. The sequence was very similar to that of *B. cereus.* The multiple sequence alignment of the PHA synthase showed that the PHA synthase differs from PHA synthases of other organisms. The analysis of the nucleotide sequence of the cloned *phaC* gene revealed that *phaC* gene is polymorphic in *Bacillus*. The PHA synthase of *Bacillus* represent the class IV PHA synthase. The activity of this protein does need a *phaR* protein subunit.

phaA gene, which codes for the enzyme β-ketothiolase was also characterised. The gene was 1173 long and was a complete ORF of 390 amino acids. The nucleotide sequence was analysed and it was similar to many *Bacillus* 3-acyl CoA thiolases. This enzyme β-ketothiolase initiates the scl PHA biosynthesis by condensing two molecules of acetyl CoA molecules to form acetoacetyl CoA. During PHV synthesis this enzyme also act on propionyl CoA along with one molecule of acetyl CoA to form 3-ketovaleryl CoA. The genome of Bacillus possesses three to four homologous gene sequences and it resembles β-ketothiolase (*bktB*) *of R. eutropha.* In *R. eutropha* it is reported that the *bktB* gene product has more affinity towards propionyl CoA than towards acetoacetyl CoA. It is concluded that *Bacillus* also contains more than one β-ketothiolases and these different genes may possess different substrate specificities. The cloned genes of *Bacillus* were complete and could be utilized to construct recombinant organisms. Since the *Bacillus* sp 256 is able to synthesize PHV, the β-ketothiolase gene from it is potent enough to produce PHV in recombinant organisms. *PhaC* gene can be utilized only along with a *phaR* gene, since the activity of PHA synthase needs *phaR* protein subunit.

4.0 INTRODUCTION

Polyhydroxyalkanoate (PHA) has been considered as an alternative thermoplastic for petroleum derived synthetic plastics. Different constituents of PHA in bacteria have been identified as various hydroxyalkanoic acids with 3-14 carbon atoms. The two major classes of PHA are short chain length PHA (scl-PHA, contains 3-5 carbon atoms) and medium chain length PHA (mcl-PHA, made up of 6-14 carbon atoms). The different monomers of PHA can form copolymers. The copolymers of short chain length-co-medium chain length (scl-co-mcl) PHA have thermoplastic to elastomeric properties. PHA containing both scl-PHA and mcl-PHA has been identified in several natural organisms (Liebergesell *et al*, 1991; Kobayashi *et al*, 1994; Kato *et al*, 1996; Brandl *et al*, 1989; Chen *et al,* 2001; Lee *et al*, 2000).

Heteropolymeric PHAs are commercially more valuable because they are less crystalline and possess lower melting temperature, which can be readily processed for broader range of application (Doi *et al*. 1995). At present, the PHA heteropolymers produced on commercial scale are co polymers containing different molar ratios of hydroxybutyrate and hydroxyvalerate - P(HB-co-HV). Other copolymers like polyhydroxy(butyrate-co-hexanoate)-P(HB-co-HX) have also been reported and its mechanical properties have been compared to that of commercial polymer such as low density polyethylene (Chen *et al*, 2001).

In recent years, quantitative and qualitative changes in PHA production in microorganisms have been achieved by using a combination of metabolic engineering and molecular microbiology techniques. Several recombinant host strains and mutants were developed in order to produce PHA in an efficient way. PHA biosynthesis genes have been isolated and characterized from natural PHA producing organisms and the relevant genes have been expressed in heterologous systems. Among the different heterologous systems, development of recombinant *E. coli* is more suitable because, it

possess fast growth, can be grown to high cell density, lacks PHA-depolymerase and has labile cell wall (Fidler and Dennis, 1992).

In the present chapter heterologous cloning of PHA biosynthesis genes in *E. coli* and *B. subtilis* are described. The recombinant *E. coli* strain *JC7623ABC1J4* was developed by cloning *phaA* and *phaB* genes from *Bacillus sp* 256 (coding for β-ketothiolase and acetoacetyl CoA reductase respectively, involved in the scl-PHA biosynthesis) and *phaC1* and *phaJ4* genes from *P. aeruginosa* (PHA synthase and (R) specific enoyl CoA hydratase, respectively) for scl-co-mcl PHA copolymer production. Recombinant strain of *B. subtilis* was developed by transforming the strain with an expression vector containing *phaC1* and *phaJ4* genes.

4.1 MATERIALS AND METHODS

4.1.1 Heterologus cloning in E. coli

Various strains and plasmids used for cloning experiments, their source and properties are given in the Table 35.

Table 35: Bacterial strains and plasmids used for experimentation

Strains/Plasmids	Relevant properties	Source
E. coli DH5α	SupE44LacU169(ϕ80 lacZΔM15)GsdR17 recA1 end A1 gyr A96 thi-1 rel A1	Lab collection
Bacillus subtilis Strain	Standard strain	Bacillus Genetic Stock Centre (BGSC), Ohio, USA
E. coli JC7623 F	lacZ+, leu-6, his-4, ara-14, recB21, recC22, sbcB15, λ^-	Vector Collection Center, National Institute of Genetics Shizuoka-ken. Japan
pBRINT-Cm	Cm ^{r} Amp ^{r}	-As above-
pTZ557R/T	Amp ^{r}	MBI Fermentas Lithuania
pTZphaB	Amp ^{r} *PhaB*+	Lab construct
pTZphaA	Amp ^{r} *PhaA*+	Lab construct
pCPC1J4	Amp ^{r} *PhaC1*+ *PhaJ4*+	Lab construct
pBRB-Cm	Cm ^{r} Amp ^{r} *PhaB*+	Lab construct
pBRBA-Cm	Cm ^{r} Amp ^{r} *PhaB*+ *PhaA*+	Lab construct
pBPC1J4	Amp ^{r} *phaC1*+ *phaJ4*+	Reeta Davis and Chandrashekar A, CFTRI, Mysore, India
pSGABant	Cm ^{r} Amp ^{r} *PhaB* $^{anti\ S}$ *PhaA* $^{anti\ S}$	Lab construct
pTZphaA nest	Amp ^{r} *PhaA* partial	Lab construct
pMUT-HA ket	Amp ^{r} Erm ^{r} *PhaA* partial	Lab construct

The strains were maintained and cultivated in Luria Bertani medium (Himedia, Mumbai, India) and were sub cultured once in a month.

4.1.2 Designing of primers

Sets of oligonucleotide primers were designed for the successful cloning and expression of selected *Bacillus* PHA biosynthesis genes in *E. coli* (Table 36). The forward primers for *phaB* and *phaA* genes were modified by inserting a ribosome-binding site (RBS, underlined) at the upstream of the start ATG. In order to facilitate the cloning, an *EcoRI* site and a *SmaI* site were added (italics) at the 5' end of phaA forward primer. The reverse primers were designed by selecting the 3' end of the coding region of the both genes.

Table 36: Modified primers for *E. coli* expression

Primer	DNA target	Sequence (5'-3')
PhaBFX	*PhaB*	5'..TAA AGAAAACAGCTAATGGTTCAATTAAATGGAAAAGTA..3'
PhaBR	*PhaB*	5'..TTACATRTATAAACCGCCGTTAATG..3'
PhaAFX	*PhaA*	5'..AGACGT*CCCCGGGGAATTC*TAAAGAAAACAGCTAATGAG-AGAAGCTGTCATTGTT..3'
PhaAR	*PhaA*	5'..CAGCGT*GGTACCCTCGAG*TTAAAGTAATTCAAATACT..3'

4.1.3 PCR amplification and cloning of PHA genes

Polymerase chain reaction was used to amplify PHA biosynthesis genes from the *pTZphaA* and *pTZphaB* clones using the modified specific primers. The PCR conditions were the same, which were used to amplify these two genes, described in the chapter 3 (pp.119, 129, section: 3.1.4). Amplicons for both the genes were purified and cloned, as mentioned earlier in chapter 3, in to two separate pTZ57R/T vectors.

4.1.4 Construction of *pBRB-Cm* and *pBRAB-Cm* vectors

pBRB-Cm and *pBRBA-Cm* vectors were constructed by ligating *phaB* and *phaA* genes, from *pTZPhB* and *pTZPhA* plasmids, respectively, in to *E. coli* integration vector *pBRINT-Cm* (Fig. 44). The overall strategy of construction of *pBRBA-Cm* vector is illustrated (Fig. 45).

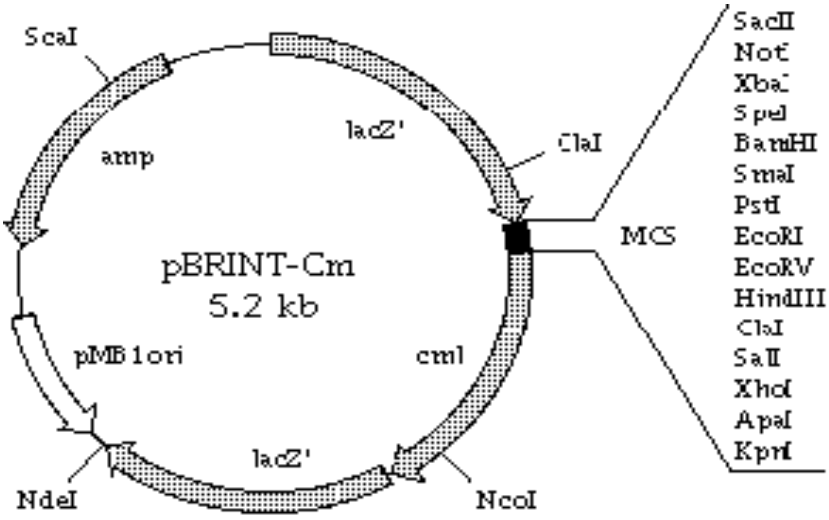


Fig. 44: Map of *pBRINT-Cm* vector

The *phaB* gene was released from *pTZPhB* construct by double digestion with *XbaI* and *BamHI*. The fragment released from this reaction was ligated with *BamHI*/ *XbaI* site of *pBRINT-Cm* plasmid. The ligation mixture was used to transform *E. coli* DH5α competent cells and the transformants were selected on chloramphenicol plates (10μg/ml). The plasmids were isolated by alkali-lysis method (Birnboim and Doly, 1979) and recombinant plasmid was selected on an agarose gel. The construct was named as *pBRB-Cm.* The presence of the *phaB* gene in *pBRB-Cm* was confirmed by restriction digestion and PCR methods. For constructing *pBRBA-Cm* vector the *phaA* gene was cloned into the *pBRB-Cm* vector. The *phaA* gene was released from the *pTZphA* vector by double digestion with *E.coRI / XhoI* enzymes.

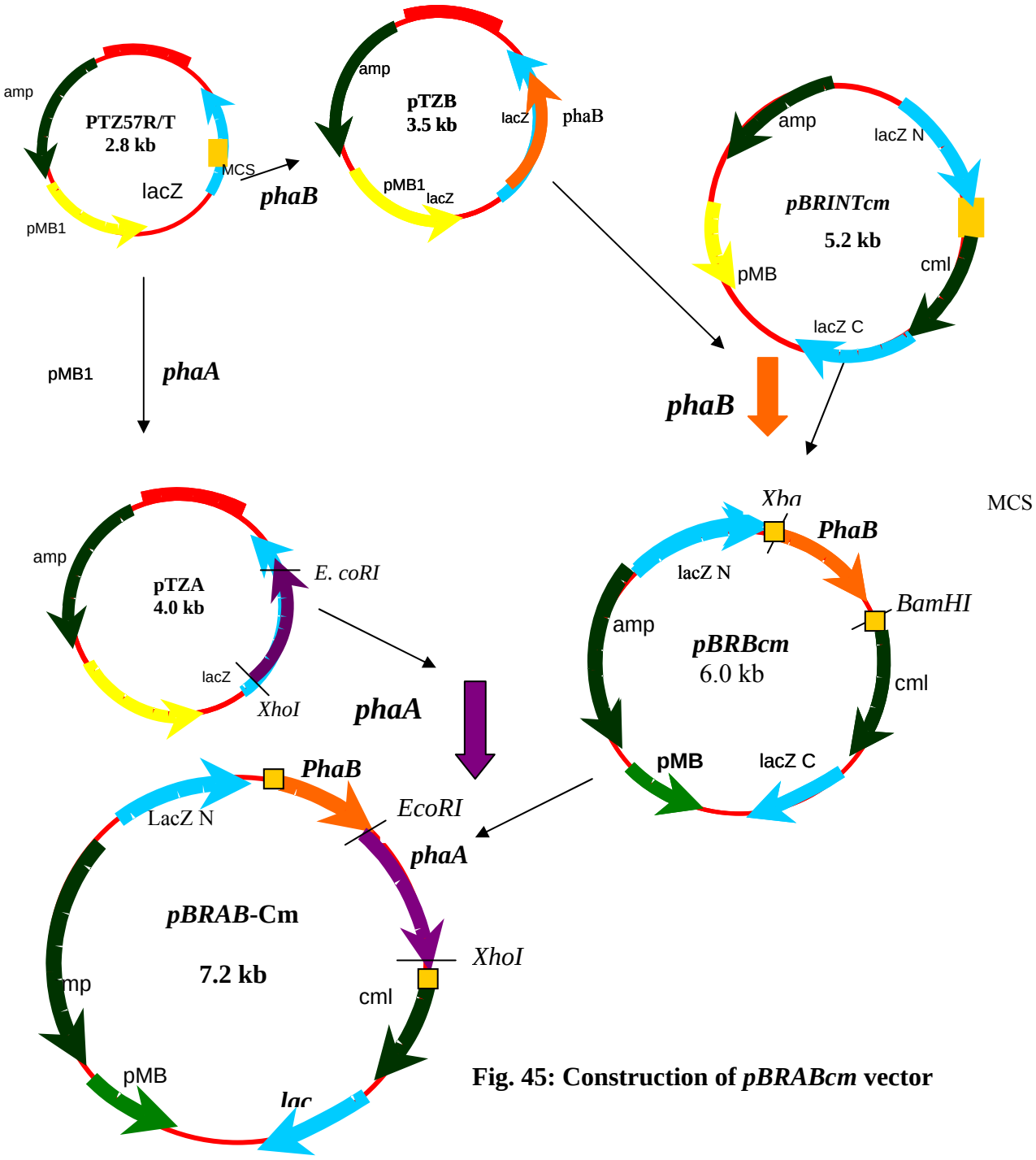


Fig. 45: Construction of *pBRABcm* vector

The following constituents were added in a micro centrifuge tube in the order stated:

Constituents	Volume (μl)
Nuclease-free water	33.0
TY^+ tango 10 x buffer	10.0
Plasmid DNA	5.0
XhoI	1.0
E.*coRI*	1.0
Final volume	50.0

The contents of the tube were mixed gently by pipetting and the tube was centrifuged briefly at 10, 000 x g to collect the contents at the bottom of the tube. The tubes were incubated at 37^0C for 4 - 8 hrs. The samples were analyzed by agarose gel electrophoresis along with 100bp DNA ladder. The released phaA gene insert was separated on an agarose gel. The insert was excised from the gel and purified by using QUIAquick gel extraction kit by following the manufacturer's instruction (Quiagen, Germany). The purified *phaA* gene was ligated with the *pBRB-Cm* construct treated with the same enzymes. The recombinant plasmid was multiplied in *E. coli* DH5α and the insert was released by digestion with *XbaI* and *KpnI.* PCR amplification of cloned genes using the *pBRAB* vector as template with gene specific primers was also carried out.

4.1.5 Transformation of *E. coli JC7623* and selection of integrant (*JC7623AB*)

Transformation of *E. coli* JC7623 was carried out using *pBRBA-Cm* plasmid by $CaCl_2$ method (Sambrook and Russel, 2001). The transformants were selected on chloramphenicol resistance. The integration of the plasmid in the bacterial chromosome was confirmed by Xgal -IPTG selection method. The white colonies were selected and

the presence of two genes in the genomic DNA of *E. coli* strain JC7623 was again confirmed by PCR and chloramphenicol resistance. The white colonies were selected and the recombinant strain was named *JC7623AB*.

4.1.5.1 Construction of *E. coli* strain *JC7623ABC1J4*

In order to construct the strain *JC7623ABC1J4,* recombinant strain *JC7623AB* was subjected to a second transformation using *pBSC1J4* vector (Fig. 46), collected from our laboratory containing PHA synthase gene (*PhaC1*) and (R)-specific enoyl CoA hydratase (*PhaJ4*) from *Pseudomonas aeruginosa.* The transformation was conducted by $CaCl_2$

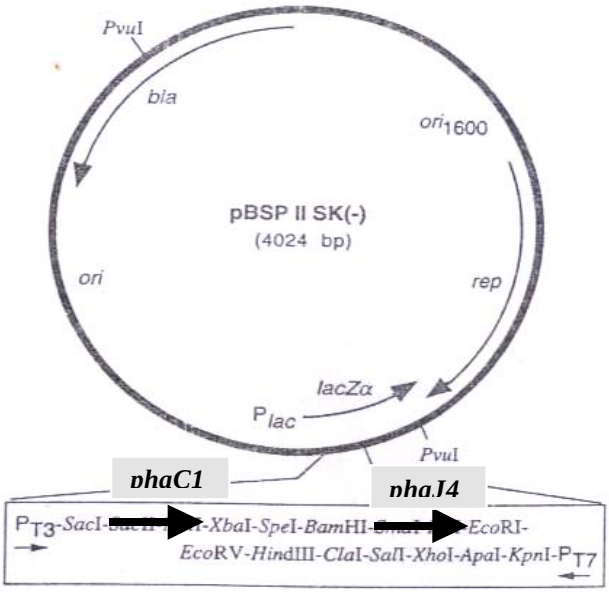


Fig. 46: *pBPC1J4* map

method. Ampicillin at a conc. of 100 µg/ml was used to select the transformants. The recombinant was named as *JC7623ABC1J4*. The strain was maintained on LB ampicillin agar plates.

4.1.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The expression of the cloned genes in recombinant *E. coli* strain *JC7623ABC1J4* was analysed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis. *E. coli* strains

were cultivated in Luria Bertani medium and the induction was carried out after attaining growth (OD 0.6 at 600nm). The crude extract of *E. coli* strains (Host, recombinant and other controls) was prepared after seven hours of induction using sonication in lysis buffer. The supernatant was collected and subjected to electrophoresis in 15% w/v acrylamide gel after determining the protein concentration in the supernatant (Bradford 1976).

Chemicals and solutions:

Separating gel buffer:

Sodium dodecyl sulphate 1 gm

Tris 45.40 gm

Dissolved in 500 ml double distilled water, pH 8.9

Stacking gel buffer

Sodium dodecyl sulphate 0.40 gm

Tris 6.06 gm

Dissolved in 190ml of double distilled water, pH was adjusted to 6.8 with 1N HCl, then made up to 200 ml.

Tank Buffer

Glycine 8.64 gm

Tris 1.8gm

Sodium dodecyl sulphate 0.6gm

Dissolved in 600ml double distilled water, pH was adjusted to 8.3 with 1N HCl

Sample Buffer (5X)

60mm tris HCl, pH 6.8

25% Glycerol

2% sodium dodecyl sulphate

14.4mm 2-Mercaptoethanol

0.1% Bromophenol blue and made up to 10 ml

Stock acrylamide for separating gel:

Acrylamide 30.0 gm

Bisacrylamide 0.4 gm

The above materials were dissolved in 50 ml of double distilled water and was made up to 100 ml. Solution was filtered through a Whatman No.1 filter paper.

Stock acrylamide for stacking gel:

Acrylamide 15 g

bis-acrylamide 0.4 g

The materials were dissolved in 30 ml of distilled water and made up to 50 ml. The solution was filtered through Whatman No.1 filter paper and stored at 4°C in a dark brown bottle.

Ammonium persulfate (APS)

Ammonium persulfate (100 mg) was dissolved in 1ml of distilled water. This solution was prepared fresh every time.

4.1.6.1 Preparation of separating gel (30 ml)

Following solutions were prepared for the experiment:

Separating gel buffer: 15 ml, acrylamide stock for separating gel: 12 ml, Ammonium persulfate: 50 µl, TEMED: 50 µl were poured between two clean glass plates and layered with 5ml of n-butanol and allowed to polymerize for 30 min. After polymerization n-butanol was removed and the gel surface was rinsed with water. The comb was inserted carefully into gel sandwich until bottom of teeth reached top of front plate.

4.1.6.2 Preparation of stacking gel (10 ml)

Following solutions were mixed together: Stacking gel buffer: 1.25ml, stock acrylamide for stacking gel: 0.75ml, Ammonium persulfate: 50 µl, TEMED: 50 µl, distilled water 6 ml. The solution was poured over the separating gel and allowed to polymerize for 30 min.

