Polyhydroxyalkanoate from *Bacillus* sp.: its production, isolation and characterization

A thesis

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by

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Dedicated to.....

JANANI
Who brought me into this world...

JANMABHUMI
Motherland

JAGANMATHE
Mother of universe

Special acknowledgement to…

Shamala madam

Wind beneath the wing……

Dr. T. R. Shamala
Scientist F-Gr IV (5),
Department of Food Microbiology

CERTIFICATE

It is certified that the thesis entitled **"Polyhydroxyalkanoate from *Bacillus* sp.: its production, isolation and characterization"** which is submitted to the University of Mysore, Mysore, for the award of **Doctor of Philosophy (Ph. D.) degree in MICROBIOLOGY** is based on research work carried out by **Mrs. M. S. Divyashree,** under my guidance during the period from April 2005 to March 2008 at the Department of Food Microbiology, Central Food Technological Research Institute, Mysore, India. The candidate was supported by senior research fellowship from Council of Scientific and Industrial Research, New Delhi, India, during the above-mentioned period.

Place: Mysore
Date: 5-12-2008

T. R. Shamala
(Guide)

DECLARATION

I, Mrs. M. S. Divyashree, declare that the data presented in the thesis entitled **"Polyhydroxyalkanoate from *Bacillus* sp.: its production, isolation and characterization"** which is submitted to the University of Mysore, Mysore, for the award of **Doctor of Philosophy (Ph. D.) degree in MICROBIOLOGY** is based on the research work carried out by me, under the guidance of **Dr. Mrs. T. R. Shamala**, Scientist-F (Gr-IV-5), Department of Food microbiology, Central Food Technological Research Institute, Mysore, India, as Senior Research Fellow of Council of Scientific and Industrial Research, New Delhi, India, during the period April 2005 to March 2008.

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:

M. S. Divyashree

Research Fellow

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Thank you one and all……

Divyashree M. S.

INDEX

List of abbreviations

ATPS	Aqueous Two-phase System
BLAST	Basic Length Alignment Sequence Test
DSC	Differential Scanning Calorimetry
K	Kinetic constant
Mcl-PHA	Medium Chain length Polyhydroxyalkanoate
NAD	Nicotinamide Adenosine Dinucleotide oxidized
NADH	Nicotinamide Adenosine Dinucleotide reduced
P(HB-co-HO-co-HD)	Poly(hydroxybutyrate-co-hydroxyoctanoate-co-hydroxydecanoate)
P(HB-co-HV)	Poly(hydroxybutyrate-co-hydroxyvalerate)
PE	Polyethylene
PEG	Poly Ethylene Glycol
PHA	Polyhydroxyalkanoate
P(HB)	Polyhydroxybutyrate
P(HV)	Polyhydroxyvalerate
PP	Polypropylene
PVA	Poly Vinyl Alcohol
PVAC	Poly Vinyl Acetate
Scl-PHA	Short Chain length polyhydroxyalkanoate
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
TCA	tri carboxylic acid
X_m	Maximum biomass concentration
X_P	Product (PHA-Polyhydroxyalkanoate)
X_R	Residual biomass
Y_{PS}	Yield of product based on substrate
Y_{RN}	Yield of biomass on nitrogen concentration
μ_m	Growth rate of the organism

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Abstract

Synthetic plastics are widely used for the manufacture of packaging materials, various household and industrially important articles. However they are recalcitrant to microbial degradation and they persist in the soil leading to environment pollution. To overcome the problem of plastic pollution, attempts are being made to replace synthetic plastics by various biopolymers and bacterial polyhydroxyalkanoate (PHA) is one amongst them. PHA is composed of hydroxy fatty acids and it represents a rather complex class of storage polymers synthesized by a various bacteria and are deposited as unique water insoluble cytoplasmic nano sized inclusions. *Bacillus* spp. represent industrially important group of organisms that are used for various metabolite production and hence attempt was made to isolate this bacterium for polymer synthesis. Based on 16SrRNA, isolated *Bacillus* sp. was identified as *B. flexus* and it was found to posses 97% homology with *B. megaterium* it shared the same cluster with *B. simplex*. This study has led to a finding of a new unexplored *B. flexus* strain, which has the ability to produce PHA at 50-60% level in the biomass. *B. flexus* grew optimally in media containing different salts of nitrogen, amino acids, carbon sources, plant oils, free fatty acids, economic media components such as palm oil effluent, molasses, corn starch, whey, rice and wheat bran extracts as sources of carbon/nitrogen/nutrients. PHA isolated from the cells was characterized by FTIR/GC/GC-MS/NMR spectroscopy/DSC. Homopolymer of polyhydroxybutyrate was synthesized in *B. flexus* cells fed with only sucrose as main carbon source and PHA copolymer of polyhydroxy(butyrate-co-valerate-co-octanoate) was produced in palm oil effluent containing medium and polyhydroxy(butyrate-co-octanoate-co-decanoate) with rice bran oil as co carbon substrate. This study has shown that based on the fatty acid supplemented in the medium, medium chain length PHA such as octanoate and decanoate can be synthesized by *Bacillus* spp also. Response surface methodology was used for optimization of nutritional parameters for optimum growth and polymer yield. Maximum production of biomass (7 g/l) and PHA (2.2 g/l) were obtained with 11.6 g/l of KH_2PO_4, 4.7 g/l of ammonium phosphate and 31 g/l of sucrose. The growth kinetics of *B. flexus* under nutrient limitation was studied by using a simple model

giving mathematical description of kinetics of microbial growth, substrate consumption and product formation.

PHAs are accumulated intracellularly and hence their extraction from the biomass is a critical step for economic production. Numerous separation processes are employed for the recovery of PHA. These involve extraction by organic solvents, which is hazardous and explosive. In the present study, novel physical, chemical and biological methods were assessed for isolation of the intracellular PHA. Analysis of cell wall of *B. flexus* indicated that the type of nutrients used for cultivation significantly influences its composition. Cells grown in inorganic medium contained lower quantities of amino acids and lacked diaminopimilic acid in the cell wall and cells lysed easily and this can be further exploited for easier recovery of the intracellular PHA. Amongst different alkalis tested for cell hydrolysis, NH_4OH efficiently digested non-PHA cellular material at pH 11, to give 50% of PHA with 98% purity. The enzyme produced by *Microbispora* sp. hydrolysed the cells of *B. flexus* to release the intracellular PHA. The enzyme was identified as a protease of 40kDa and it was purified to homogeneity. Aqueous two-phase system was successfully employed as a non-organic solvent method for the isolation of PHA from other cellular materials. Exposure of cells to gamma irradiation indicated that the irradiation resulted in cell lysis leading to easy PHA extractability, low degree of cross-linking, and improvement in molecular weight as well as tensile strength of the polymer. Lambda lytic gene was integrated into the amylase gene (*amyE*) locus of the chromosomal DNA of *B. flexus*. This caused autolysis of the recombinant cells when xylose was supplemented into the medium. The study has focused on economic production of PHA by a newly isolated strain of bacterium, which is so for not reported as PHA producer, and newer aspects of PHA isolation methods have been worked out.

Introduction and Review of literature

Introduction

Plastics are utilized in almost every industry ranging from automobile to food and medicine. Plastics have acquired significance since 1940s and have replaced glass, wood and metal in packaging applications. These materials have molecular weight ranging from 50-100 kDa and can be chemically modified to various strength and shapes (Madison and Huisman, 1999). They are resistant to environmental stress and hence they are widely used as packaging materials. The synthetic polymers can be easily molded into almost any desired shape including fibers and thin films. They have high chemical resistance and are used widely in the manufacture of several durable goods.

Plastics being xenobiotic are recalcitrant to microbial degradation (Flechter, 1993) and they persist in the soil for a long time leading to pollution problems (Atlas, 1993). According to an estimate, plastics are produced every year which amounts to 80kg million tones in USA, 60kg million tones in European countries and 20kg million tones in India (Kalia *et al.,* 2000). Plastic materials used for packaging purposes are discarded into landfills and into marine environments. The accumulated plastic in landfills cannot be disposed off by burning because it releases harmful chemicals like HCl and HCN during incineration. Recycling also presents some major disadvantages in sorting out variety of plastics depending on further application. Because of their persistence in our environment they adversely effect the environment including wildlife and imparts unaesthetic look to cities, beaches and forests. The rapid increase in production and consumption of plastics has led to the serious plastic waste problems, so called 'White Pollution'. Ecofriendly bioplastics, which are durable, environmentally degradable, recyclable and produced from renewable resources (Luzier, 1992), can replace synthetic plastics, to overcome the problem of pollution caused by non-degradable plastics.