4.1.6.3 Sample preparation

To the sample (200µl of the *E. coli* total cell extract or any other sample) sample buffer was added (to get 1X sample buffer in a mixture), vortexed thoroughly and boiled for 1 min, cooled and spun. 50 µl of sample was loaded (depending on the protein concentration) into each well.

Electrophoresis conditions:

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

4.1.6.4 Staining and destaining of the gel

The gels were stained in 0.05% (w/v) Coomassie brilliant blue R-250 in acetic acid/methanol/water (10:25:65%v/v), for 0.5 –18 h and destained repeatedly in the same solution without dye (methanol can be replaced with ethanol).

4.1.6.5 Documentation of gel

The gel was documented in a Chemiluminescence detector machine (Bio-Rad, USA).

4.1.6.6 Cultivation of recombinant *E. coli*

Cultivation of the recombinant strain *JC7623ABC1J4* is dealt in detail under chapter 5.

4.1.7 Heterologous cloning in *Bacillus subtilis*

Heterologous cloning in *B. subtilis* was carried out by introducing *Pseudomonas aeruginosa* PHA biosynthesis genes namely *phaC1* (PHA synthase) and *phaJ4* (R-specific enoyl CoA hydratase in to *B. subtilis* by electroporation. The plasmid clone *pCPC1J4* was introduced into *B. subtilis* by electroporation and the transformants were selected on kanamycin resistance. The recombinant *B. subtilis* colonies were checked for the presence of plasmid. The recombinant *B. subtilis* was subjected for PHA production study. The biomass obtained was hydrolyzed and analysed for PHA by gas chromatography.

4.1.7.1 *Bacillus subtilis 168* Electro competent cells preparation (Mod. Silo-suh *et al,* 1994)

- A colony of *B. subtilis* was inoculated in to 10 ml of sterile LB medium contained in a 125 ml conical flask; the flask was incubated overnight at 28 ^{0}C at 300 rpm.

- The culture (0.5 ml) was inoculated in to 50 ml sterile LB broth contained in a 500 ml conical flask and the flask was incubated, at 28 ^{0}C at 300 rpm.

- The growth of the culture was monitored until it reached OD600 =0.3 (cell density of 1x 10^7 cells / ml).

- The culture was chilled in ice for 10 min.

- The cells were transferred in to 50 ml chilled sterile oakridge tube

- The cell pellet was collected by centrifugation at 4 ^{0}C for 10 min (10,000 X g) in a chilled rotor.

- The supernatant was discarded and the pellet was suspended in 10 ml sterile ice-cold electroporation (EP) buffer and cells were pelletted as mentioned above.

- The supernatant was discarded and the pellet was suspended in 5 ml sterile ice-cold EP buffer.

- The pellet was collected by centrifugation at 4 ^{0}C for 10 min. (10,000 X g) in a chilled rotor.

- The supernatant was discarded and the pellet was suspended in 0.5 ml EP buffer. The cells were placed on ice and used immediately.

4.1.7.2 Electroporation

The electroporation was carried out in a Gene pulsor machine (Bio-Rad, Germany) as per the procedure detailed below.

a) For each sample to be electroporated, DNA was pipetted (0.05-1µg in p to 10µl in water or TE buffer) in to a sterile 1.5ml micro centrifuge tube. The tubes were placed on ice and 100µl of electro competent cells were added to each *pCPC1J4* plasmid sample; mixed and incubated on ice for 5-10 min.

b) 100µl of plasmid/cell suspension was transferred in to chilled 0.2 ml cuvette that was placed on ice and mixed.

c) For each sample to be electroporated 2 ml LB broth was taken in a 17x100mm sterile tube containing 2 ml of LB broth at room temperature.

d) From the home screen on gene pulsor Xcell, *Bacillus cereus* protocol was accessed; DNA sample was transferred in to electroporation cuvette and the suspension was tapped, the cuvette was placed in shock pod, the chamber was closed and pulsed once.

e) The cuvette was removed from the chamber and the cells were immediately transferred to LB broth contained in the 17x100mm tube.

f) The pulse parameters were checked and recorded. The time constant was about $8.6^{m\ sec.}$ and the voltage was about 1kV and the field strength could be calculated as actual volts. kV/cuvette gap in cm).

g) The cells were incubated for 1 to 1.5 hrs at 37 ^{0}C at 250 rpm. The aliquots of electroporated cells were plated on LB agar plates containing kanamycin (50µg/ml) and incubated overnight at 28 ^{0}C.

4.1.7.3 Solution and reagents

EP Buffer (0.5 m M K_2HPO_4-KH_2PO_4, 0.5 mM $MgCl_2$, 272 mM sucrose) 54.5 ml 1M sucrose, 100µl of 1M $MgCl_2$, 190µl of 0.1M KH_2PO_4, 810µl of 0.1M K_2HPO_4. The volume was made to 200 ml (with milli Q water), filter sterilized and stored at 4 ^{0}C.

4.1.7.4 Isolation of plasmids

Alkali lysis method was carried out to isolate plasmids from recombinant *B. subtilis* (Birmboin and Dolly, 1979) with minor modifications (To the solution I30 mg/ml lysozyme powder was added and the mixture was incubated at 37 ^{0}C for 30 min).

4.1.7.5 PHA production by recombinant *B. subtilis*

Preparation of inoculum

Single colony of recombinant *B. subtilis* was inoculated to 5ml sterile PHA broth and the tube was incubated overnight at 30 ^{o}C in a shaker at 250 rpm. This was inoculated 50 ml production medium containing 5mg % of kanamycin.

Medium

PHA production was carried out in PHA production medium. The composition of the medium is given below:

Components	**g/l**
Na_2HPO_4 2 H_2O	4.4
KH_2PO_4	1.5
$(NH_4)_2SO_4$	1.5
$MgSO_4$ 7 H_2O	0.2
Sucrose	20.0
(pH 7.0)	

Nonanoic acid was added to the medium at a concentration of 3%.

Cultivation of the strain

PHA production was carried out in shake flask culture. The inoculum was transferred to 50 ml PHA production medium containing 50µg/ml kanamycin and incubated at 30 ^{0}C for 72 h at 250rpm. The fatty acid was added after initiating growth of the bacteria.

Polymer extraction

The intracellular polymer was extracted by sodium hypochlorite digestion method.

4.1.8 Gas chromatography

GC analysis was carried out using methanolyzed cells (page No. 56).

4.1.8 Gene Disruption studies in *Bacillus*

Various methods are used to disrupt genes to study their functions. Methods used are transposon-mediated mutagenesis, homologous recombination, site directed mutagenesis, anti sense technology etc. In the present study an attempt has been made to disrupt the gene function by anti sense technology.

Antisense is usually considered as a mechanism for sequence- specific messenger RNA (mRNA) recognition that leads to the degradation of the target mRNA. Regulation of gene expression by antisense RNA was first discovered in bacteria as naturally occurring phenomenon. The antisense technology is based on blocking the information flow from DNA via RNA to protein by the introduction of an RNA strand complementary to (part of) the sequence of the target mRNA. These antisense RNA base pairs targeted to mRNA leads to formation of double stranded RNA. The double stranded RNA formation can impair mRNA maturation and / or translation or alternatively lead to rapid degradation of mRNA.

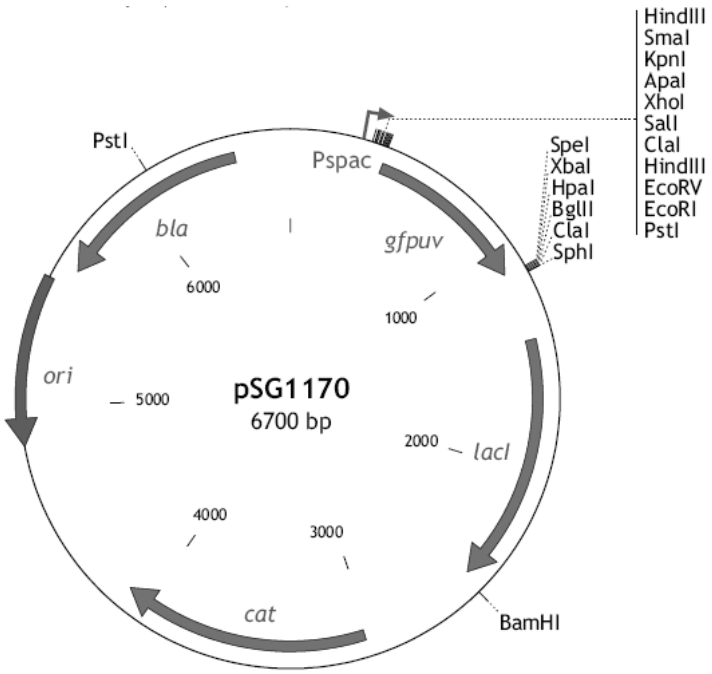


Fig 47: *B. subtilis* integration vector pSG1170

PhaA and *phaB* genes from *Bacillus sp* 256 were cloned into a *B. subtilis* integration vector *pSG1170* (Fig 47) in a reverse orientation.

4.18.1 Construction of *pSGABant* vector

A ~2 k bp DNA fragment containing *phaA* and *phaB* gene from *Bacillus sp* 256 was released from the plasmid *pBRAB*-Cm by double digestion as described below:

Constituents	Volume (µl)
Nuclease-free water	28.0
TY^+ tango 10 x buffer	10.0
Plasmid DNA (*pBRAB-Cm*)	10.0
XbaI	1.0
KpnI	1.0
Final volume	50.0

The above components were taken in tube, mixed gently by pipetting and the tube was centrifuged briefly at 10, 000 x g to collect the contents at the bottom of the tube. The reaction was carried out at 37 ^{0}C for 4 - 8 hrs. The samples were analyzed by agarose gel electrophoresis along with 100bp DNA ladder.

The vector pSG1170 was also subjected to double digestion by the same manner and the vector was separated from released GFP fragment (~1 kbp long) by agarose gel electrophoresis. Both the insert and the vector were excised from agarose gel and purified using gel extraction kit (Quiagen).

4.1.8.2 Ligation and transformation

The following components were pipetted into a thin-walled 0.2 ml PCR reaction tube:

Double digested pSG1170	:	5.0 µl
Insert DNA	:	15.0 µl
10X Ligase Buffer	:	3.0 µl
PEG 4000 solution	:	2.0 µl
T4 DNA Ligase, 5U/µl	:	1.0 µl
Deionized water (to make upto 30.0 µl)	:	4.0 µl

The reaction components were mixed by brief spin. The samples were incubated overnight at 22 ^{0}C. The ligation mixture was used to transform competent cells of *E. coli* DH5α. The transformants were selected on ampicillin containing LB agar plates. The plasmids were isolated from the transformed *E. coli* by alkali lysis method (Birnbiom and Doly, 1979) and the recombinant plasmid was screened on agarose gel.

4.1.8.3 Transformation of *Bacillus sp* 256

The transformation was carried out by elctroporation using Gene pulsor machiene (Bio-Rad, Germany) as mentioned earlier (pages 167-169; 4.1.7.1, 4.1.7.2). The transformed cells were plated on LB gar plates containing chloramphenicol at 5µg/ ml concentration.

4.1.9. Construction of *pMUT-HA ket* vector

pMUTIN-HA vector was selected for tagging pha gene (Fig. 48). This vector is an integration vector for *B. subtilis*, designed to tag genes with haemoagglutinin peptide sequence HA (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala). Anti HA antibodies are available commercially. The vector possesses *bla* gene for ampicillin resistance and IPTG inducible pspac promoter. In order to make a fusion protein of *Bacillus* ketothiolase protein with HA peptide 723 bp fragment of *phaA* gene was cloned in to *HindIII KpnI* site of the *pMUTIN-HA* vector.

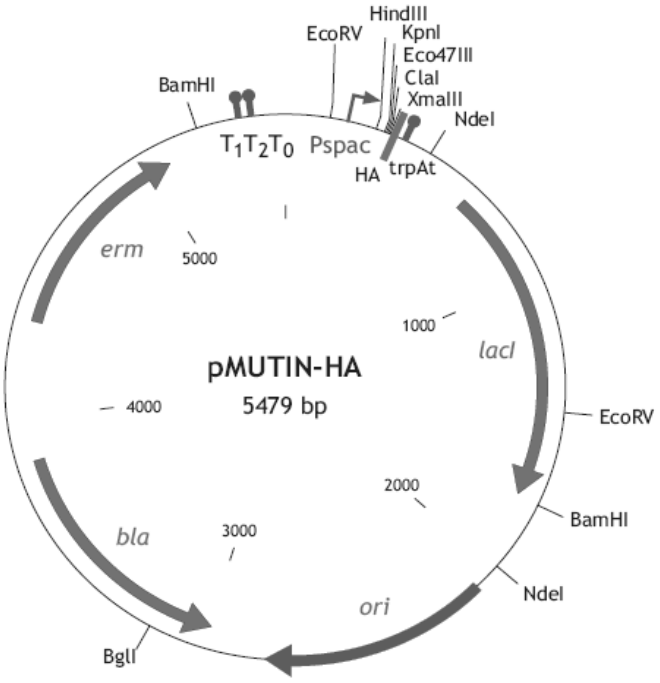


Fig 48: *B. subtilis* vector *pMUTIN-HA*

~750 bp insert was released from *pTZAnest* plasmid (*pTZ57R* vector containing 723 bp long phaA gene fragment from the start site) by separate restriction digestions. The insert was separated on agarose gel. The insert was excised from the agarose gel and purified by using gel extraction kit. The purified insert and vector were subjected to ligation as follows:

Double digested pMUTIN HA vector	:	2.0 µl
Insert DNA	:	10.0 µl
10X Ligase Buffer	:	2.0 µl
PEG 4000 solution	:	1.0 µl
T4 DNA Ligase, 5U/µl	:	1.0 µl
Deionized water (to make upto 20.0 µl)	:	4.0 µl

The mixture was incubated at 22 ^{0}C for 8 h and was used to transform competent cells of *E. coli* DH5α. The transformanats were selected on ampicillin plates. The plasmids were isolated by alkali lysis method. The recombinant *pMUT-HA ket* plasmids were screened based on their mobility on agarose gel.

4.1.9.2 Transformation of *Bacillus sp* 256

The transformation was carried out by electroporation using Gene pulsor machiene (Bio-Rad, Germany) as mentioned earlier. The transformed cells were plated on LB erythromycin plates (0.3 μg/ ml)

4.2 RESULTS

4.2.1 Heterologous cloning in *B. subtilis*

Electrocompetant cells of *B. subtilis* were prepared and the electroporation was carried out in a Gene pulsor machine (Bio-Rad, Germany). The transformants were selected on the basis of kanamycin resistance. The plasmid isolated from the recombinant showed the correct size with control plasmid and there was no plasmid in control untransformed *B. subtilis* (Fig. 49).

4.2.1.1 PHA production by recombinant *B. subtilis*

The bacterium grew well in sucrose medium while the growth was slow with poor biomass in PHA production medium containing nonanoic acid. The recombinant strain produced PHA in a range of 2-5 % on the basis of dry weight biomass. The biomass was hydrolyzed and subjected to gas chromatography. The GC graph showed the presence of PHB and mcl- PHA in the polymer of recombinant strain, which was absent in the control untransformed *B. subtilis* that was grown in the same medium. (Figs. 50-51).

4.2.2 Construction of *pBRBA-Cm* plasmid

The genes coding for β-ketothiolase and acetoacetyl CoA reductase from *Bacillus sp* 256 were amplified and cloned in to *pTZ57R/T* vector separately (*pTZPhaA* and *pTPhaB*). For constructing *pBRB-Cm* vector the *phaB* fragment was released from *pTZPhaB*, the released insert was of expected size ~ 800 bp. The *pBRB-Cm* construct was screened on the basis of the mobility in an agarose gel (Fig. 52). The *pBRBA-Cm* construct was made by cloning the *phaA* insert released from the *pTZPhaA* plasmid in to *pBRB-Cm* vector. The insert was nearly 1.2 kb in size (Fig. 53).

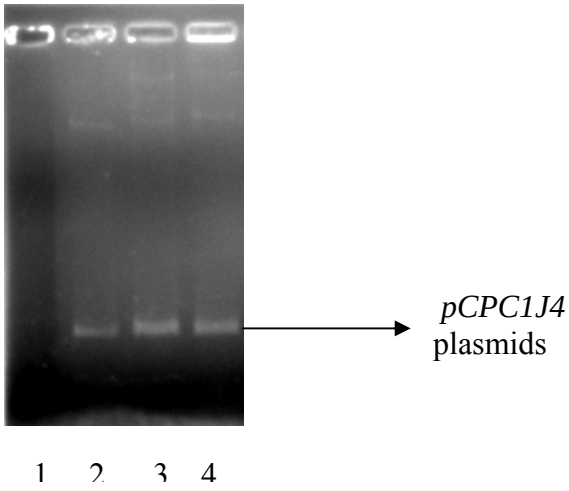


Fig. 49: Plasmid isolated from the recombinant *B. subtilis* 168
Lane1= Control host *B. subtilis*,
Lanes 2&3=pCPC1 from transformant *B. subtilis*
Lane 4= pCPC1J4 control plasmid

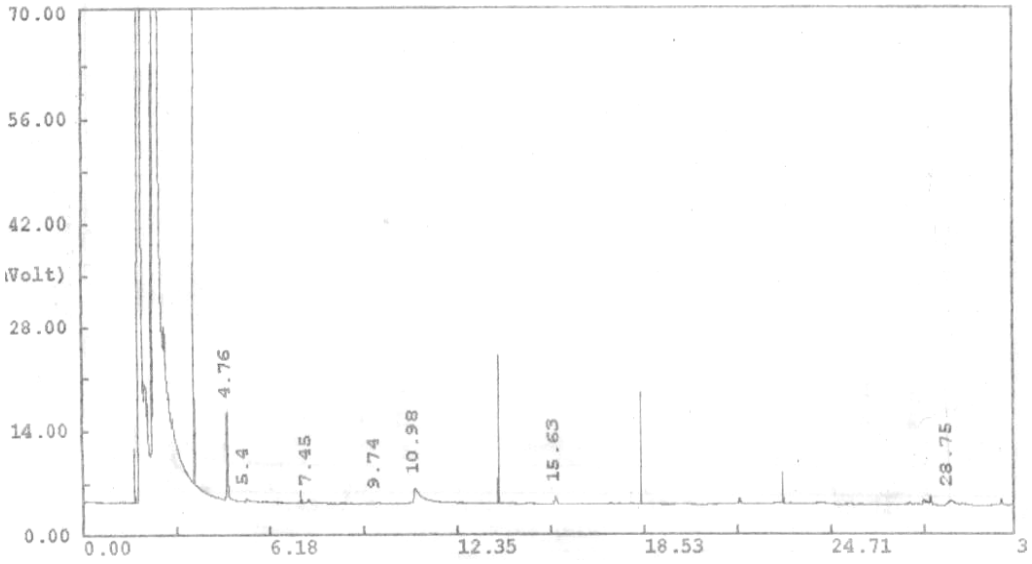


Fig 50: GC profile of PHA from *B. subtilis* (control) cultivated in nonanoic acid medium

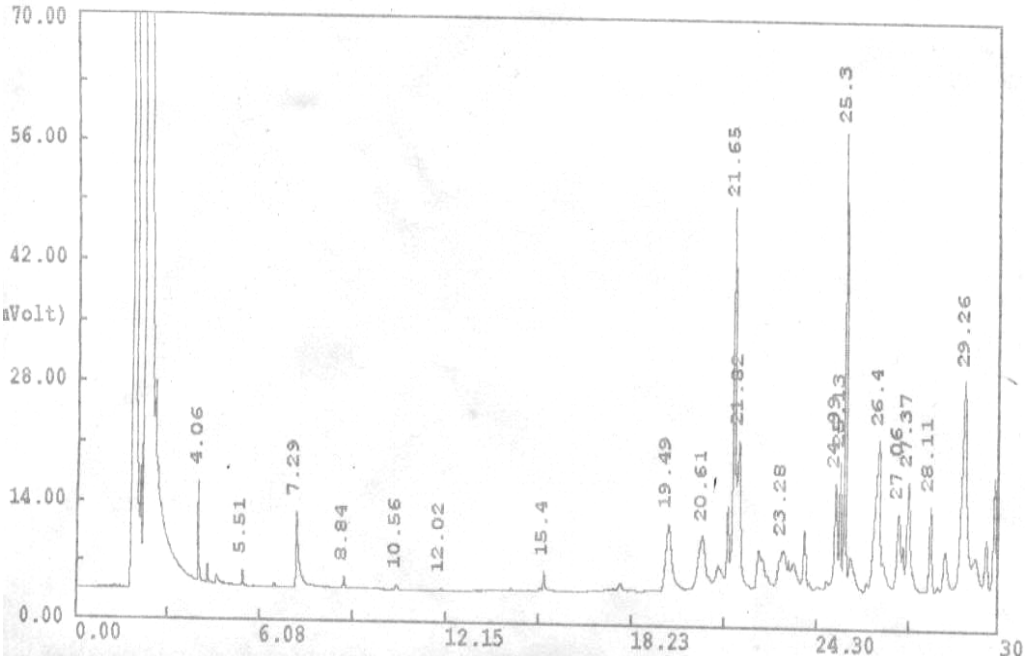
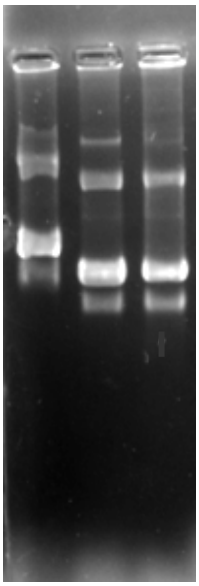


Fig. 51: GC profile of PHA from recombinant *B. subtilis* cultivated in nonanoic acid medium

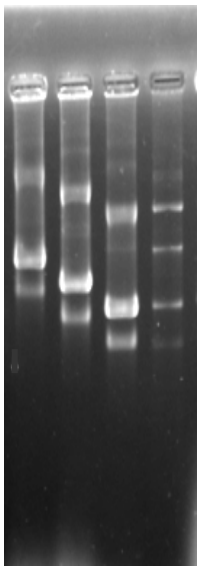
4.2.2 Expression of *pBRBA-Cm* and *pBPC1J4* in *E. coli JC7623*

All the four genes *phaA*, *phaB*, *phaC1* and *phaJ4*, introduced in the *E. coli* were under the control of IPTG inducible *lacZ* promoter and the induction was carried out after six hours of growth. The expression of pha genes in recombinant *E. coli* was confirmed by SDS PAGE analysis. The SDS PAGE analysis showed the successful expression of the cloned genes under the control of *lacZ* promoter (Fig. 54). Protein bands of ~43 kDa and ~27kDa were observed in the recombinant *E. coli* JC7623*BA* strain while two additional protein bands of expected molecular weight (representing PHA synthase and enoyl CoA hydratase) was observed in strain *JC7623ABC1J4*. The control lane showed the absence of these bands. The expression of pha genes and their activity were again confirmed by subjecting the recombinant *E. coli* for PHA production. Cultivation of the strain and PHA production are described in chapter 5.