Biopolymers

Polymers are the most abundant molecules in living matter. The term "Biopolymer" is used to refer polymers formed in nature during the growth cycles of all the organisms. Biopolymers that occur in nature are: Polynucleotides, polyamides, polysaccharides, polyisoprenes, lignin, polyphosphates and polyester such as

polyhydroxyalkanoate (PHA). They are susceptible to enzymatic degradation because the enzymatic polymerization is responsible for their synthesis (Lenz, 1993).

Polyesters

Polyesters are a group of polymers formed by the polycondensation of carboxylic acid with hydroxy alcohols.

Natural polyesters

Natural polyesters are produced by a wide variety of microorganisms, plants and insects, for protection or as a reserve food material, which play vital role in living organism. Most of them are synthesized by plants as structural components of the cuticle covering the aerial parts of plants, such as cutin and suberin, or by microorganisms as intracellular reserve compounds.

Cutin

Cutin is polyester composed of hydroxy and epoxy fatty acids, which acts as a barrier between aerial parts of higher plants and their environment. It constitutes the structural component of the cuticles of higher plants.

Suberin

Suberin is found in the epidermis and hypodermis of the roots of the plants. It is a polymer containing aromatics and polyester functions, which consists of phenolic and aliphatic domains, is associated with a complex mixture of lipids, collectively known as "wax".

Poly(malic acid)

Poly(malic acid) is made up of malic acid repeating units, which are linked through ester bonds between the hydroxy and the carboxy groups. It is produced by variety of myxomycetous fungi.

Polyhydroxyalkanoates (PHA)

In bacteria PHA is synthesized as intracellular reserve materials. PHAs have gained more importance due to their close analogy to plastic from different sources namely microorganisms, recombinant bacteria and transgenic plants and other methods are being investigated to exert control over the quality, quantity and economics of PHA production. Biodegradability makes them attractive substitute for non-degradable synthetic plastic (Reddy *et al.,* 2003).

PHA can accumulate in many Gram-positive and Gram-negative bacterial cells as discrete granules (Kshama, 2005). It is not restricted to any particular physiological group. PHAs have been detected in photosynthetic and aerobic groups, lithotrophes, organotrophes and cyanobacteria. Under optimum conditions, PHA may be accumulated to 80% of the cell dry weight. Since prokaryotes do not store fat in the form of triglycerides, PHA appears to be an alternative to the storage of lipid (Doi and Steinbüchel, 2002).

Over 150 different PHAs have been isolated from various bacteria in which the monomer is 3-hydroxy alkanoate. Different types of PHAs are synthesized by feeding fatty acids or other aliphatic carbon precursor substrates. This has given rise to an unexpected diversity in the number of PHAs that can be synthesized.

Considerable attention is directed towards commercial application of PHA because it can be produced from renewable resources. Application includes packaging manufacture of bottles and medical applications. Detailed account on PHA is dealt with in later chapters.

Synthetic biodegradable polyesters

There are a few synthetic polyesters that are truly biodegradable. These have an aliphatic ester linkage in the main chain. Backbones of these polymers are found to be susceptible for hydrolysis. It was found that fungi more readily degrade polyesters derived from diacids of medium size monomer.

Polyglycolic acid (PGA)

PGA is a linear, aliphatic polyester and its copolymer poly(glycolic acid-co-lactic acid) (PGA/PL) is used as absorbable surgical sutures. They hydrolyze easily in aqueous environments such as body fluids. After hydrolysis the ultimate product is metabolized to carbon dioxide and water or are excreted via the kidney.

Polycaprolactone (PCL)

Polycaprolactone is available commercially and it is synthesized from the ring-opening polymerization of caprolactone. It is widely used in drug delivery systems. Its degradation *in vivo* is much slower than that of polyhydroxy acids.

Polyamides

Polyamides contain amide linkage that is found in polypeptides. They are known to degrade slowly and the degradation by enzymes and microorganisms for low molecular weight oligomers has been reported. Benzyl, hydroxy and methyl substituents can enhance biodegradation.

Polyvinyl alcohol (PVA)

Polyvinyl alcohol (PVA) is one of the readily biodegradable of vinyl polymers in wastewater or activated sludge. The microbial degradation involves the initial enzymatic oxidation of the secondary alcohol groups in PVA to ketone groups and then hydrolysis of the ketone groups occurs, which involves bacteria such as *Flavobacterium* sp. and *Acinetiobacter* sp. (Chandra and Rustgi, 1998).

Polyvinyl acetate (PVAC)

Polyvinyl acetate undergoes biodegradation more slowly. Copolymers of ethylene and vinyl acetate were susceptible to slow degradation in soil.

Environmental problems that can be alleviated by biodegradable plastics

It is hoped that biodegradable plastics would divert part of bulky plastic wastes from land filling, which is increasing pressure on landfills. It will also facilitate organic waste management by eliminating the cost involved in removing the collection bags before entering compost facilities. Biodegradable plastics of renewable sources will contribute to a more sustainable society by conserving the non-renewable resources namely the petroleum products.

Biodegradable polymers are a newly emerging field. A vast number of biodegradable polymers have been synthesized recently and some microorganisms that are capable of degrading them have been identified. Environmental pollution by synthetic polymers has assumed dangerous problem. As a result, attempts have been made to minimize these problems by utilization of biodegradable polymers.

The three types of biodegradable plastics introduced are:

1. Photodegradable
2. Semi-biodegradable
3. Completely biodegradable.

Photodegradable plastics have light sensitive groups incorporated directly into the backbone of the polymer as additives. Extensive ultra-violet radiation can disintegrate their polymeric structure that will encourage bacterial growth and biodegradation. Semi-biodegradable plastics are the starch-linked plastics where starch is incorporated to hold together short fragments of polyethylene. Bacteria present in the soil will act on the starch and releases the other polymer which remain as non degradable. The third type is new and promising because it is completely biodegradable by bacteria. They are polyhydroxyalkanoate (PHA), polysaccharides and some of the synthetic biodegradable polyesters with aliphatic ester linkages in the main chain such as polycaprolactone (PCL), polylactide (PLA), poly vinyl alcohol (PVA), etc., copolymers or blends of these polymers (Reddy *et al.,* 2003).

New challenges on waste management

As defined by the United Nations Environmental Program (UNEP), proper waste management is the practical step, which ensures that the wastes are managed in such a manner, which will protect human health and the environment against the adverse effects. The impact of biodegradable plastics on prevailing waste management technologies and practice that can really reduce the pressure on landfills and facilitate organic recycling is the new challenge ahead.

Biodegradable plastics are mainly driven by the situation under which plastic products are non-recyclable. Composting is the most relevant waste treatment technology for biodegradable plastics. It has been identified that the reduction of contaminant level is crucial for the success of composting.

Biodegradable plastics can benefit waste management only if collected and treated separately. In addition, it is vital to make sure that there is no mixture with conventional plastic waste destined for recycling. To fulfill these requirements, it is essential that biodegradable materials are labeled and identified in a sensible and easily recognizable way.

Application of environmentally benign technology such as biodegradable plastics in many developing countries are mostly driven by the government's command or ban on other hazardous material or a semi-compulsory recommendation of certain technology (Ren, 2003).

Wide acceptance of biodegradable plastics will bring higher requirements on integrated waste management, ranging from clear labeling. Technology development in the field will make the biodegradable plastics of renewable origin more comparable with conventional plastics in energy consumption and cost, leading to successful commercialization of biodegradable plastics.

Public concern over the harmful effects of olefin-derived plastics has stimulated inter disciplinary research by microbiologists, chemists and molecular biologists on the biosynthesis, degradation and physical properties of these natural biodegradable thermoplastics produced by bacteria (Page, 1989). Industrial application of bioplastics has been hindered by its low thermal stability and brittleness on storage.