1 2 3

Fig. 52: *pBRB-Cm* construct
Lane 1 recombinant *pBRB*
Lanes 2 & 3 control plasmid *pBRINT-Cm*



1 2 3 4

Fig. 53: *pBRINT* clones
Lane 1 *pBRBA-Cm*
Lane 2 *pBRB-Cm*
Lanes 3 &4 *pBRINT-Cm*

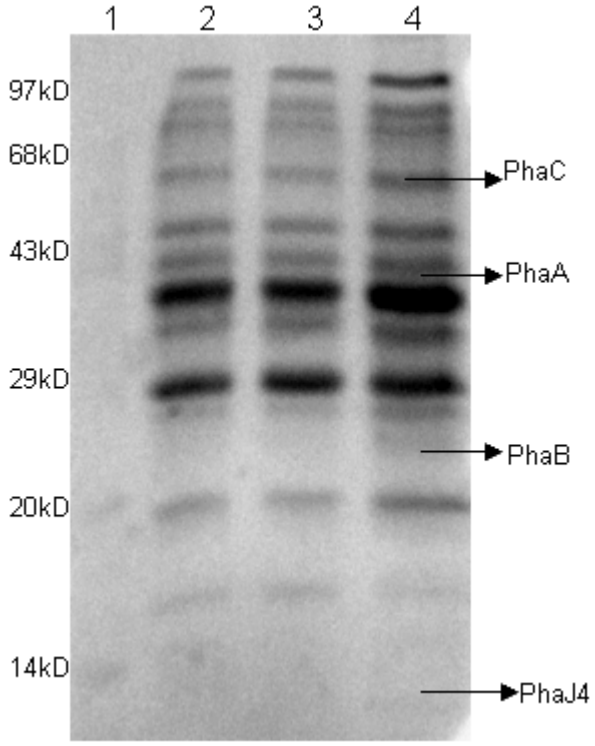


Fig. 54: SDS PAGE of recombinant *E. coli* lysate

Lane 1= Standard molecular marker
Lanes 2 & 3= Protein profile of host organism
Lane 4=Protein profile of recombinant *E. coli* strain

4.2.3 Construction of *pSGABant* vector and gene disruption in *Bacillus*

pSGABant vector was constructed by cloning *phaA* and *phaB* genes into *KpnI XbaI* site of *pSG1170* vector in a reverse orientation. The *phaAB* insert was released from the *pBRBA-Cm* vector by restriction digestion with *KpnI XbaI* was of expected size (Fig 55). The insert was cloned into *B. subtilis* integration vector *pSG1170* after removing GFP gene from it. The recombinant plasmid migrated slowly compared to the control plasmids on an agarose gel (Fig 56). The transformation of *Bacillus sp* 256 resulted in few colonies on chloramphenicol plates. Colonies were sub cultured in PHA and LB broth containing the antibiotic. The cells failed to grow further in media from the plates.

4.2.4 Construction of *pMUT-HA ket* plasmid

DNA insert containing partial *phaA* gene of *Bacillus sp* 256 (Fig 57) was cloned in to *Hind III KpnI* site of *pMUTIN HA* plasmid as a fusion protein construct. The recombinant *pMUT-HA ket* plasmids showed slower movement on agarose gel (Fig 58). Transformation of *Bacillus sp* 256 did not result in any colony on plates.

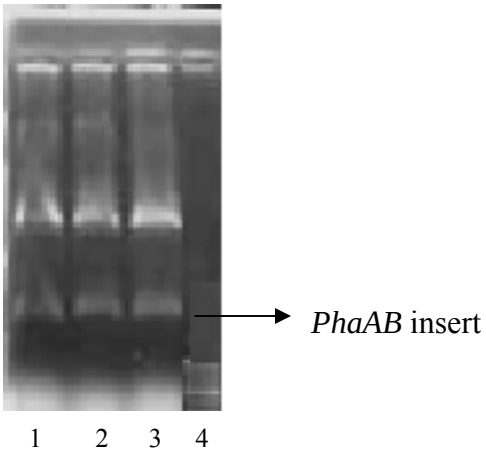


Fig 55: *phaAB* insert release from *pBRBA*-Cm
Lanes 1, 2, & 3 = phaAB insert release from *pBRBA*-Cm; Lane 4 =100 bp DNA marker

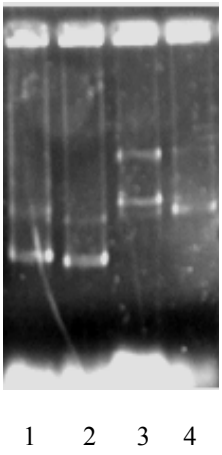


Fig 56: *pSGABant* vector
Lane 1= control plasmid
Lane 2 =self ligation
Lanes 3 & 4 =recombinant *pSGABant* plasmids

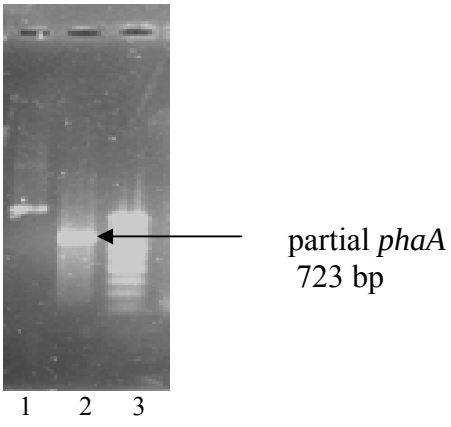


Fig 57: PCR amplification of partial *phaA*
Lane 1= *phaA* full gene
Lane 2= partial *phaA*
Lane3= 100 bp DNA marker

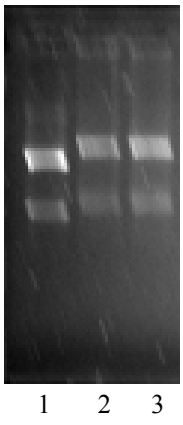


Fig 58: Recombinant *pMUT-HA ket*
Lane 1= Control pMUTIN-HA
Lanes 2 &3 recombinant pMUT-*HA ket*

4.3 DISCUSSION

Heterologous cloning and expression of genes isolated from microorganisms have been practiced since long to study the functional expression of the genes. Among the different host organisms, *E. coli* is the most exploited one, while *B. subtilis* and *Saccharomyces sp* have been used as alternative hosts. Heterologous expression of PHA biosynthesis genes was first carried out in *E. coli* (Schubert et al, 1988), wherein the PHA biosynthesis genes from *R. eutropha* was cloned and expressed in *E. coli*. The use of *B. subtilis* as PHA production host has been reported earlier in which *B. subtilis* 1A304 was transformed with PHA biosynthesis genes from *B. megaterium* (Law et al 2003). The recombinant *B. subtilis* grown on malt wastes produced 5% PHA in the cells. In our study *B. subtilis* was used as a host for cloning PHA biosynthesis genes from *P. aeruginosa*. *B. subtilis* strain, which basically does not produce PHA, was transformed with a plasmid containing *phaC1* and *phaJ4* genes (*pCPC1J4*) from *Pseudomonas sp*. To develop recombinant *B. subtilis* we used *pCE20* plasmid (Fig. 59), which is a shuttle vector in *E. coli* and *B. subtilis*. The vector is provided with two multiple cloning sites, under the control of *pspac* and *spo* promoters that is designed for expression in *E. coli* and *B. subtilis,* respectively. *pCPC1J4* the derivative of *pCE20*, which contained *phaC1* and *phaJ4* genes of *P. aeruginosa*, was under the control of *spo* promoter for constitutive expression in *B. subtilis*. The transformant *B. subtilis* produced PHA (5%) in medium containing nonanoic acid. The PHA produced was a copolymer of PHB and mcl PHA.

Pseudomonas PHA synthase is known to lead to synthesis of mcl-PHA of 6-14 carbon length. The enzyme can attract 3-hydroxyacyl CoA of different chain length, derived from fatty acid β-oxidation and de novo biosynthesis, with the help of two enzymes such as (R) specific enoyl CoA hydratase, 3-Hydroxyacyl CoA reductase and 3-Hydroxy acyl CoA ACP reductase, coded by the genes *phaJ, FabG* and *FabD,* respectively. In the present study *phaC1* gene of *P. aeruginosa* along with *phaJ4* gene was cloned for heterologous PHA production in *B. subtilis*. The results of the study

showed that the *phaJ4* gene product enantiomerically converted trans-Δ-S-enoyl Co A formed in the β-oxidation, of fatty acids provided, to R-3-hydroxyacyl CoA (which can be polymerized by PHA synthase). The biomass produced was low in kannamycin containing medium. Even though the polymer yield was low, this is the first report on the use of *B. subtilis* as a host for expressing Pseudomonas genes for PHA production.

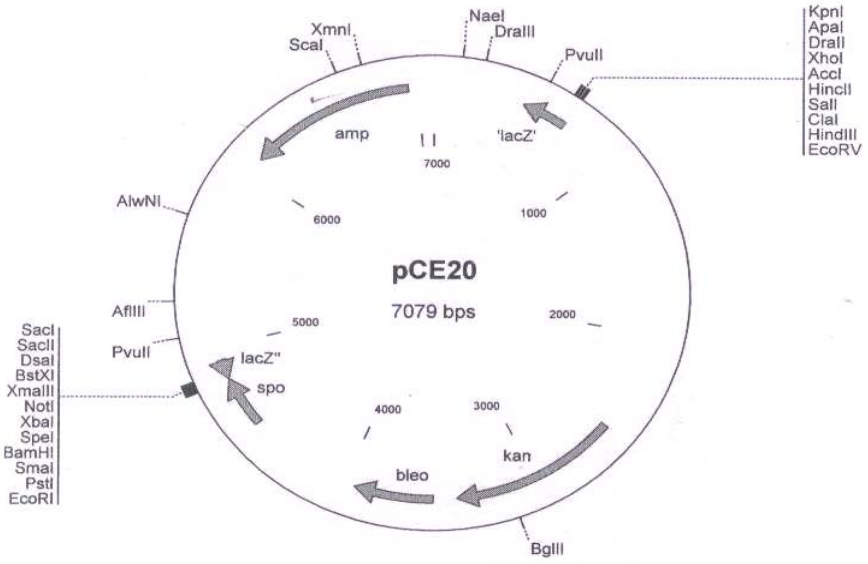


Fig. 59: *pCE20* vector map

Different pathways are involved in bacteria for scl and mcl PHA production. The cascade of the enzymatic reactions involves the use of different precursor substrates. Scl-PHA biosynthesis is a three-step enzymatic reaction, which uses acetyl CoA as the precursor molecule. No other pathway is known to involve these enzymes whereas mcl-PHA biosynthesis pathway genes are related to fatty acid metabolism in bacteria except PHA synthase. The substrate specificity of PHA synthase enzyme is an important factor to be considered for PHA copolymer production by recombinant organisms.

To clone and express *phaA* and *phaB* genes from *Bacillus* in *E. coli*, *pBRINT-Cm* vector was used (Fig. 44), which is a promoter less vector with chloramphenicol resistance marker. The vector is specifically designed for chromosomal integration of cloned genes on *lacZ* gene by homologous recombination (Balbas *et al*, 1996). The vector is provided with MCS and a chloramphenicol marker, which are flanked by N terminal and C terminal region of *lacZ* gene.

The selected host for gene expression and PHA production was *E. coli* strain JC7623 (ATCC47022). The use of this *E. coli* strain as host enabled the chromosomal integration of the genes; it is based on the inability of the strain to maintain ColE1 derivatives (Balbas *et al*, 1996). The integration target of the *pBRBA-Cm* clone was *lacZ* gene, where recombination and double cross over occurred (Fig. 60). The integrated genes were under the control of IPTG inducible natural *lacZ* promoter. The successful integration of the pha genes was confirmed by blue white selection, resistance to chloramphenicol, PCR methods and inability of the organism to grow in lactose medium. The SDS PAGE analysis reconfirmed the expression of these genes from the IPTG inducible natural *lacZ* promoter of *E. coli* strain JC7623.

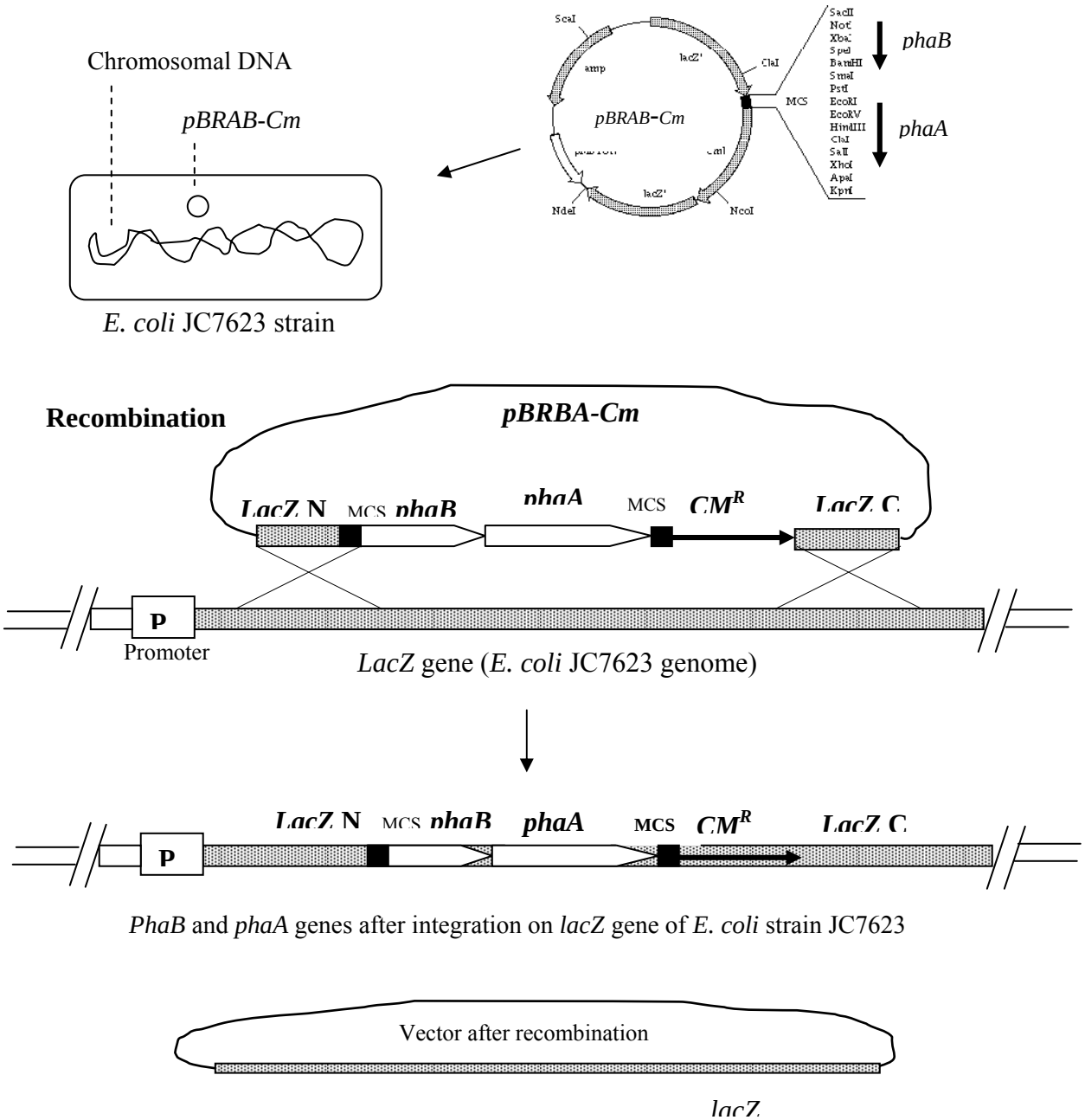


Fig. 60: The mechanism of genomic integration of *pBRBA*-Cm vector into *E. coli* JC7623

The present experiment showed that the PHA synthase of *P. aeruginosa* could also polymerize 3-hydroxybutyryl CoA monomers. An attempt was made here to provide 3-hydroxybutyryl CoA and 3-hydroxyvaleryl CoA monomers to PHA synthase of *P. aeruginoasa* by cloning the concerned genes. By cloning *phaA* and *phaB* genes from *Bacillus sp* 256 and *phaC* and *phaJ4* from *P. aeruginosa*, a PHA biosynthesis pathway was engineered in *E. coli* for scl-co-mcl PHA co polymer production. The details of PHA production are dealt with under chapter 5.

Transformation of *Bacillus sp* 256 with *pSGABant* and *pMUT-Haket* plasmids were not stable. The reasons for not getting a stable transformation in *Bacillus sp* 256 may be due to the presence of a strong nuclease system to degrade the invading foreign DNA or due to the instability of the plasmids, owing to the lack of integration of the plasmid in the genomic DNA.

4.4 CONCLUSIONS

A combination of metabolic engineering and molecular techniques has been applied over the years to enhance PHA production in recombinant microorganisms in an efficient way. Several host strains, predominantly *E. coli* strains, have been used to clone and express PHA biosynthesis genes isolated from a variety of PHA producing microorganisms. Heterologous cloning and expression of PHA biosynthesis genes was initiated with the cloning of *R. eutropha* genes in *E. coli* for PHA production. Alternatively homologous expression also been applied to enhance PHA production by the natural organisms, which carry an extra copy of gene/s.

Recombinant strain of *B. subtilis* was developed by transferring *phaC1* and *phaJ4* genes, coding for PHA synthase and (R) specific Enoyl CoA hydratase. The recombinant strain was subjected to PHA production in medium containing nonanoic acid. The strain produced low amounts of intracellular PHA (5%). The biomass produced was less when compared to its growth in the medium without antibiotic. The PHA synthase of *P. aeruginosa* is recognized to polymerize monomers of 6-14 carbon chain length produced in various steps in fatty acid metabolism with help of some genes such as *phaJ*, *FabG* etc. Here the recombinant strain was equipped with *phaJ4* gene, which is responsible for enantiomeric conversion of β-oxidation intermediates of fatty acids to PHA biosynthesis pathway. The results of this study suggested that the presence of PHA synthase gene and (R) specific enoyl CoA hydrates enabled the *B. subtilis* strain to produce PHA from fatty acids.