Review of literature

Polyhydroxyalkanoates (PHAs) are the polymers of hydroxyalkanoates that accumulate as storage material in many microorganisms such as *Bacillus* spp., *Rhizobium* spp, *Alcaligenes* spp., *Pseudomonas* spp., *Azotobactor* spp. etc. They are attaining considerable attention as biodegradable polymers and are emerging as a new branch of research field (Salechizadch and Loosdrecht, 2004).

A wide variety of microorganisms spread over, more than 90 genera of bacteria are known to produce PHA (Steinbuchel, 1991; Findlay and White, 1983). Lemoigne first discovered PHA in 1926 in *Bacillus megaterium* as hydrophobic inclusions in the cytoplasm (Eversloh *et al* 2001; Anderson and Dawes, 1990). These inclusion bodies are of high industrial significance, which are optically active, easily biodegradable with a melting point 180 ^{0}C and have properties similar to polyethylene (PE) and polypropylene (PP) (Lee, 1996; Sasikala and Ramana, 1996). However their high price compared to thermoplastic has limited their use in many areas. To reduce the cost and make PHAs competitive with the conventional plastic, continued efforts are being made to improve the strain, growth conditions, enhancement of PHA production, fermentation parameters, separation, recovery and also in the genetic engineering aspects (Lee, 1996).

The cost of PHA depends on many factors such as substrate used, productivity and the recovery process. PHA content of the biomass also affects the cost, as increase in PHA content from 50% to 88% leads to lowering of recovery cost (Lee and Choi, 1998). High cost of PHA is due to the use of costlier substrate for fermentation and large amounts of digesting agents that are used to lyse the cell wall to release the polymer. The price can be lowered from \$16 kg^{-1} to \$ 8 kg^{-1}, by scale up process and by using cheaper substrates.

Occurrence of PHA in microorganisms

Bacteria are capable of accumulating 25-80% PHA of their cellular dry weight; *Cupriavidus necator* (formerly called as *Alcaligenes eutrophus/Ralstonia eutropha)* is one of the high PHA yielding bacterium, PHA synthesizing genes of which are completely sequenced and cloned. Various groups of microorganisms such as phototrophs, chemotrophs and a few fungi are also known to produce PHA. But

Enterobacteria are among the organisms, which do not form PHA, but PHA producing gene from *Alcaligens eutrophus* has been cloned and expressed in *E. coli* (Slater *et al.,* 1988, Schubert *et al.,* 1998). Many phototrophic organisms like *Cyanobacteria* and chemotrophs are known to accumulate PHA in considerable amount. Free living and symbiotic nitrogen fixing bacteria namely *Azotobacter* spp., *Rhizobium* spp. also accumulate high concentration of PHA (65-70%) (Kshama and Shamala, 2003). PHA accumulations by bacteria such as *Halophilic* and *Archaebacteria* have also been reported.

Structure and classification of PHA

PHAs are formed as intracellular inclusions under unbalanced growth conditions, which is described as poly (β-hydroxyalkanoate) containing different alkyl groups. Several poly(β-hydroxyalkanoate) and copolymers can be produced under controlled growth conditions, which has wide applications in medical and packaging areas (Brandl *et al.,* 1990).

As many as 150 different hydroxyalkanoic acids have been identified and grouped as follows:

1. 3HA from 3-hydroxy propionic acid (3HP) to 3-hydroxy hexanoic acid (3HHx).
2. Unsaturated 3-hydroxyalkanoic acids with one double bond (3-hydroxy-4-pentanoic acid, 3-hydroxy-7-cis tetra decanoic acid) or two double bonds (3-hydroxy 5,8-cis-cis-tetra decanoic acid) in the R pendent group (R-is, side chain of HA).
3. 3HA with methyl group at various positions of the R-pendent group.
4. Non-3HA such as 4-hydroxybutyric acid (4HB) or (4-hydroxy valeric acid).
5. 3HA with different functional groups including free carboxyl groups such as malic acid, esterified alkyl groups (3-hydroxy succinic acid methyl ester acetoxy groups and other functional groups.
6. Hydroxyalkanoic acid with modification at third carbon atom, which contribute to the backbone of the polyester are 3-hydroxyalkanoic acids with a double bond such as 3-hydroxy-2-butenoic acid and various 3HA with methyl group (Steinbuchel and Valentine, 1995).