The recombinant *E. coli* strain *JC7623ABC1J4* was developed by cloning *phaA* and *phaB* genes from *Bacillus sp* 256 (coding for β-ketothiolase and acetoacetyl CoA

reductase respectively, involved in the scl-PHA biosynthesis) and *phaC1* and *phaJ4* genes from *P. aeruginosa* for scl-co-mcl PHA copolymer production. Expression of the four cloned genes was confirmed by SDS gel electrophoresis. The *E. coli* strain grew well in PHA production medium and was selected for further studies. Growth and PHA production by recombinant *E. coli* are described under chapter 5.

5.0 INTRODUCTION

Polyhydroxyalkanoates are carbon and energy storage compounds, produced by many eubacteria and some archaea (Steinbuchel *et al*, 1995). PHAs are biodegradable, biocompatible and possess various plastic and elastomeric material properties depending on their monomer constituents (Park *et al,* 2005). The chemical or biological hydrolysis of PHA result in optically pure (*R*)-form hydroxycarboxylic acids, which is commercially highly important for the manufacture of antibiotics, vitamins, perfumes and pheromones (Steinbuchel and Valentine, 1995).

Depending on the type of the monomer units in the polymer PHA shows variety of physical properties. Based on the monomer composition of the PHA, PHA producing microorganisms are divided in to two groups. The first group, represented by *R. eutropha* in which the PHA is synthesized via acetoacetyl-CoA to 3-hydroxybutyryl-CoA giving rise to a polymer of butyric acid monomers (PHB). With regard to other constituents of scl-PHA, such as 3-hydroxyvalerate, addition of precursors or substrates and conversion towards their corresponding coenzyme are essentially needed. The second group is represented by *P. aeruginosa,* where PHAs (mcl-PHA) synthesized from the fatty acid metabolism. The mcl-PHA is formed of monomer units, which contains 6 to 14 carbon atoms (DeKonig, 1993). They are flexible and have several applications.

The PHA synthase is the key enzyme in the PHA biosynthesis pathway, which has broader substrate specificity, which polymerizes various monomer compounds derived from different pathways in to PHA. In scl-PHA biosynthesis pathway other enzymes involved, are NADPH dependent acetoacetyl CoA reductase and β-ketothiolase. The PHA production pathway and the gene organization in *R. eutropha* has been studied in detail and the first metabolic pathway towards the scl-PHA formation in *E. coli* was established by cloning the whole *phb* gene operon into *E. coli* (Schubert *et al*, 1988).

Later on many PHB biosynthesis genes from various bacteria have been identified and functionally expressed in *E. coli*, which lead to scl-PHA formation (Li *et al,* 2006).

In recent years molecular techniques have been applied to enhance PHA production in microorganisms. Several mutants with altered phenotypes in PHA synthesis have been developed as optimal recombinant host strains. Over-expression of *pha* genes in the natural PHA producer has not resulted in significant difference in polymer accumulation. Natural producers, like *R. eutropha*, are well adapted for intracellular PHA accumulation and it can store PHA up to 90% of its dry weight. But the natural producers are not suitable for industrial production of PHA due to their long generation time and rigid cell wall, which make the extraction more tedious. Natural isolates possess PHA depolymerase which lead to intracellular degradation and utilization of PHA.

Various *E. coli* strains have been used in industrial scale production process as PHA production host. Commonly used strains are *E. coli* XL1 Blue, *E. coli* HMS174, *E. coli* GCSC4401, *E. coli* RS3097 etc. It has been reported that the PHB production reached up to 90% (w/w) of the cellular dry weight using recombinant *E. coli* harboring *R. eutropha* PHA biosynthesis genes (Lee and Chang, 1995). Productivity 4.63 g/l/h of PHA has been reported in a fed batch culture of recombinant *E. coli* XL 1 Blue strain containing *A. latus* PHA biosynthesis genes using glucose as substrate (Choi *et al,* 1998). The mcl-PHA also has been industrially produced using recombinant *E. coli,* harboring *P. aeruginosa* PHA biosynthesis genes, from decanoate (Qi, *et al,* 1998). In the present study *E. coli* JC7623 strain has been used for cloning of PHA biosynthesis genes from *Bacillus* sp and *P. aeruginosa* for scl-co-mcl PHA production. The host and gene combination used are being reported for the first time for PHA production. The chapter deals with the production of PHA by recombinant *E. coli JC7623ABC1J4* in the presence of various carbon substrates and optimization of fermentation conditions.

5.1 MATERIALS AND METHODS

5.1.1 PHA production by recombinant *E. coli* strains

5.1.1.1 Microorganisms and maintenance

Three recombinant strains of *E. coli*, namely *fadBC1J4*, *JC7623C1J4*, *JC7623ABC1J4 were* selected to study the PHA production. *FadB C1J4* was developed in our laboratory (Reeta and Chandrashekar 2006), by transforming *E. coli fadB* mutant (strain with defective β-oxidation (S) specific trans enoyl CoA hydratase gene, *fadB*) with plasmid bearing *P. aeruginosa phaC1* and *phaJ4* genes (*pBSPC1J4*). JC7623*C1J4* is a transformed *E. coli* JC7623 with *pBSPC1J4* plasmid. Strain *JC7623ABC1J4* is transformed *E. coli* JC7623 bearing PHA biosynthesis genes: *phaB* (acetoacetyl CoA reductase) and *phaA* (β-ketothiolase) from *Bacillus sp* and *phaC1* (PHA synthase) and *phaJ4* (R specific enoyl CoA hydratase) from *P. aeruoginosa*.

All recombinant strains of *E. coli* were maintained on Luria Bertani (LB) (Himedia Mumbai India) plates/slants at 4 ^{0}C, containing 100 µg/ml ampicillin. The slants were subcultured once a month.

5.1.1.2 Production medium

Modified synthetic medium of Wang and Lee (1998), was used for experiments:

Composition	g/l of distilled water
KH_2PO_4	13.5
$MgSO_4$ $7H_2O$	1.4
Citric acid	1.7
$(NH_4)_2HPO_4$	4.0
Glucose (sterilized separately)	20.0
Yeast extract (Optional)	2.0
Tryptone	2.0
Trace metal solution	1 ml
pH (7.0); ampicillin 10 mg% (w/v)	

Trace metal solution (In a Liter of 5 M HCl): 10 g $FeSO_4$. $7H_2O$; 2 g $CaCl_2$ $2H_2O$; 2.2 g $ZnSO_4$ $7H_2O$; 0.5 g $MnSO_4$ $4H_2O$; 1 g of $CuSO_4$ $5H_2O$; 0.1 g $(NH_4)_6Mo_7O_{24}$ $4H_2O$; and 0.02 g $Na_2B_4O_7$ $10H_2O$. By varying the co carbon source in the synthetic medium, three different media were formulated and used: a) medium containing citrate without tryptone, b) citrate and butyrate with tryptone c) butyrate without citrate for PHA production by the recombinant *E. coli* strains.

a) LB medium with 2% (w/v) glucose and 10 mg% ampicillin was used for comparison.

5.1.1.3 Inoculum preparation and viable plate counts

The strains were cultivated in 5 ml of LB broth containing ampicillin (100 mg/l) at 37 ^{0}C, 250 rpm for 12-18 h.

Total plate counts or viable cell count or cell forming units of inoculum was estimated by plating diluted fresh growing culture on nutrient medium. One ml of the broth was serially diluted six times in test tubes containing 9 ml of sterile saline and homogenized. 0.1 ml sample from individual dilutions were plated in duplicates on nutrient medium contained in petriplates. Plates were incubated at 37 ^{o} C for 24-48 h and the number of colonies developed was counted. Number of viable cells was presented as colony forming units or viable cell counts/ml.

5.1.1.4 Utilization of various carbon sources for PHA production

Recombinant *E. coli* strain *JCABC1J4* was cultivated in shake flasks in triplicates a) LB medium with 2% (w/v) glucose and 10 mg% ampicillin and b) modified synthetic medium of Wang and Lee (1998) as mentioned above with different co carbon substrates such as butyric acid, valeric acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid and decanoic acid. These organic acids were added (0.2 g %, w/v) separately to synthetic medium with tryptone to study the PHA production by recombinant strain. The flasks were inoculated (10 %, v/v) with 8 h old inoculum and incubated at 37 ^{0}C at 250 rpm for 48 h. The filter-sterilized solutions of fatty acids were added (0.2 g %, w/v) in 5 ml after 24 h of growth.

5.1.1.5 Estimation of biomass and PHA

Dry weight of the cells was estimated by centrifugation of fermented broth at 8000 rpm for 15 min followed by washing and drying of the sedimented cells at 50 ^{0}C to a constant weight. The PHA content of recombinant *E. coli* was determined after sodium hypochlorite hydrolysis and chloroform extraction (Williamson and Wilkinson, 1958; Law and Slepecky, 1961). PHA obtained was quantified as percentage of dry weight of biomass.

5.1.1.6 Residual sugar analysis

Residual sugar in the centrifuged fermented broth was estimated by DNS method (Miller, 1959), which is described in detail in materials and methods (page 51)

5.1.1.7 Statistical analysis

Statistical analysis of the results was carried out using computer based Microsoft excel programme with nonbiased or n-1 method.

5.1.1.8 Gas Chromatography

GC analysis was performed, using a methanolysed mixture of lyophilized cells and purified polymer, in a Fisons GC analyser with benzoic acid as internal standard (Pages 56-57).

5.1.1.9 NMR analysis

^{1}HNMR of the polymer was carried out in deuterated chloroform at 400 MHz on an AMX 400 spectrophotometer. [P(HB), P(HB-co-HV) from Sigma Aldrich, USA] were used as standards.

5.1.1.10 Scanning Electron Microscopy

To study the morphology of the cells, culture broth was centrifuged and the cells were washed twice in phosphate buffer 0.1M (pH 6.5). The washed cells were fixed in 1-2%

glutaraldehyde overnight. The cells were centrifuged and successfully washed with 10-100% gradient of ethanol followed by methanol. The sample was then dried in a desiccator. The dried cells were gold sputter coated and analyzed in a scanning electron microscope (LEO-435 VP scanning electron microscope). Scanned image was captured at a magnification of 5000 X to study the morphology of the cells.

5.1.2. Analysis of the *Bacillus* genome

Genomic blast method was performed using *R. eutropha* β-ketothiolase (bktb) sequence. The amino acid sequence was blasted against various *Bacillus* genome sequences. The *Bacillus* β-ketothiolase sequences were taken for designing gene specific primers. The β- ketothiolase amino acid sequence was used to construct phylogenetic tree with the help of MEGA-2 software along with different β-ketothiolase sequences from the gene bank data.

5.1.3 Optimization using Response Surface Methodology (RSM)

RSM was used for optimization of growth and PHA production by recombinant E coli (Tables 37, 40-43).

5.1.3.1 Microorganism and inoculum

Initial studies revealed that the recombinant *E. coli* strain *JC7623ABC1J4* was a better strain for biomass and PHA production. This strain was used for medium optimization studies. Method of maintenance of the strain and inoculum preparation used was as described above.

5.1.3.2 Experimental design

As reported earlier (Triveni *et al,* 2001) a central composite rotatable design (CCRD) with five variables, at five levels (citric acid, KH_2PO_4, glucose, $(NH_4)_2HPO_4$ and inoculum) was used to study the response pattern and optimum combination of the variables used (Table 37).

Table 37: Variables and their levels for CCRD

Components	Symbols	-2	-1	0	1	2	Mean	St. Deviation
KH_2PO_4	X_1	8.50	11.00	13.50	16.00	18.50	13.50	2.50
$(NH_4)_2HPO_4$	X_2	1.00	2.50	4.00	5.50	7.00	4.00	1.50
Glucose	X_3	5.00	13.75	22.50	31.25	40.00	22.50	8.75
Citric acid	X_4	0.00	0.88	1.75	2.63	3.50	1.75	0.88
Inoculum	X_5	10.00	70.00	130.00	190.00	250.00	130.00	60.00

CCRD was arranged to fit the regression model using multiple regression program (Table 40). The CCRD combines the vertices of the hypercubes whose coordinates are given by a 2*n* factorial design to provide for the estimation of curvature of the model (Joglekar and May 1987). Six replicates (treatments 27–32) were included for estimation of a pure error of sum of squares (Table 40).

5.1.3.4 Statistical analyses

A second order polynomial equation was employed to fit the experimental data presented in Table 38. The proposed model for the responses *Y*1, *Y*2, *Y*3 and *Y*4 was $Y1 = a0 + a1x1 + a2x2 + a3x3 + a4x4 + a12x1x2 + a13x1x3 + a14x1x4 + a23x2x3 + a24x2x4 + a34x3x4$, where *Yi* (*i* = 1–4) is the predicted response for biomass and PHA yield, *a*0 is the value of the fitted response point of design, *ai*, *aii*, *aij* the linear, quadratic and cross-product terms, respectively. A non-linear mathematical optimization procedure (Saxena and Rao 1996) of the Quattro Pro software (Quattro Pro, Version 4.0, Borland International, Inc. USA) was used for the optimization of the fitted polynomials for biomass and PHA yields (Tables 39-40; Floros and Chinnan 1988). Responses obtained were compared with the predicted models (Table 41). The fitted polynomial equation was expressed as surface plots through which it was possible to visualize the relationship between the response and the experimental levels of each factor used in the experiments.

5.1.3.5 Analysis

Estimation of biomass and PHA were carried out as per methods described above.

5.1.4 Cultivation of recombinant *E. coli* strain *JC7623ABC1J4* in a fermentor

E. coli strain *JC7623ABC1J4* strain was cultivated in a jar fermentor (Bioflo 110, New Brunswick Scientific Co. USA) of 3 l capacity with 2 l of medium. The medium contained (g/l) 13.5 KH_2PO_4, 1.4 $MgSO_4$ $7H_2O$, 1.7 citric acid, 4.0 $(NH_4)_2HPO_4$, 2 tryptone and 20 glucose. Inoculum was prepared by transferring a single colony of the recombinant strain in to 5 ml LB broth with 10 mg % ampicillin. The tube was incubated at 200 rpm at 37 ^oC for 4 h and the content was transferred in to 50 ml of LB broth with 10 mg % ampicillin. The flask was incubated at 37 ^oC, 200 rpm for 6 h and 200 ml of such inoculum was transferred into 2 l of sterile medium contained in the fermentor. The inoculum contained $2x10^2$ viable cells/ ml. Fermentation was carried out at pH 6.8 and dissolved oxygen was maintained at 40 % saturation level by varying the rpm using cascade mode. Estimation of biomass, residual sugar and extraction of PHA were carried out as mentioned above.

5.2 RESULTS

5.2.1 Comparison of recombinant *E. coli* strains for PHA production

Three recombinant *E. coli* strains (*fadBC1J4*, *JC7623C1J4*, *JC7623ABC1J4*) were cultivated in PHA production medium having different combinations of nutrients. Data in Table 38 shows that among the three media, the medium containing citric acid and butyrate in the presence of tryptone was suitable for growth and accumulation of PHA for all three *E. coli* strains. *E. coli* strain *JC7623ABC1J4* produced highest amount of PHA in all the media. The PHA yield reached maximum of 51 % when it grew in medium containing citrate, tryptone and butyrate.

Table 38: Effect of co carbon sources and tryptone on PHA production by recombinant strains

Strain	Co-carbon sources	Biomass g/l	PHA % of biomass	Residual sugar g%
1	Citrate (lacks tryptone)	1.7	32	0.4
2	Citrate (lacks tryptone)	1.1	15	1.0
3	Citrate (lacks tryptone)	1.1	48	0.6
1	Citrate + Butyrate	1.8	33	0.4
2	Citrate + Butyrate	1.9	35	1.7
3	Citrate + Butyrate	1.8	51	0.6
1	Butyrate (lacks citrate)	1.4	28	0.4
2	Butyrate (lacks citrate)	1.6	36	1.6
3	Butyrate (lacks citrate)	1.5	38	0.9

1 = Strain *fadBC1J4*: *FadB* mutant harboring *PhaC1* and *PhaJ4* of *P. aeruginosa*
2 = Strain JC7623*C1J4* : JC7623 having *PhaC1* and *PhaJ4* of *P. aeruginosa*
3 = Strain *JC7623ABC1J4*: JC7623 harboring *PhaC1* and *PhaJ4* of *P. aeruginosa* and *PhaA* and *PhaB* from *Bacillus sp* 256.

The *fadBC1J4* strain did not show any variation in PHA yield in the presence or absence of tryptone in the medium while inclusion of the tryptone induced higher PHA yield by strain *JC7623ABC1J4.* The presence of citric acid and tryptone was essential for higher PHA production by *JC7623ABC1J4* strain. This strain was selected for further studies.

Sudan black B stained *E. coli* strain *JC7623ABC1J4* cells showed bluish-black colored intracellular PHA granules compared to gram stained cells (Figs. 61 and 62). The recombinant *E. coli* cells were subjected to Scanning Electron Microscopy (SEM) before and after PHA accumulation. The SEM images showed a clear difference between the PHA accumulating cells and normal *E. coli* cells. The PHA accumulating cells were swollen due to PHA granules present in the cells (Fig. 63). The scanning electron microscopy of *E. coli* cells after 48 h of fermentation showed pit formation in the cell walls (Fig. 64). The pitting may indicate autolysis of the cells after the fermentation.

5.2.2 PHA production by recombinant *E. coli* strain *JC7623ABC1J4*

Strain *JC7623ABC1J4* was further cultivated in the presence of various fatty acids to produce scl-co-mcl-PHA. The results obtained are tabulated in the Table 39. Growth of the recombinant strain in LB medium (82 mg %) was less compared to that of synthetic medium (212 mg %). Supplementation of tryptone to medium resulted in higher biomass production (467 mg %) compared to yeast extract. In the presence of glucose only PHB was synthesized. Butyric acid supported highest yield of PHA (53 % of biomass) compared to other fatty acids. The strain was able to produce 2, 2, 1, 3, 1, 4 and 4-mol % of hydroxyvalerate when the cells were grown in butyrate, valerate, hexanoate, heptanoate, octanoate, nonanoate and decanoate, respectively. Quantitative and qualitative analysis of the polymer by GC indicated that the molar percentage of scl:mcl PHA varied, depending on the fatty acid supplemented to the glucose containing medium as a co-substrate, from 91:9 to 24:76 (Table 39; Figs. 65-69). Concentration of mcl-PHA increased on supplementation with heptanoic, octanoic and decanoic acids.

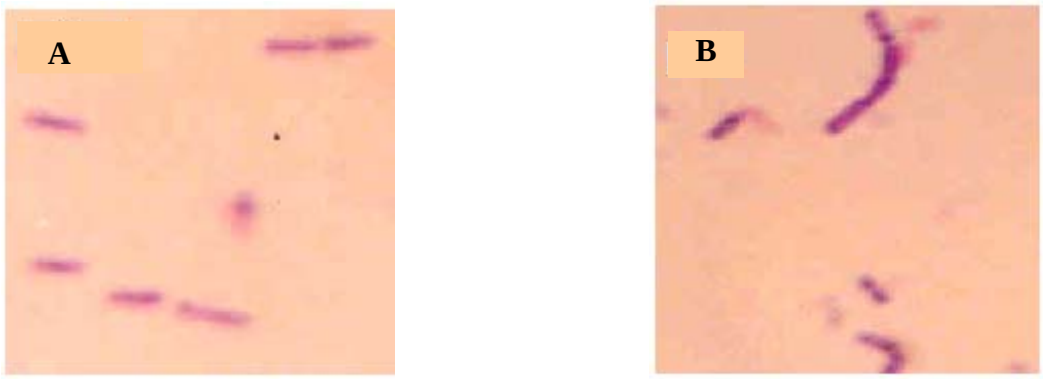


Fig. 61: Gram stained *E. coli* strains
A = E. *coli* strain JC7623 (host)
B = *E. coli* recombinant strain *JC7623ABC1J4*

Fig. 62: Recombinant *E. coli* strain *JC7623ABC1J4* with PHA granules stained with Sudan black B

Table 39: Production of PHA by recombinant *E. coli (JC7623ABC1J4)* in complex and defined synthetic medium

Medium	Co-carbon substrates (0.2 g%, w/v)	Biomass (mg/100ml)	PHA (% of biomass)	Residual sugar (g%)	Scl: Mcl PHA (Mol%)*	Mol% of HB:HVin the polymer
A	Nil	212±17.7	30±5.65	0.90	100:0	100:0
B	Nil	467±15.55	38±2.82	0.04	100:0	100:0
B	Butyric acid	470±5.65	53±2.82	0.16	91:9	89:2
B	Valeric acid	490±14.14	45±1.41	0.19	100:0	98:2
B	Hexanoic acid	412±16.97	48±3.53	0.26	100:0	99:1
B	Heptanoic acid	290±14.14	24±1.41	1.02	83:17	80:3
B	Octanoic acid	230±14.14	36±0.70	0.86	24:76	23:1
B	Nonanoic acid	211±15.55	41±0.70	0.70	73:27	69:4
B	Decanoic acid	249±1.41	36±0.70	0.83	71:29	67:4
C	Nil	82±8.48	47±1.41	1.20	ND	ND
B**	Nil	59±6.5	24±2.08	1.30	100:0	100:0
B**	Butyric acid	394±16.4	19±1.5	0.20	92:8	100:0

A=Synthetic medium with yeast extract (0.2 g%, w/v) and glucose (2 g%, w/v); B=Synthetic medium with tryptone (0.2%, w/v) and glucose (2 g%, w/v); C= Luria Bertani broth with glucose (2 g%, w/v); *By GC analysis; **Recombinant strain *JC7623C1J4* used for comparison.

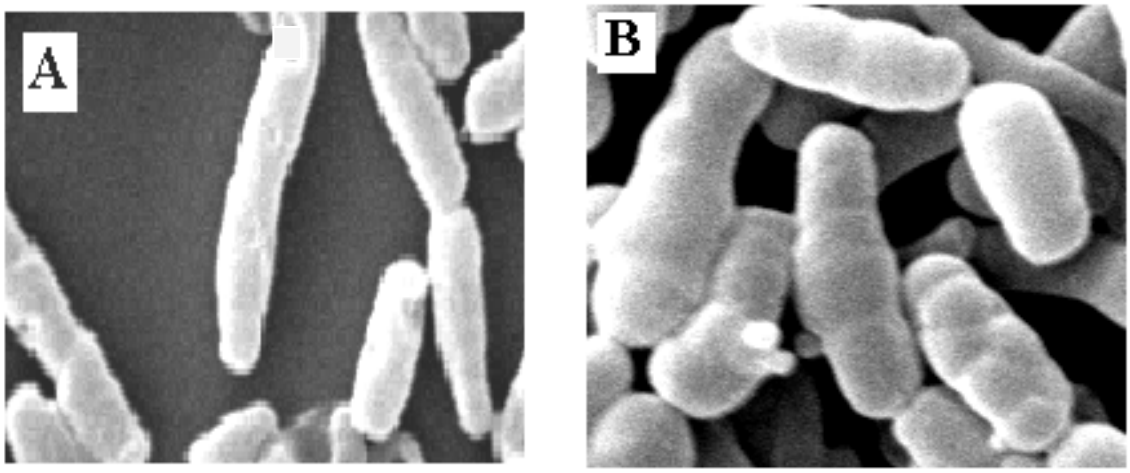


Fig. 63: Scanning Electron Microscopy photographs of recombinant *E. coli* cells (harboring *PhaA, PhaB, PhaC1* and *PhaJ4* genes in host JC7623): A) Cells prior to granule formation B) Cells with PHA granules

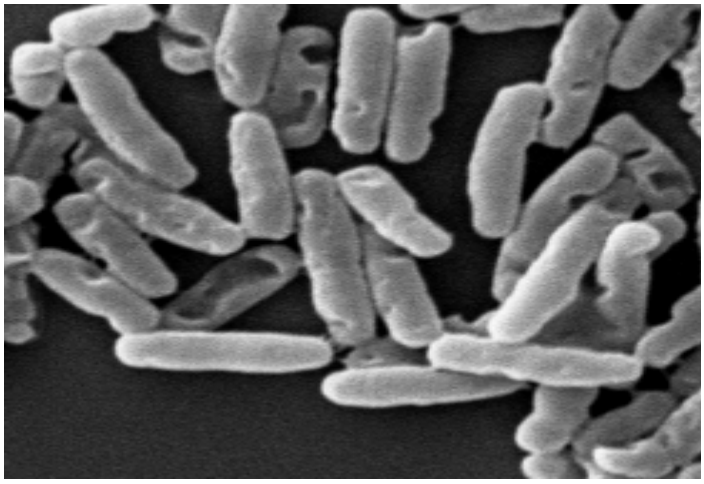


Fig. 64: Scanning Electron Microscopy photographs of recombinant *E. coli* (*JC7623ABC1J4*) harboring *phaC1, PhaJ4, PhaA* and *PhaB* genes (Magnification, 5K X). Pitting of the cells indicate autolysis after 48 h of growth.

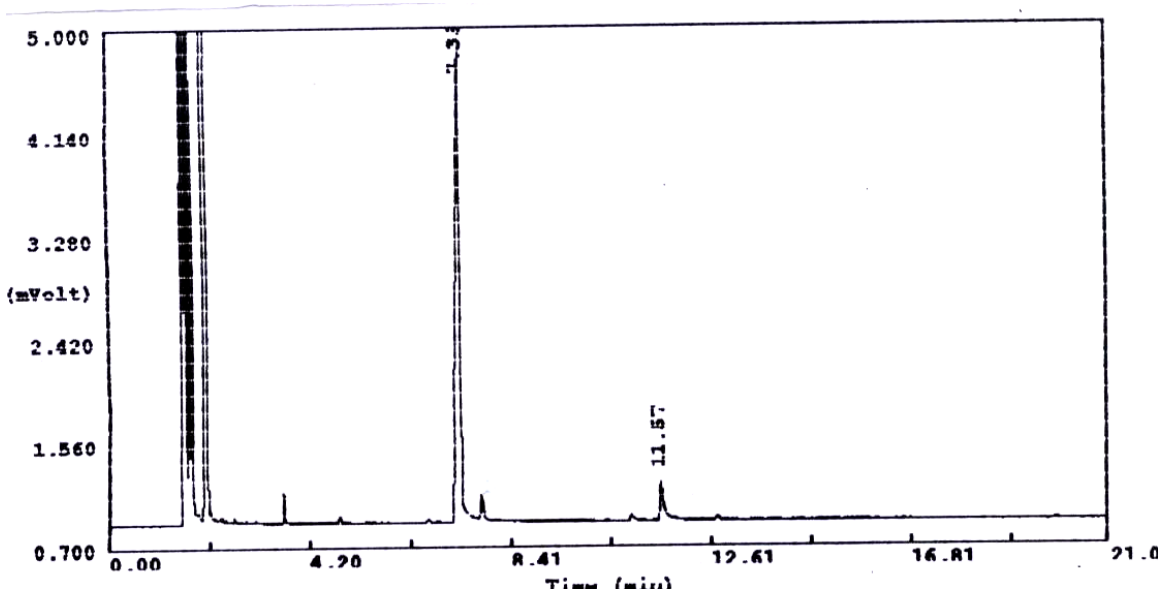


Fig. 65: GC profile of standard poly -3(HB-co-HV)

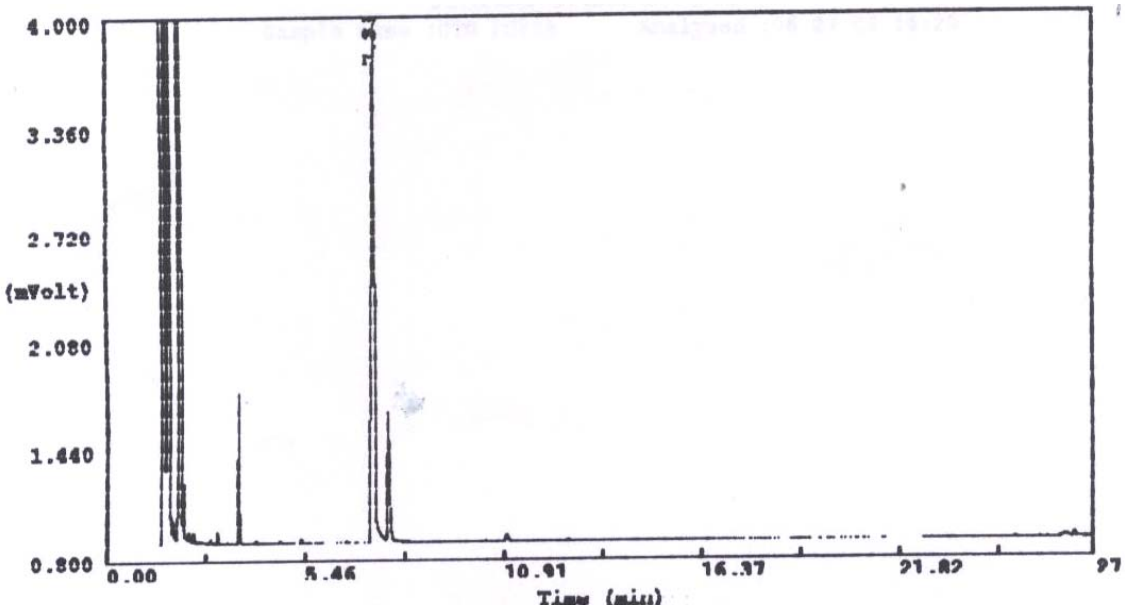


Fig. 66: GC profile of PHA produced by *JC7623ABC1J4* strain on glucose

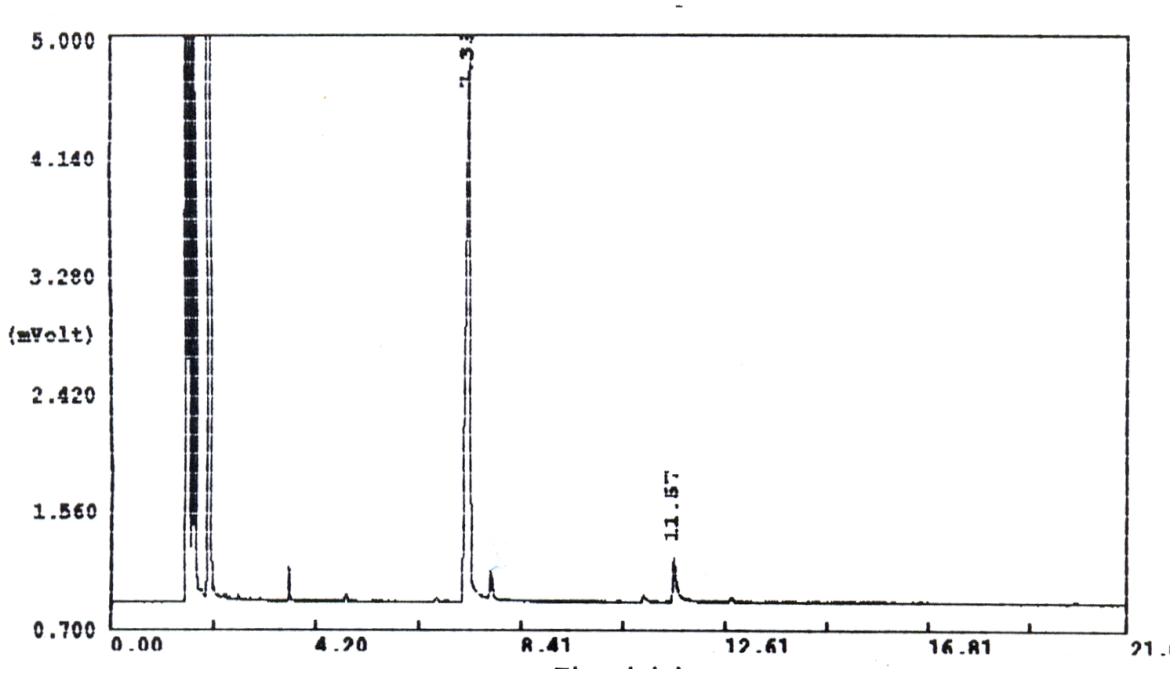


Fig. 67: GC profile of the PHA synthesized by recombinant *E. coli* (*JC7623ABC1J4*) on butyric acid

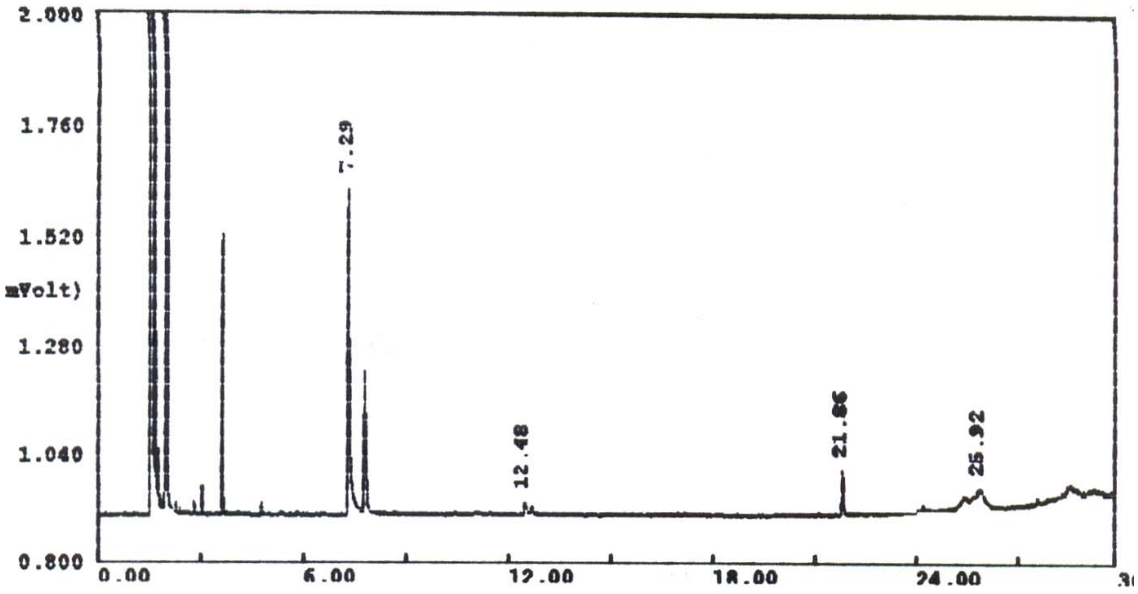


Fig 68: GC profile of the PHA synthesized by *JC7623ABC1J4* strain on nonanoic acid

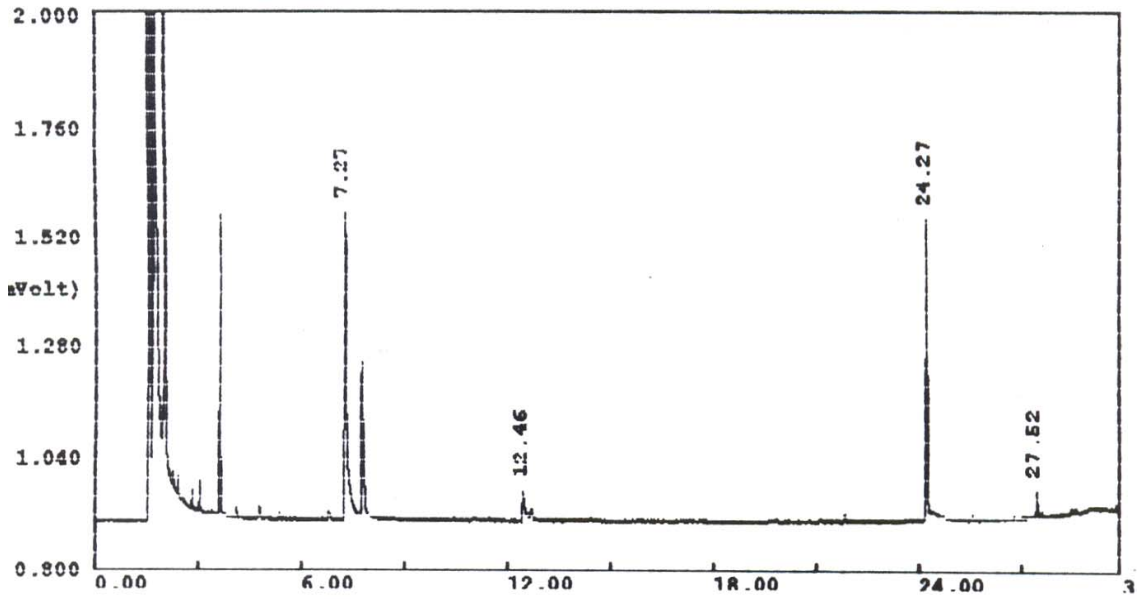


Fig. 69: GC profile of the PHA synthesized by *JC7623ABC1J4* strain on decanoic acid

5.2.3 Characterization of PHA

^{1}H NMR was used to characterize PHA obtained after cultivation of recombinant *E. coli JC7623ABC1J4*. Standard P(HB-co-HV) was used as standard (Fig. 70). Characteristic signals assigned to PHB was a doublet at 1.29 ppm which is attributed to the methyl group, a doublet of quadruplet at 2.5 ppm which is due to methylene group and a multiplet at 5.28 ppm which is characteristic of methyne group. A triplet at 0.9 ppm and a methylene resonance at 1.59 and methyne resonance at 5.15 indicated presence of valerate. Analysis of PHA from recombinant *E. coli* showed resonance of HB, HV (Fig. 71). In addition to this, ^{1}H NMR spectra of PHA indicated the synthesis of polyhydroxybutyrate (PHB) and higher alkanoate copolymers in the presence of glucose and fatty acids whereas only PHB was synthesized in the presence of glucose as sole carbon substrate (Fig. 68). ^{13}C NMR was also used for the analysis of PHA (Figs. 72-74). Data shown in the NMR spectra are in accordance with data reported in the literature (Labuzek and Radecka, 2001; Pedro, 2003).

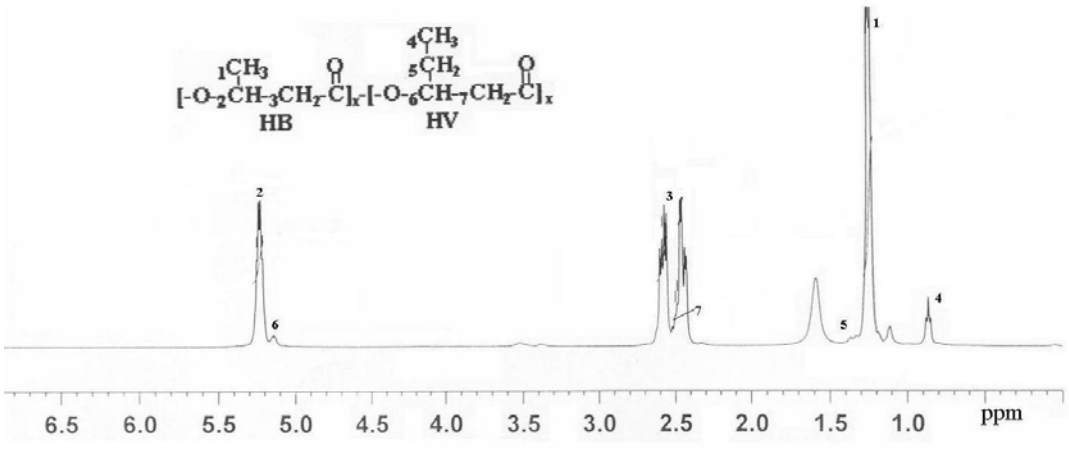


Fig. 70: ^{1}H NMR spectrum of standard P(HB-co-HV)

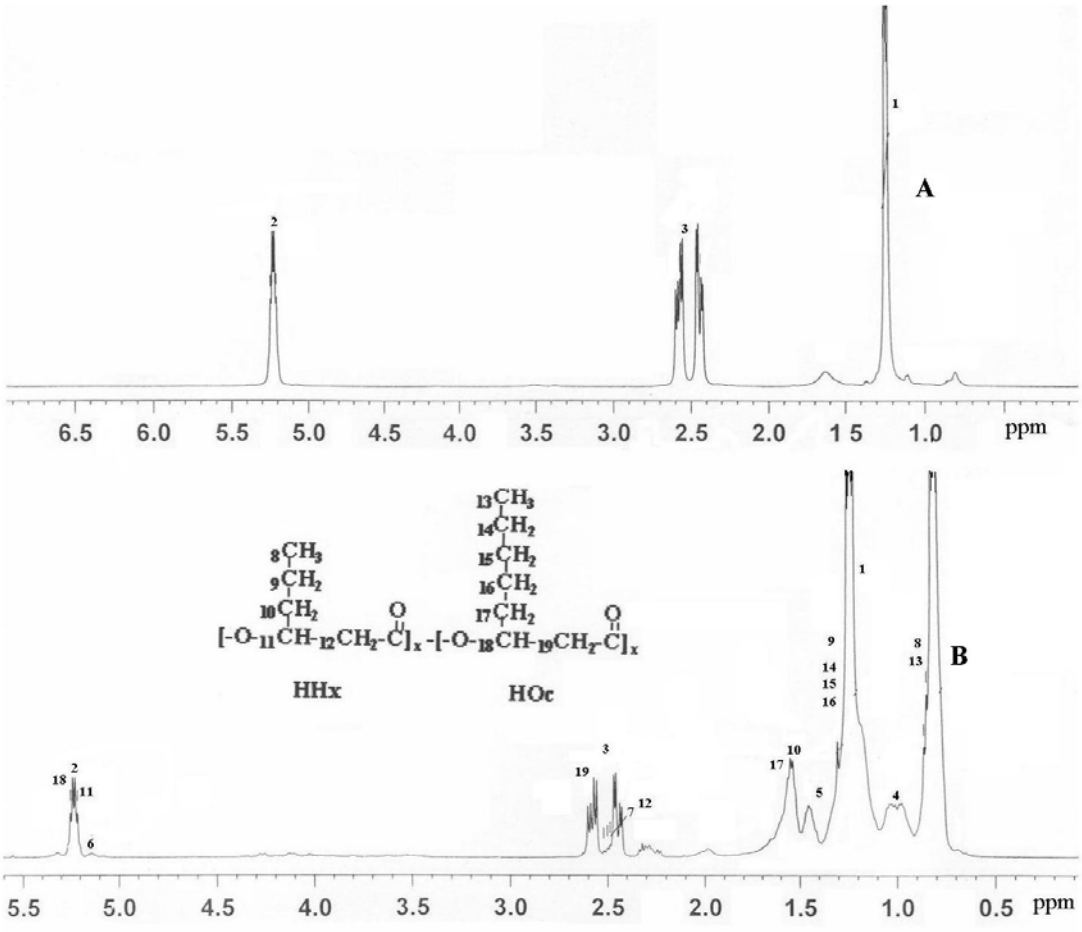
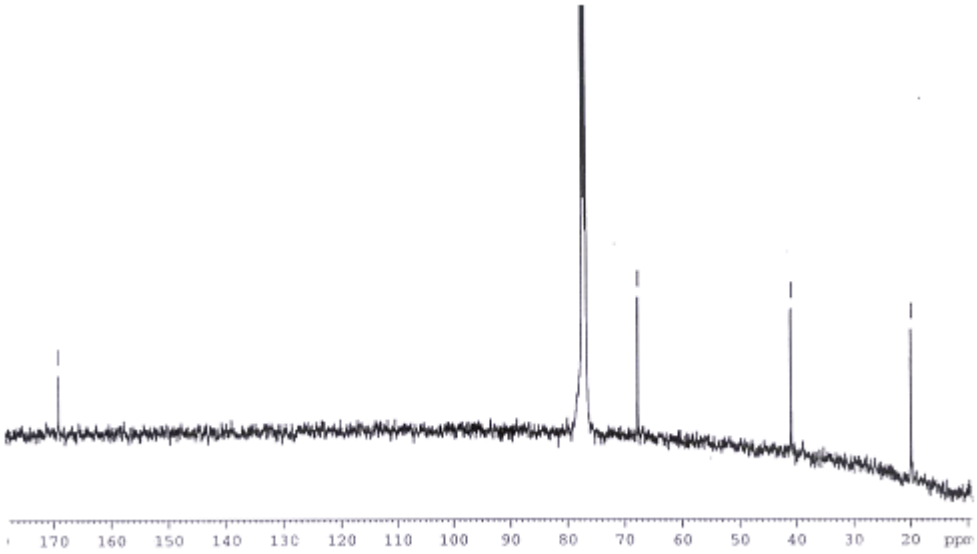


Fig. 71: ^{1}H NMR spectra of PHA produced by recombinant *E. coli* (strain *JC 7623ABC1J4)*: A) Grown on glucose; B) Cultivated on glucose and octanoic acid



Fig, 72: ^{13}C NMR spectrum of standard PHB

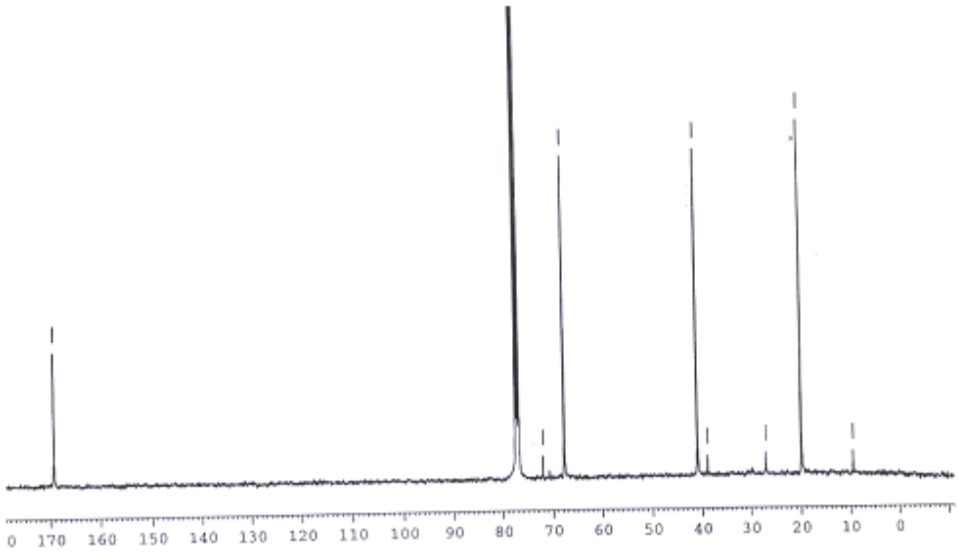


Fig. 73: ^{13}C NMR spectrum of standard P(HB-co-HV)

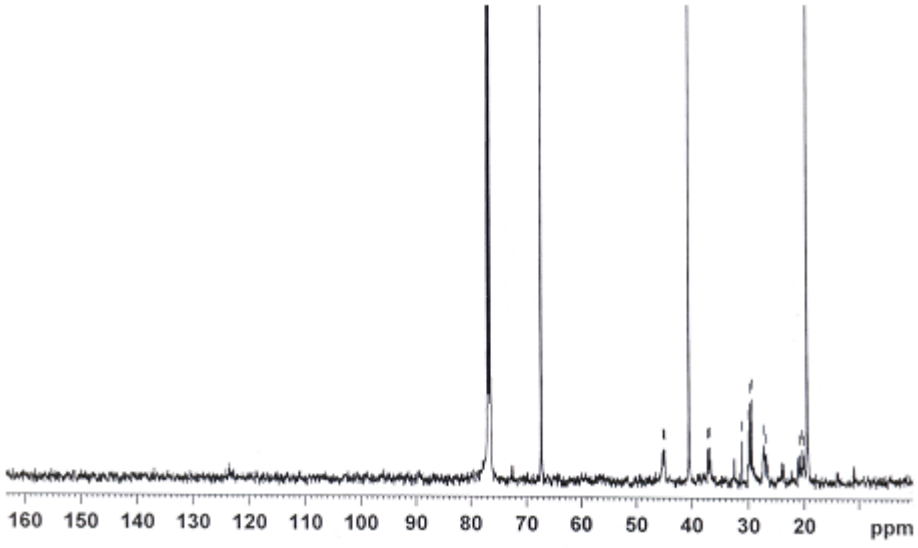


Fig. 74: ^{13}C NMR spectrum of PHA from recombinant *E. coli*

5.2.4 Optimization of PHA production by recombinant *E. coli JC7623ABC1J4* strain using response surface methodology

PHAs are accumulated in the bacterial cells due to nutrient depletion conditions. In the present experiments it was observed that the recombinant strains do not require such conditions for PHA production. The responses obtained indicated that the nutrients do affect the overall growth by way of increase and decrease in the nutritional status (Table 40). The maximum biomass (6.56 g/l) and PHA yield (2.6 g/l) were observed in the treatment number 20 (KH_2PO_4 13.5 g/l, $(NH_4)_2HPO_4$ 7 g/l, glucose 22.5, citric acid 1.75 g/l and inoculum 130 ml/l) where the $(NH_4)_2HPO_4$ concentration was moderately higher than the other components. The responses measured in the experiment were PHA and biomass. The effect of variables on these responses is tabulated by the coefficient of second order polynomials (Table 41). Less significant terms were omitted using t-test and the responses under different combinations were analyzed analysis of variance (ANOVA) appropriate to experimental design (Table 42). Feasible optimum conditions are tabulated in Table 43. Response surface graphs obtained based on the coefficients are shown Figs. 75-78.

5.2.4.1 Effect of glucose and KH_2PO_4 on biomass and PHA

Different concentrations of KH_2PO_4 did not show any difference in PHA yield (Fig 75)

5.2.4.2 Effect of glucose and ammonium phosphate on biomass and PHA production

Fig 76 show that the increase in concentration of glucose and ammonium phosphate gave higher yield of biomass and PHA where as growth and polymer production were least at minimum concentrations of glucose (5g/l) and ammonium phosphate (1g/l). Maximum yields of biomass (12g/l) and PHA (4.2 g/l) were observed when the media was provided with highest concentration of glucose (40 g/l) and ammonium phosphate (7 g/l).

5.2.4.3. Effect of citric acid and glucose on biomass PHA production

Interactions of different concentrations of citric acid and glucose showed that biomass and PHA yields decreased when the citric acid concentration was high in the medium (Fig. 77). However a combination of lower concentration of citric acid and higher concentration of glucose helped growth of cells and polymer production (biomass 10 g/l and PHA 5.3 g/l).

5.2.4.4. Effect of inoculum and glucose on biomass and PHA

Various concentrations of inoculum was used in the experiment and its interactions with glucose showed that increasing the inoculum beyond 10 ml% (10 ml to 130ml) did not result in enhanced growth or polymer production (Fig. 78). Initial concentration of glucose 5g/l in the medium resulted in 3g/l biomass and 1.2 g/l of PHA. The highest biomass (9 g/l) and PHA (5.5 g/l) was observed in medium with higher glucose levels.

5.2.5 Cultivation of recombinant *E. coli* strain *JC7623ABC1J4* in a fermentor

Fermentation of *JC7623ABC1J4* strain, using glucose as sole carbon source, resulted in the accumulation of intracellular PHA. The PHA accumulation was initiated at about 12 h of cultivation and reached maximum after 48 h (Fig. 79). At the end of fermentation period maximum biomass (10 g/l) and PHA (4 g/l) were obtained. Concentration of PHA was 40 % of dry cell biomass.

Table 40: Treatment schedule for five-factor CCRD and response in terms of biomass and PHA yield

Exp No.	KH_2PO_4	$(NH_4)_2PO_4$	Glucose	Citric acid	Inoculum Conc.	Biomass yield (g/l)		PHA yield (g/l)	
	X_1	X_2	X_3	X_4	X_5	Observed	Predicted	Observed	Predicted
1	-1	-1	-1	-1	1	1.020	1.046	0.380	0.494
2	1	-1	-1	-1	-1	0.540	0.450	0.042	0.000
3	-1	1	-1	-1	-1	5.400	5.624	2.120	2.094
4	1	1	-1	-1	1	1.800	2.072	0.700	0.800
5	-1	-1	1	-1	-1	4.000	3.850	2.010	1.912
6	1	-1	1	-1	1	1.660	1.558	0.680	0.706
7	-1	1	1	-1	1	0.990	1.204	0.400	0.472
8	1	1	1	-1	-1	4.380	4.476	2.000	1.886
9	-1	-1	-1	1	-1	1.080	1.026	0.250	0.246
10	1	-1	-1	1	1	1.660	1.654	0.520	0.640
11	-1	1	-1	1	1	2.500	2.808	0.800	0.966
12	1	1	-1	1	-1	5.080	5.272	2.000	1.980
13	-1	-1	1	1	1	1.420	1.354	0.520	0.612
14	1	-1	1	1	-1	5.380	5.198	1.980	1.882
15	-1	1	1	1	-1	0.660	0.792	0.380	0.332
16	1	1	1	1	1	2.200	2.384	0.640	0.718
17	-2	0	0	0	0	2.180	1.985	0.780	0.680
18	2	0	0	0	0	3.380	3.325	1.020	1.044
19	0	-2	0	0	0	2.800	3.241	1.280	1.274
20	0	2	0	0	0	6.050	5.365	2.040	1.970
21	0	0	-2	0	0	2.580	2.267	0.700	0.548
22	0	0	2	0	0	2.420	2.483	0.800	0.880
23	0	0	0	-2	0	0.940	0.821	0.680	0.714
24	0	0	0	2	0	1.000	0.873	0.580	0.474
25	0	0	0	0	-2	3.320	3.363	1.000	1.274
26	0	0	0	0	2	0.702	0.211	0.400	0.050
27	0	0	0	0	0	2.400	1.775	0.560	0.526
28	0	0	0	0	0	1.600	1.775	0.540	0.526
29	0	0	0	0	0	1.920	1.775	0.400	0.526
30	0	0	0	0	0	1.460	1.775	0.480	0.526
31	0	0	0	0	0	1.960	1.775	0.560	0.526
32	0	0	0	0	0	1.060	1.775	0.540	0.526

Table 41: Estimated coefficients of the fitted second order polynomial representing the relationship between the response and the process variable

	Biomass yield			PHA Yield		
	Estimated coefficients	Standard error	t-value	Estimated coefficients	Standard error	t-value
a_0	1.775**	0.188	9.441	0.526**	0.076	6.921
a_1	0.335**	0.096	3.490	0.091**	0.039	2.333
a_2	0.531**	0.096	5.531	0.174**	0.039	4.462
a_3	0.054	0.096	0.563	0.083*	0.039	2.128
a_4	0.013	0.096	0.135	-0.060	0.039	-1.538
a_5	-0.788**	0.096	-8.208	-0.306**	0.039	-7.846
a_{11}	0.220*	0.087	2.529	0.084**	0.035	2.400
a_{22}	0.632**	0.087	7.264	0.274**	0.035	7.829
a_{33}	0.150	0.087	1.724	0.047	0.035	1.343
a_{44}	-0.232**	0.087	-2.667	0.017	0.035	0.486
a_{55}	0.003	0.087	0.034	0.034	0.035	0.971
a_{12}	0.137	0.118	1.161	0.099*	0.048	2.063
a_{13}	0.467**	0.118	3.958	0.142**	0.048	2.958
a_{14}	0.731**	0.118	6.195	0.292**	0.048	6.083
a_{15}	-0.178	0.118	-1.508	-0.051	0.048	-1.063
a_{23}	-0.919**	0.118	-7.788	-0.387**	0.048	-8.063
a_{24}	-0.278**	0.118	-2.356	-0.097*	0.048	-2.021
a_{25}	-0.174	0.118	-1.475	-0.111**	0.048	-2.313
a_{34}	-0.183	0.118	-1.551	-0.119**	0.048	-2.479
a_{35}	-0.189	0.118	-1.602	-0.132**	0.048	-2.750
a_{45}	0.277**	0.118	2.347	0.118**	0.048	2.458

*Significant at 5% level, ** Significant at 1% level

Table value of $t_{0.05, 11} = 1.796$ and $t_{0.01, 11} = 2.178$.

Table 42: Analysis of variance for the fitted second order polynomial model and lack of fit for biomass yield as per CCRD

		Biomass yield			PHA Yield		
Source of variation	df	Sum of squares	Mean sum of squares	F value	Sum of squares	Mean sum of squares	F value
First order terms	5	24.117	4.823	22.188	3.425	0.685	165.469
Second order terms	15	45.430	3.029	13.932	7.690	0.513	123.850
Total	20	69.547			11.114		
Residual							
Lack of fit	6	1.536	0.256	1.178	0.394	0.066	15.878
Pure error	5	1.087	0.217		0.021	0.004	
Total error	11	2.623			0.415		
	31	72.170			11.529		
Coefficient of Determination (R2) =				0.964			0.964

* Significant at 1% level; ^{ns} Not significant

Table 43: Feasible optimum conditions and predicted and experimental value of response at optimum condition

	KH_2PO_4	$(NH_4)_2HPO_4$	Glucose	Citric acid	Inoculum Conc.
Maximum Biomass					
	x_1	x_2	x_3	x_4	x_5
Coded Value	2.000	2.000	-2.000	1.575	-2.000
Uncoded	18.500	7.000	5.000	3.129	10.000
	Experimental Value		Predicted Value		
Biomass Yield (Y1)	11.0		12.579		
PHA Yield (Y2)	4.0		5.319		
Maximum PHA Yield					
	x_1	x_2	x_3	x_4	x_5
Coded Value	-2.000	-2.000	2.000	-2.000	-2.000
Uncoded	8.500	1.000	40.000	0.000	10.000
	Experimental Value		Predicted Value		
Biomass Yield (Y1)	8.8		10.149		
PHA Yield (Y2)	4.4		5.702		

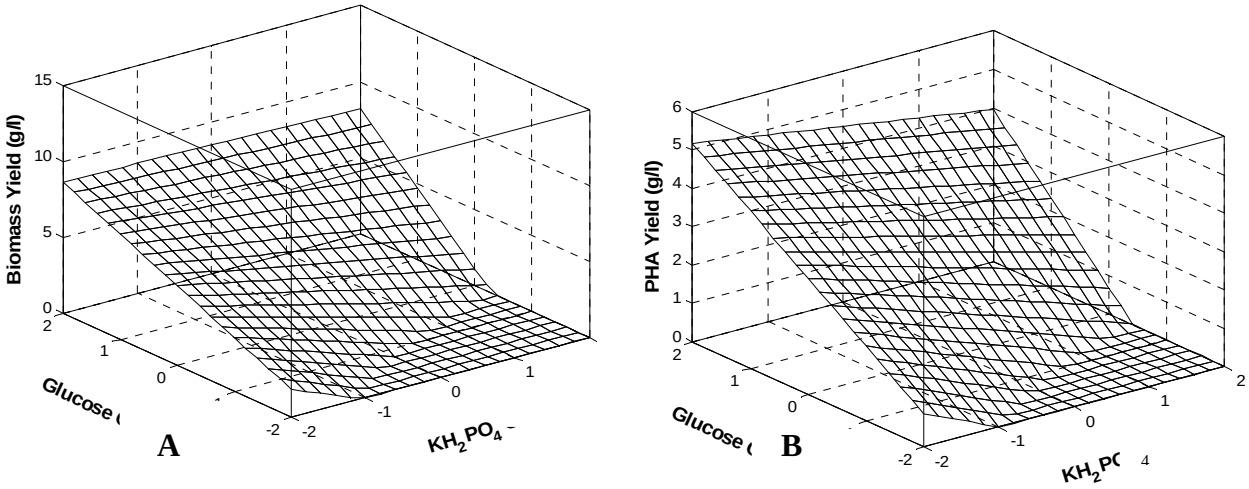


Fig. 75: Effect of interaction of glucose and KH_2PO_4 on biomass (A) and PHA (B)

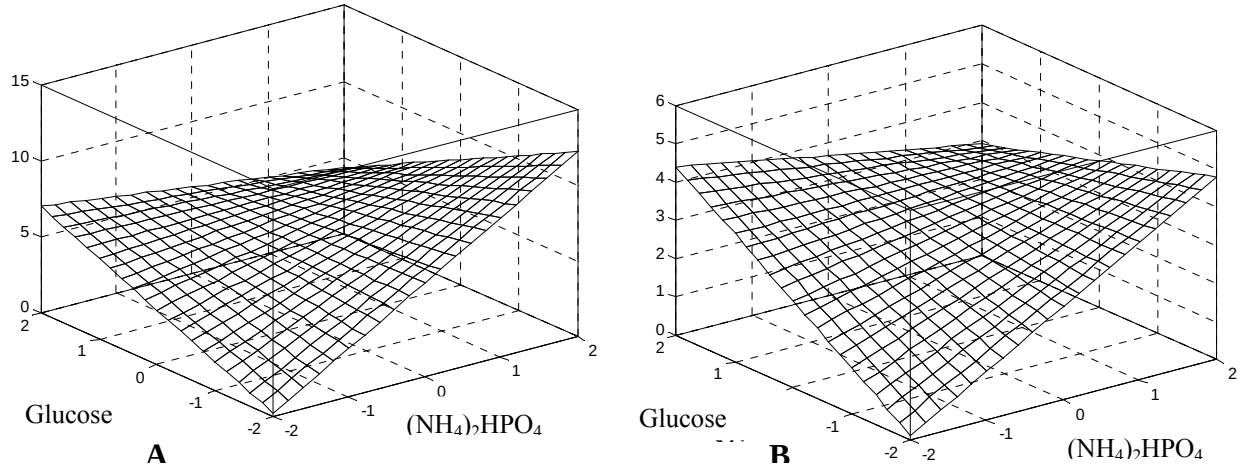


Fig. 76: Effect of interaction of glucose and $(NH_4)_2HPO_4$ on biomass (A) and PHA (B)

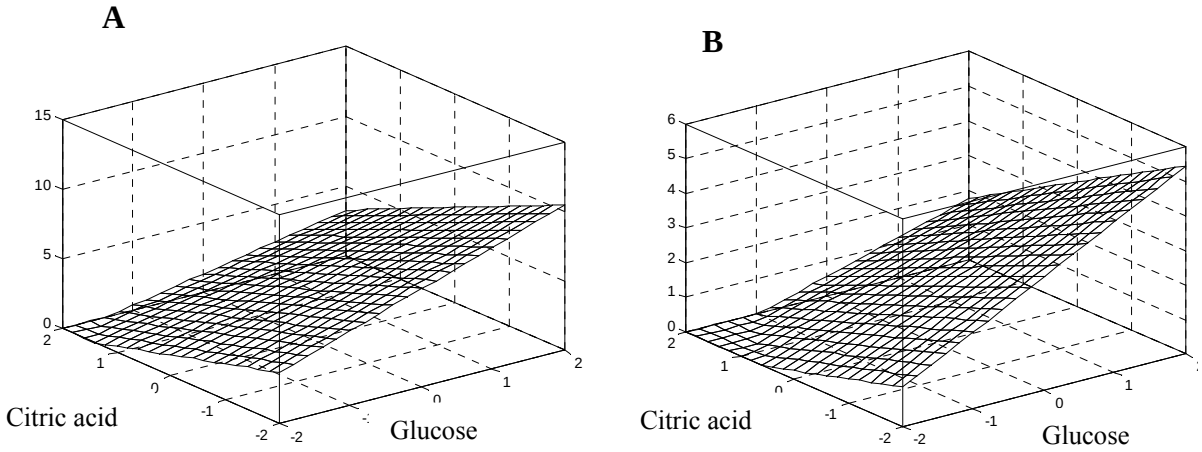


Fig. 77: Effect of interaction of citric acid and glucose on biomass (A) and PHA (B)

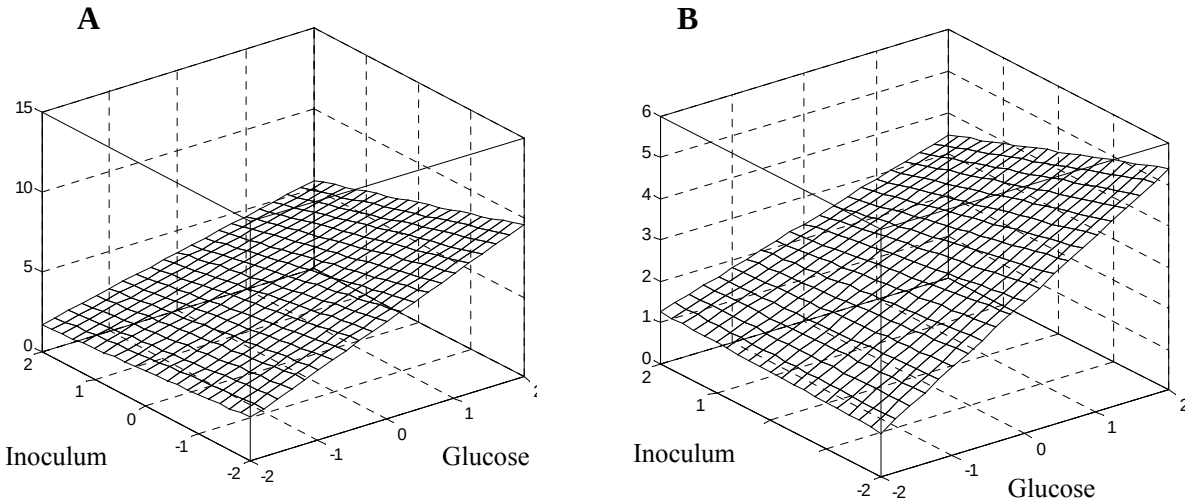


Fig.78: Effect of interaction of inoculum and glucose on biomass (A) and PHA (B)

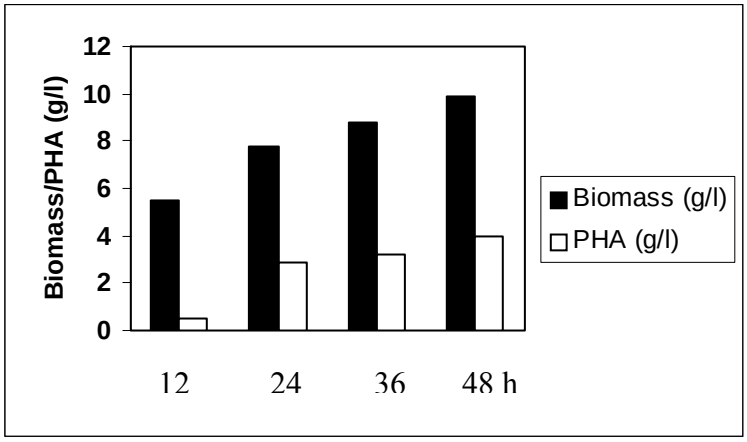


Fig. 79: PHA production by recombinant *E. coli* in a fermentor with glucose as a substrate

5.3 DISCUSSION

Monomer composition of PHA is one of the important criteria for its use as commodity plastic. Scl-PHAs have properties close to conventional plastics while the mcl-PHAs are regarded as elastomers and rubbers. PHB homopolymer is known to be brittle and polymers with low mol% of scl-PHA tend to fall into the category of elastomers, which have limited use. Heteropolymers are formed by polymerization of different types of monomers. Copolymers of PHA can be formed containing 3-hydroxybutyrate (HB), 3-hydroxyvalerate (HV), 3-hydroxyhexanoate (HH) or 4-hydroxybutyrate (4HB) monomers etc. Copolymer of P (HB-co-HV) or scl-co-mcl PHA with low mol% of mcl monomers has properties similar to polypropylene and polyethylene, which has several potential uses (Nomura *et al,* 2004). In the present study, a recombinant *E. coli* strain *JC7623ABC1J4* was developed by cloning *phaA*, *phaB* from *Bacillus sp* (involved in scl-PHA synthesis)*; phaC1* and *phaJ4* from *P. aeruginosa* (genes involved in mcl-PHA synthesis*)*. The strain produced PHB from glucose as main carbon substrate and scl-co-mcl PHA copolymer from glucose and fatty acids.

Inclusion of tryptone and citric acid in the medium seemed to have a role in growth and PHA accumulation by the recombinant strains. When tryptone was absent in the medium the bacteria showed lesser growth. Tryptone is a hydrolysis product of casein and is rich in nitrogen for bacterial growth. The addition of tryptone in the medium increased the biomass of all the three recombinant strains of *E. coli*, while the PHA concentration remained unaffected. The data in table 38 shows that in the absence of citrate the intracellular concentration of PHA was reduced. The microorganisms can utilize citric acid as a metabolite to derive energy. The metabolism of citric acid via TCA cycle can yield coenzymes like NADH and NADPH, which is essential for PHA production. The NADPH molecule is the coenzyme of acetoacetyl CoA reductase enzyme in the scl-PHA biosynthesis pathway, so it is considered as one of the limiting factors of PHA synthesis in microorganisms. The excess level of intracellular NADH can also induce PHA production by feedback inhibition of TCA cycle and directing the acetyl

CoA to PHA biosynthesis pathway. The highest production of PHA was obtained when the media contained citric acid and butyrate.Bacteria can oxidize butyrate and yield acetyl CoA molecule which leads to synthesis of scl-PHA.

In eubacteria *PhaA* and *phaB* genes are involved in scl-PHA biosynthesis pathway. The products of these genes, β-ketothiolase and acetoacetyl CoA reductase catalyze first two enzymatic steps of scl-PHA biosynthesis pathway with an end product 3-OH butyryl CoA, the monomer of scl-PHA. The activity of these two enzymes depends on the availability of acetyl CoA molecules in the cellular environment. In bacteria acetyl CoA molecules can be derived mainly from fatty acid beta-oxidation and glycolytic pathway. The acetyl CoA enters in to PHA biosynthesis pathway with help of the enzymes coded by *phaA* and *phaB* genes. The presence of mcl-PHA biosynthesis genes such as *phaC1* and *phaJ4* along with *phaA* and *phaB* enabled the recombinant strain to follow a novel PHA biosynthesis pathway (Fig. 80) for PHA copolymer production. The β-ketothiolase and acetoacetyl coA reductase can yield 3-OH butyryl CoA as scl-PHA fraction of the copolymer whereas mcl fraction can be derived from beta oxidation cycle with the help of enoyl CoA hydratase, which enantiomerically convert trans-Δ (S) enoyl CoA to (R) 3-hydroxy acyl CoA. Both the 3-OH butyryl CoA and (R) 3-hydroxy acyl CoA can be polymerized by the broad spectrum PHA synthase enzyme in to the polymer. The material properties of PHA are altered by change in the monomer units by employing PHA synthase having different substrate specificities (Liebergesell *et al*, 2000). The recombinant *E. coli* produced copolymers of scl-co-mcl-PHA from fatty acids like butyric acid, hexanoic acid, hexanoic acid, octanoic acid etc. The scl-co-mcl PHA copolymer production by the strain *JC7623ABC1J4* from a variety of fatty acids (Table 37) is indicative of the ability of the strain to utilize the novel engineered pathway for copolymer production.

The PHA production studies revealed that the recombinant *E. coli* is able to synthesize a copolymer of P (HB-co-HV). In eubacteria it is reported that the PHV can be derived from citric acid cycle- via methylmalonyl CoA pathway, aliphatic fatty acid

degradation pathway (β-oxidation) and through amino acid pathway with a common precursor propionyl CoA.

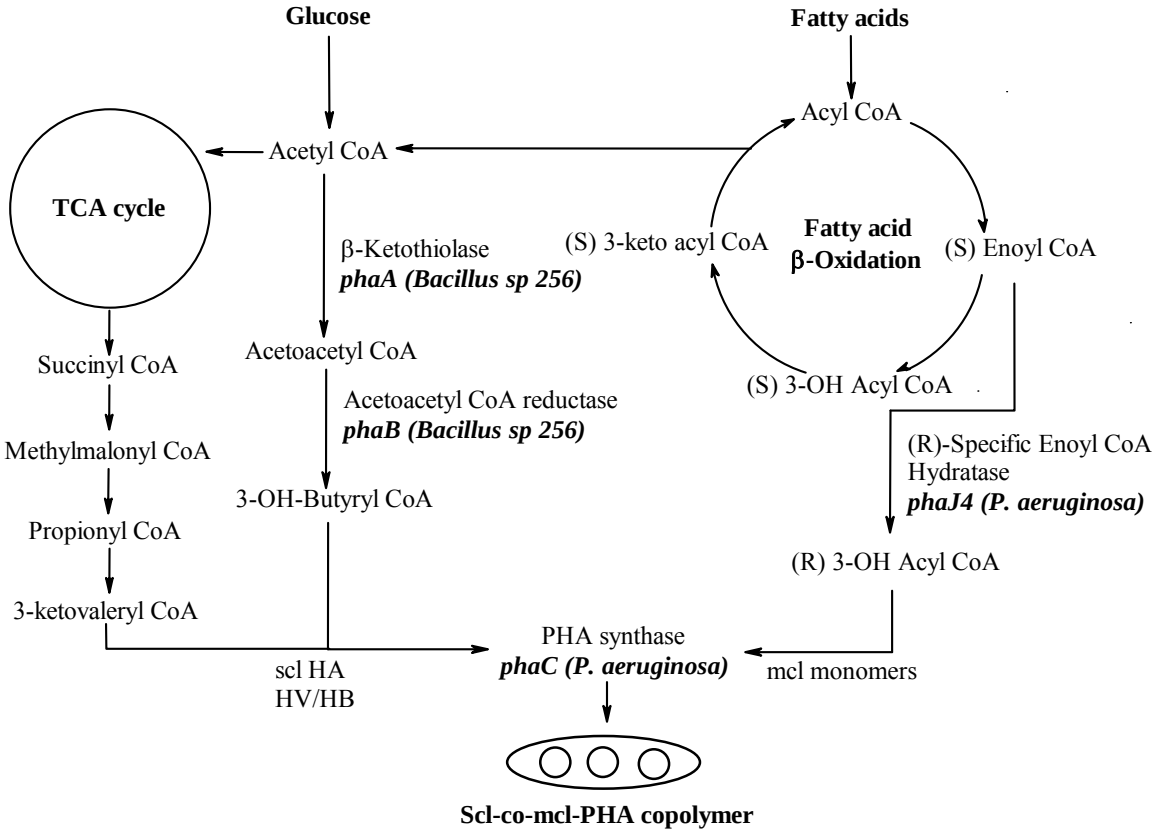


Fig. 80: Novel engineered pathway for scl-co-mcl PHA copolymer production by *E. coli* strain *JC7623ABC1J4*

The recombinant *E.coli* harboring the β-ketothiolase gene from the *Bacillus sp* 256 is able to add HV monomers in the PHA along with other monomers. Analysis of the *Bacillus* genome revealed the presence of four β-ketothiolases one of which was similar to that bktB of *R. eutropha*, reported to be involved in PHV production (Slater *et al,* 1998). It is known that the intermediates of beta-oxidation cycles with five carbon atoms of acyl chain may be converted to 3HV, if the respective enzymes are present

(Steinbuchel and Eversloh, 2003). Valerate may be synthesized from the decarboxylation of amino acids present in the tryptone, from citric acid via succinyl CoA or through β-oxidation pathway of odd aliphatic fatty acid. Our data indicate that the PHV production in the recombinant *E. coli* may be possible through the breakdown of fatty acids as evidenced by the increase in the proportion of PHV when the recombinant cells were grown in the presence of higher fatty acids such as heptanoic acid and nonanoic acid. The absence of PHV synthesis in the medium containing only glucose and citric acid suggested the same conclusion.

Aliphatic fatty acids with a higher carbon chain length and odd number of carbon atoms like valeric acid, heptanoic acid, nonanoic acid etc are also propiogenic substrates. Intermediate of β-oxidation cycles with five carbon atoms of acyl chain may be converted to 3HV, if the respective enzymes are present (Steinbuchel and Eversloh, 2003). It can be concluded that the cloned β-ketothiolase was able to take up propionyl CoA to produce HV. PHB production from glucose suggests that this ketothiolase can condense two molecules of acetyl CoA also. This shows the broad substrate specificity of the enzyme.

In order to facilitate scl-mcl-PHA production PHA synthase (*phaC*) and (R) specific enoyl CoA hydratase have been cloned to construct recombinant *E. coli* system (Timm *et al*, 1990). In the present study the production of scl-PHA from glucose alone suggests that the *Bacillus* genes (*phaA* and *phaB*) enabled the bacteria to use scl-PHA biosynthesis pathway for the production of this homopolymer. Maximum production of PHA from glucose was 40% of biomass and the reasons might be a) non-availability of NADPH b) lowed affinity of PHA synthase of *P. aeruginosa* towards scl-PHA synthesis. The production of scl-PHA and scl-co-mcl-PHA copolymer suggests that the *P. aeruginosa* PHA synthase has different substrate specificity. The mol% of mcl-PHA was high when the recombinant *E. coli* was provided with octanoic acid-decanoic acid. From Table 37 it is clear that there is an affinity for enoyl CoA hydratase towards the higher alkanoates to provide more mcl in the copolymer when compared with lower alkanoates.

The recombinant strain produced PHA with different mol% of mcl-PHA fraction when it grew in media containing fatty acids like butyric acid, heptanoic acid, octanoic

acid, nonanoic acid and decanoic acid. The (R) specific enoyl CoA hydratase (*phaJ4*) present in the recombinant strain can divert some molecules of enoyl CoA to mcl-PHA biosynthesis pathway by converting the (S) enoyl CoA to (R) 3-hydroxyacyl CoA. At the same time some part of the (S) enoyl CoA can be converted in to (S) 3-hydroxyacyl CoA by (S) 3-hydroxyacyl CoA hydratase enzyme involved in the beta-oxidation of fatty acid yield acetyl CoA molecules, which can enter in scl-PHA biosynthesis pathway. So the ratio of scl-mcl fraction in the PHA copolymer also depends on the competition between (R) specific enoyl CoA hydratase (mcl-PHA biosynthesis) and (S) 3-hydroxyacyl CoA hydratase (beta oxidation).

Response surface methodology (RSM) has been used for optimization of processing parameters. By using this method factors and interactions that affect the desired response in bacterial fermentations have been studied to optimize the desired yields (Triveni *et al,* 2001; Kshama *et al*, 2004). In the present study the experimental results indicated that RSM is an efficient method to obtain optimization data for biomass and PHA production by recombinant bacteria. A complex interaction was observed between factors and their variables, which resulted in quantitative changes in biomass and PHA. From the RSM studies it was possible to conclude that the recombinant could yield high biomass under ammonium phosphate concentration. Optimized biomass (11 g/l) and PHA (4 g/l) were obtained at 18.5 (g/l), KH_2PO_4, 5 (g/l) glucose; 7(g/l) $(NH_4)_2HPO_4$; 3 (g/l) of citric acid and 10 % inoculum.

5.4 CONCLUSIONS

Recombinant *E. coli JC7623ABC1J4* possessing PHA synthesis genes of *phaA*, *phaB* from *Bacillus sp* (involved in scl-PHA synthesis)*; phaC1* and *phaJ4* from *P. aeruginosa* (genes involved in mcl-PHA synthesis*)* was used in the cultivation experiments. The complementation of PHA genes from different hosts in *E. coli* has demonstrated that the system can be used to produce P(HB-co-HV)-co-mcl PHA copolymers. Production of mcl-PHA appeared to be dependent on the flux through the β-oxidation pathway and scl-PHA was through the intervention of actyl CoA. The flexibility in carbon source usage could allow for the production of PHA with different mol% of scl or mcl monomers. The strain was able to produce 2, 2, 1, 3, 1, 4 and 4-mol % of hydroxyvalerate when the cells were grown in butyrate, valerate, hexanoate, heptanoate, octanoate, nonanoate and decanoate, respectively, as co carbon substrates in the medium. The PHA yield reached maximum of 51 % when it grew in medium containing butyrate. Quantitative and qualitative analysis of the polymer by GC indicated that the molar percentage of scl:mcl PHA varied, depending on the fatty acid supplemented to the glucose containing medium as a co-substrate, from 91:9 to 24:76. Concentration of mcl-PHA increased under supplementation of heptanoic, octanoic and decanoic acids. ^{1}H NMR spectra of PHA indicated the synthesis of polyhydroxybutyrate (PHB) and higher alkanoate copolymers in the presence of glucose + fatty acids whereas only PHB was synthesized in the presence of glucose as sole carbon substrate. In PHA synthesis, the acetyl CoA enters in to biosynthesis pathway with the help of the enzymes coded by *phaA* and *phaB* genes. The presence of mcl-PHA biosynthesis genes such as *phaC1* and *phaJ4* along with *phaA* and *phaB* enabled the recombinant strain to follow a novel PHA biosynthesis pathway

Response surface methodology (RSM) was used as an efficient method to obtain optimization data for biomass and PHA production by recombinant bacteria. A complex interaction was observed between factors and their variables, which resulted in quantitative changes in biomass and PHA. From the RSM studies it was possible to conclude that the recombinant could yield high biomass under higher levels of

ammonium phosphate. Optimized biomass (11 g/l) and PHA (4 g/l) were obtained a combination (g/l) of 2 tryptone; 1.4 $MgSO_4$; 18.5 KH_2PO_4; 5 glucose; 7 $(NH_4)_2HPO_4$; 3 of citric acid and 10 % inoculum.

The strain was also cultivated in a fermentor in medium containing glucose as a carbon source. At the end of fermentation period maximum biomass (10 g/l) and PHA (4 g/l) were obtained. Concentration of PHA was 40 % of dry cell biomass.

SUMMARY AND CONCLUSIONS

Plastics, occupy a unique position in the world of materials as they are durable and play a key role, in the manufacture of materials pertaining to transportation, communication, entertainment, health care products, food packaging etc. They possess many attractive properties, such as lightweight, durability and flexibility, they are produced from cost effective raw materials and hence they meet a large share of the material needs of man. The highly recalcitrant nature has lead to their presence in the environment after disposal and hence is regarded as environmental hazard. The rapid increase in production and consumption of plastics has resulted in plastic waste accumulation leading to serious pollution problems. As an alternative to this, biodegradable polymers offer the best solution to the environmental hazard posed by the conventional plastics. In the recent past, there has been growing public awareness and scientific interest regarding the use and development of biodegradable polymer materials as ecologically useful alternatives to synthetic plastics. In addition to biodegradability, such a polymer material must posses the physical and chemical properties of synthetic plastics. In this context polyhydroxyalkanoate (PHA) produced by bacteria has been identified as environmental friendly biological plastic of the future.

PHAs are structurally simple macromolecules that are synthesized by various bacteria as carbon and energy reserve of the cells. These intracellular inclusions are formed under stressed growth conditions, which occur in the presence of excess of carbon source on one hand, and a limiting nutrient condition on the other. The nutrient condition may be limitation of nitrogen, potassium, iron, magnesium, manganese, phosphate, sulphate, oxygen etc. Besides its importance as a source of energy, the intracellular presence of this polymer seems to play a significant role in the survival of microorganisms under several environmental stress conditions. Several hydroxyalkanoates units are synthesized as homopolymer or heteroplymer units by bacteria. These are broadly classified as short chain length PHA (scl-PHA; 4-5 carbon

atoms) and medium chain length PHA (mcl-PHA; 6-14 carbon atoms). The physical and mechanical properties depend on the monomer composition. Copolymers posses better thermo mechanical properties compared to homopolymers. PHAs are used for medical and industrial applications. They are used for manufacture of surgical sutures, drug delivery systems, biodegradable carrier for herbicides, packaging containers, bags, films etc. PHA producing microorganisms include various genera that are taxonomically placed in different groups. This includes species of *Azotobacter, Chromobacterium, Methylobacterium, Micrococcus, Pseudomonas,* etc. Microorganisms accumulate PHA from 1-80% of their cell dry weight. *Ralstonia eutropha* is known to synthesize up to 80% PHA under specific growth conditions. Polyhydroxybutyrate (PHB), which is a commonly found homopolymer of PHA, was first identified during 1926 in *Bacillus megaterium*. The members of the genus *Bacillus* that produce PHA include *B. megateium, B. cereus, B. anthracis, B. holodurans, B. thuringiensis* etc. In the present work different *Bacillus spp* were isolated from soil samples collected from various parts of the country. The isolates were screened for PHA production by sudan black staining. PHA production studies were carried out in shake flask culture in PHA production medium. Among 38 different *Bacillus spp* tested for PHA production, *Bacillus sp* 256 produced highest amount of the polymer (55% of biomass) and hence it was selected for further studies. The selected isolate was characterized by morphological, biochemical and molecular methods. *Bacillus* 256 was gram positive, rod shaped and non motile. The bacterium produced endospores at apical position and showed positive reaction for catalase, nitrate reduction, and oxidase tests. It could not hydrolyse starch, gelatin and casein. The results of the morphological studies suggested that the isolated bacterium belonged to the genus *Bacillus.* The strain showed similarities with *B. endophyticus* because: it had ellipsoidal spore situated at terminal position, nonmotile, absence of anaerobic growth, Voges-Proskauer negative, oxidase positive, not able to hydrolyze casein, gelatin and starch; acid production from arabinose, glucose, manitol, maltose, mannose, rhamnose, xylose; etc. The strain produced pale pink pigmentation on PHA agar slants and the pigment produced was not diffusible. On nutrient agar the colonies

were slimy; the cells were resistant to ampicillin and also grew in the presence of lysozyme. Some of the morphological or biochemical characters can be confused with that of *B. licheniformis* hence the bacterium was further examined by16S rRNA to determine its relationship at the genomic level. One portion of 16SrRNA gene was amplified from the genomic DNA of *Bacillus sp* 256 by PCR. The DNA fragmnet was cloned and sequenced. The DNA sequence was found to be very conserved and it was analysed using various online softwares. The sequence was aligned with about 80 published and unpublished *Bacillus* related 16S rRNA gene sequences. Sequence of 16SrRNA gene was similar to that of *Bacillus endophyticus* (99%), *Bacillus sp*. 19490 (99%), *Bacillus sp*. GB02-16/18/20 (97%), *Bacillus sp*. MSSRF (96%) etc. Only recently, *B. endophyticus* has been isolated and characterised from the inner tissues of cotton plants. Therefore the identification of this bacterium based on morphological and biochemical studies and comparision of the data with earlier report would indicate that it is similar to *B. licheniformis* in sevral aspects. The literature data on phylogenetic tree indicates that *B. licheniformis* is closer to *B. endophyticus* in the evolutionary position. Based on these factors the isolated culture has been tentatively identified as *B. endophyticus*. Our study is the first report to show that this endophytic bacterium exists in the soil. This is similar to other endophytic bacteria that have been isolated as free living forms from the soil. Importance of *Bacillus* in food fermentation has been known since long time. The genus is industrially important for the production of extracellular amylases, proteases. The genus also includes several species that are pest control agents. In the present study it has been shown that *B. endophyticus* can exist outside as a free living form in the soil and can produce industrially important polymer such as polyhydroxyalkanoate. The amount of PHA produced is relatively high (55% of biomass) and it appeared encouraging to study various aspects of PHA production and characterize the PHA synthesis genes using this new strain.

Production of PHA has gained industrial importance for its use as biodegradable polymer applications or as biocompatible plastics. Homopolymers or heteropolymers

may be obtained depending on the co carbon substrates used in the fermentation and the capacity of the microorganisms to metabolize the substrate for polymer production. *Bacillus spp* produce PHA due to limitation of potassium and phosphorous in the medium. In order to increase the PHA yield it is essential to optimize the nutrient and growth conditions. Different carbon and nitrogen sources were tested for PHA production by *Bacillus sp* 256. Among various N_2 sources studied, $(NH_4)_2$ H PO_4 was found to be a better nitrogen source for PHA production (biomass 1.6 g/l and PHA 69% of biomass) Amongst the carbon sources, sucrose gave higher biomass (1.6 g/l) and PHA yield (55% of biomass). The bacterium was also cultivated in media containing molasses, cornstarch, and corn steep liquor. Maximum production of PHA (55%) was obtained in the medium containing hydrolysed cornstarch, while biomass was highest in molasses medium (2.6 g/l). Amongst the plant oils used as co carbon substrates, non-edible plant oil such as pongemia oil resulted in optimal yield of PHA in the biomass (65%). Fermentor cultivation in medium containing a) sucrose as the sole carbon source, b) sucrose as main carbon source and pongemia oil as co-carbon substrate, c) sucrose as main carbon source and saponified pongemia oil as co carbon substrate resulted in high biomass (4g/l) and accumulation of PHA (2 g/l) in pongemia oil containing medium. The results also showed that the medium containing saponified pongemia oil was a better medium for growth (4 g/l) and accumulation of PHA (2.5 g/l) by *Bacillus sp.* When only sucrose was used as carbon substrate nearly 60% degradation occurred due to prolonged fermentation (72 h) compared to 20% degradation in pongemia oil containing growth medium. This shows that supplementation of pongemia oil also prevented degradation of PHA once maximal production was achieved, which indicates that such fermentation conditions can be used for this strain for commercial process. The formation and breakdown of PHA in the cells appear to be important in defining nutritional status of the microbial cells. In *Bacillus spp.* it serves as an endogenous source of carbon and energy for cell activities and spore formation. The co substrate absorption may lead to availability of energy in the form of fatty acids to the cells which may lead to delayed degradation of PHA polymer. Hydroxyacyl coenzyme A thioesters required for quantitative and qualitative productivity

of PHA are supplied by fatty acid biosynthesis and degradation pathway. The extra metabolic flux of fatty acid degradation appears to have channelised towards enhanced copolymer production in oil fed cells, which was absent in sucrose fed cells. The overall results indicate that plant oils may be better suited for the stabilized production of PHA copolymer by *Bacillus spp*.

FTIR, GC and NMR were used to characterize the PHA obtained from the cells. FTIR is one of the powerful and rapid tools to obtain information on polymer structure. The spectrum indicated a transmittance band at 1752cm^{-1}, which is attributed to the stretching vibration of the C=O group (ester carbonyl) in the PHA polyester. Accompanying bands of the C-O-C groups appeared in the spectral region from 1150 cm^{-1} to 1300cm^{-1} . Transmittance regions from 2800 to 3100 cm^{-1} correspond to the stretching vibration of C-H bonds of the methyl (CH_3), and methylene (CH_2) groups. Other characteristic bands present for scl-PHA were 2977, 2934, 1282 (CH_3 bend), 1100, 1058 (C-O) 979 and 515.

Gas chromatography is a very efficient method for quantitative estimation as well as characterization of PHA. The results indicated that PHA extracted from media containing rice bran oil, pongemia oil and oleic acid as co substrates was a copolymer of P(HB-co-HV). The butyrate methyl ester eluted at 9.5 min and valerate methyl ester at 12.81 min. GC also showed that the copolymer (hydroxyvalerate) was about 4 and 6-mol% of the total polymer content. Molar concentration of P(HB-co-HV) was maximum in the presence of saponified pongemia oil (80:20 mol%) compared to unsaponified oil (94:6). Homopolymer of PHB was obtained from the cells fed with only sucrose.

Nuclear Magnetic Spectroscopy (NMR) is a powerful technique used for elucidating the chemical structure of the compounds. The ^1H NMR spectrum of PHA showed three characteristic groups signals of PHB: a doublet at 1.29 ppm which is characteristic of methyl group, a doublet of a quadruplet at 2.5 ppm which is attributed to

methylene group and a multiplet at 5.28 ppm characteristic of a methylene group. A triplet at 0.9 ppm and a methylene resonance at 1.59 and methyne resonance at 5.5 indicated the presence of valerate in the polymer**.**

Several pathways are involved in the synthesis of PHA. Biosynthesis of scl-PHA such as PHB is initiated by the condensation of two acetyl-CoA molecules to acetoacetyl CoA that is catalysed by the enzyme β–ketothiolase. Acetoacetyl CoA is reduced to hydroxybutyryl CoA by the NADPH dependant acetoacetyl CoA reductase. This is polymerized to PHB in the presence of PHA synthase. This path way has been studied in detail and is known to exist in bacteria such as *Ralstonia eutropha, Bacillus spp* etc. The mcl-PHA biosynthesis pathway is closely related to the β-oxidation pathway where fatty acids serve as carbon substrates. Hydroxyaceyl CoA intermediates of β-oxidation pathway function as precursors for the mcl-PHA biosynthesis. Fatty acids are activated by the acyl CoA synthetase leading to the formation of corresponding acyl-CoA thioesters. These are degraded to acetyl CoA through β-oxidation pathway. Acyl CoA is reduced to trans-2, 3- enoyl CoA that is catalysed by acyl CoA dehydrogenase. Further conversion is catalysed by multi enzyme complex. Enzymes such as (R)-specific enoyl CoA hydratase, hydroxyacyl CoA epimerase, and β-ketoacyl CoA reductase are connected with the β-oxidation pathway for the synthesis of mcl-PHA. Mcl-PHA synthesis is prevalently found in *Pseudomonas spp.*

Several genes that encode enzymes involved in PHA synthesis and degradation have been cloned and characterized from several microorganisms. These studies have shown that several pathways are involved in the synthesis of PHA each of which is optimized for the environment where these microorganisms are found. In addition to diversity in the metabolic pathway for PHA synthesis, there is a divergence in the *pha* gene loci of various bacteria. These *pha* genes are found as clusters in an operon in some bacteria and in others they occur as separate transcriptional units. Even though PHB was

first isolated from *B. megaterium,* biosynthetic mechanism involved in PHA synthesis has not been characterized in *Bacillus spp* in detail.

The pha operon of *Bacillus* consist of five genes such as *phaP*, *phaQ*, *phaR phaB* and *phaC*, where the *phaRBC* genes are in one orientation under the control of a single promoter; *phaP* and *phaQ* are in separate orientation. In *Bacillus* the gene coding for β-ketothiolase (*phaA*) is not associated with *pha* operon. In the present study PHA biosynthesis genes were cloned and sequenced. PCR cloning strategy was used to isolate the genes from *Bacillus sp*. Different primers were designed to amplify PHA biosynthesis genes such as *phaA*, *phaB* and *phaC*. All the genes characterized were of complete ORFs of expected size with a start site and a termination codon. The gene sequences were used to study the gene polymorphism and sequence homology.

The *phaB* gene *Bacillus sp* 256 was 744 bp long. The sequence showed similarity with acetoacetyl CoA reductases of various *Bacillus spp*. Multiple sequence alignment with deduced amino acid sequence of *phaB* gene showed that acetoacetyl CoA reductase of *Bacillus sp* 256 is also similar to that of many other PHA producing organisms in various aspects. This protein is almost similar in most of the PHA producing organisms with an average molecular weight of 27 k Da. The amino acid sequence was conserved towards the C terminal region of the polypeptide chain. *PhaC* gene from *Bacillus sp* was cloned and sequenced completely. The sequence was a complete ORF consisting of 361 amino acids. The PHA synthase of *Bacillus* is reported as a unique one, which requires a *phaR* protein sub-unit for its activity. The gene coding for β-ketothiolase was also cloned from *Bacillus sp* 256 and characterized. The gene was 1173 bp long complete ORF and was similar to that of *B. cereus*. The *Bacillus* ketothiolase was found to be distinct; showed lesser homology with ketothiolases from other PHA producing organisms. Genomic BLAST with *bktB* protein sequence from *R. eutropha* suggested that the ketothiolases present in *Bacillus* genome has an ascending order of similarity with *bktB* protein.

The PHA biosynthesis genes from *Bacillus* and *Pseudomonas* were selected for heterologous expression in *B. subtilis* and *E. coli* for PHA production. Plasmid bearing *phaC1* and *phaJ4* genes from *Pseudomonas aeruginosa* (*pCPC1J4*) were transferred in to *B. subtilis* strain by electroporation. The recombinant *B. subtilis* was selected on the basis of kanamycin resistance. The strain was subjected to PHA production in the medium containing nonanoic acid. The recombinant produced only 5% of PHA copolymer.

PHA biosynthesis genes from *Bacillus sp* and *Pseudomonas sp* were cloned for scl-co-mcl PHA production in *E. coli*. *PhaA* and *phaB* genes from *Bacillus sp* 256 were cloned into *E. coli* integration vector *pBRINT-Cm* (*pBRAB* construct). The *E. coli* strain JC7623 was transformed with *pBRBA* vector and the white integrant colonies (*JC7623AB*) were selected on chloramphenicol resistance. The *JC7623AB* strain was subjected to another transformation with *pBSC1J4* plasmid containing *phaC1* and *phaJ4* genes from *Pseudomonas aeruginosa* (Collected from laboratory from Reeta Davis and Chandrashekar A). The expression of all four genes was induced by isopropyl β-D-thiogalactopyranoside. The expression of the cloned genes was monitored on SDS PAGE.

Three recombinant strains such as *E. coli fadBC1J4* (*fadB* mutant LS1298 bearing *phaC1* and *phaJ4* genes from *P. aeruginosa*), *E. coli JC7623C1J4* (*E. coli* strain JC7623 containing *phaC1* and *phaJ4* genes) and *E. coli JC7623 ABC1J4* (*E. coli* strain JC7623 bearing *phaA* and *phaB* gene from *Bacillus sp* 256; *phaC1* and *phaJ4* genes from *P. aeruginosa*) were subjected for PHA production. The strain *JC7623ABC1J4* grew well in PHA production medium and produced highest amount of PHA and hence it was selected for further studies.

Recombinant *E. coli* JC7623*ABC1J4* possessing PHA synthesis genes of *phaA*, *phaB* from *Bacillus sp* (involved in scl-PHA synthesis)*; phaC1* and *phaJ4* from *P. aeruginosa* (genes involved in mcl-PHA synthesis*)* was used in the cultivation experiments. The complementation of PHA genes from different hosts in *E. coli* has demonstrated that the system can be used to produce P(HB-co-HV)-co-mcl PHA

copolymers. Production of mcl-PHA appeared to be dependent on the flux through the β-oxidation pathway and scl-PHA was through the intervention of actyl CoA. The flexibility in carbon source usage could allow for the production of PHA with different mol% of scl or mcl monomers. The strain was able to produce 2, 2, 1, 3, 1, 4 and 4-mol % of hydroxyvalerate when the cells were grown in butyrate, valerate, hexanoate, heptanoate, octanoate, nonanoate and decanoate, respectively as co carbon substrates in the medium. The PHA yield reached maximum of 51 % when it grew in medium containing butyrate. Analysis of the genome of *Bacillus* indicated the presence of four β–ketothiolase genes. Sequences of these were checked for their similarities with the β-ketothiolase (bktB) of *Ralstonia eutropha.* The similarity of the said genes with bktB in a descending order was 48%, 44%, 43%, 35% and these were designated as *PhaA1-PhaA4. PhaA2* from *Bacillus sp* 256, which encodes β–ketothiolase was cloned and sequenced completely. The sequence showed the presence of a complete open reading frame (ORF) of 1173bp in size. The deduced amino acid sequence showed maximum similarity with that of *PhaA2* from other *Bacillus spp* such as B. *cereus*, *B. anthrax* and *B. halodurans.* The sequence of *PhaA3* and *PhA4* from these species differed from that of the *Bacillus sp* 256 sequence. Our data indicate that the PHV production in the recombinant *E.coli* may be possible through the breakdown of fatty acids as evidenced by the increase in the proportion of PHV when the recombinant cells were grown in the presence of higher fatty acids such as heptanoic acid and nonanoic acid. The absence of PHV synthesis in the medium containing only glucose and citric acid suggested the same conclusion.

Quantitative and qualitative analysis of the polymer by GC indicated that the molar percentage of scl:mcl PHA varied, depending on the fatty acid supplemented to the glucose containing medium as a co-substrate, from 91:9 to 24:76. Concentration of mcl-PHA increased under supplementation of heptanoic, octanoic and decanoic acids. ^{1}H NMR spectra of PHA indicated the synthesis of polyhydroxybutyrate (PHB) and higher alkanoate copolymers in the presence of glucose and fatty acids whereas only PHB was synthesized in the presence of glucose as sole carbon substrate. In PHA synthesis, the

acetyl CoA enters in to biosynthesis pathway with the help of the enzymes coded by *phaA* and *phaB* genes. The presence of mcl-PHA biosynthesis genes such as *phaC1* and *phaJ4* along with *phaA* and *phaB* enabled the recombinant strain to follow a novel PHA biosynthesis pathway.

Response surface methodology (RSM) was used as an efficient method to obtain optimized biomass and PHA production by recombinant bacteria. A complex interaction was observed between factors and their variables, which resulted in quantitative changes in biomass and PHA. From the RSM studies it was possible to conclude that the recombinant could yield high biomass under higher levels of ammonium phosphate. Optimized biomass (11 g/l) and PHA (4 g/l) were obtained in medium containing (g/l): tryptone 2; $MgSO_4$ 1.4; KH_2PO_4; 18.5; glucose5; $(NH_4)_2HPO_4$;7; citric acid 3 and 10 % inoculum.

The recombinant strain was also cultivated in a fermentor in medium containing glucose as a carbon source. At the end of fermentation period maximum biomass (10 g/l) and PHA (4 g/l) were obtained. Concentration of PHA was 40 % of dry cell biomass.

Future studies

Bacillus sp 256 is an excellent organism for PHA production. It is able to synthesize PHA from versatile carbon sources such as sugars, fatty acids, oils, molasses and starch hydrolysates. Compared to other *Bacillus sp* that are reported in the literature, *Bacillus sp* 256 produced higher amount of PHA which was composed of P(HB-co-HV) copolymer. This study has indicated that multiple ketothiolases are present in the Bacillus genome, one of which was cloned and characterized in the present assignment. These enzymes can be studied in detail to show the reason for requirement of multiple copies of ketothiolases in the bacterium. In the present study recombinant *E. coli* strain produced moderate amount of PHA (40%), the following points may be considered to improve the strain for enhanced PHA production:- a) Intracellular NADPH level is considered as one of the limiting factors of scl PHA production and intracellular NADPH concentration can be

further increased by addition of a gene which reduces NADP molecule such as gdh gene coding for glucose 6-phosphate dehydrogenase in pentose phosphate pathway. b) The recombinant strain was observed to form filaments during fermentation; inhibition of filamentation would lead to enhancement of polymer synthesis. c) *E. coli* JC7623 strain can be subjected to mutation in fatty acid metabolism (*fad* mutation) to diverge the metabolic intermediates in to PHA biosynthesis. d) Enhancement of the ability of the strain to grow on economic substrate such as glycerol, whey etc. e) Extraction of the intracellular PHA can be simplified by cloning an inducible lytic gene in to the recombinant strain.

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