General structure of PHA

PHAs are polymers with (R)-3-hydroxy monomer unit. The monomers of short chain length PHA (scl-PHA) consist of 3-5 carbon atoms and monomers of medium chain length (mcl-PHA) consist of 6-14 carbon atoms. The molecular weight of these polymers ranges between 2 x 10^5 and 3 x 10^6 and number of monomers (n) varies from 1000 to 30,000. Structure of PHA is shown in Figs. 1 and 2.

D (-)-3-hydroxybutyric acid (3 HB) remained only known constituent of PHA until constituents other than 3HB were reported, based on chemical analysis 3 HV and 3 HHx were detected later as constituents of PHA produced by *Bacillus* species (Findlay and White, 1983). 3–hydroxy octanoic acid (3HO) was detected as constituent of PHA in *Pseudomonas oleovorans* (De Smet *et al.,* 1983). It is possible that new constituents will continue to be detected by using different precursor substrate or bacteria.

Terminology of PHA

With the discovery of new hydroxyalkanoic acids, terminologies used by different researchers are as follows:

1. Poly(hydroxy alkanoates) [P (HA)] General.
2. Poly(hydroxybutyrate-co-hydroxyvalerate) [P(HB-co-HV)] is for copolymer.
3. Poly(hydroxybutyrate-co-hydroxypropionate-co-hydroxy valerate), [P(HB-co-HP-co-HV)] for Terpolyesters.
4. Short chain length PHA (scl-PHA) 3-5 carbons.
5. Midium chain length PHA (mcl-PHA) 6-16 carbons.
6. Long chain length PHA (lcl-PHA) > 16 carbons (Sasikala and Ramana, 1996)

Non-HA with unsaturated or substituted side chains have not yet been detected and also lcl-PHA is yet to be detected (Steinbüchel and Valentine, 1995).

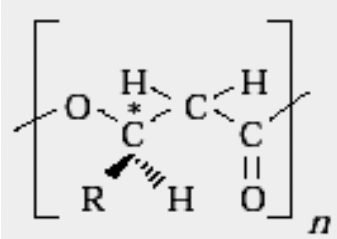


Figure 1: General structure of PHA.

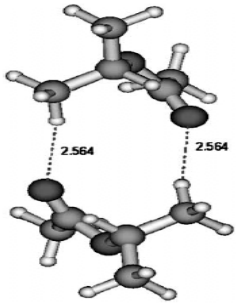


Figure 2: Model of the PHB dimer ($CH_3CH(CH_3)$-$OCOCH_2CH_3$). Dotted lines indicate C-H····O=C.
Source: Sato *et al.*, (2005)

Where R=

Hydrogen 3 – hydroxy propionate

Methyl 3 – hydroxy butyrate

Ethyl 3 – hydroxy valerate

Propyl 3 – hydroxy caproate

Butyl 3 – hydroxy heptonoate

Pentyl 3 – hydroxy octanoate

Hexyl 3 – hydroxy nonanoate

Heptyl 3 – hydroxy decanoate

Octyl 3 – hydroxy undecanoate

Nonyl 3 – hydroxy dodecanoate

Where N=

3 = 4- hydroxybutyrate(4 HB)

4 = 5- hydroxyvalerate (5HV)

Homopolymer

P(HB), containing repeating units of (R)-3HB is the most common PHA produced by many bacteria. *Ralstonia eutropha* is known to produce 80% P(HB) of the dry cell weight, using various carbon sources. Homopolymers as such lack good mechanical properties for practical application but polymer blends of P(HB) with other biodegradable polymers or with copolymers have better material property.

Copolymer

Incorporation of different hydroxyalkanoates units into the P(HB) results in PHA copolymer which has improved physical properties than the homopolymer. Many bacteria are known to synthesize copolymer with other hydroxyalkonoates of medium chain length depending on their intrinsic PHA biosynthesis pathway and the carbon source.

A copolymer of P(HB-co-HV) has been investigated most extensively among the PHA copolymers and applied to commercial products. The incorporation of hydroxy valerate (HV) into P(HB) results in P(HB-co-HV) (Marchessault, 1996). Addition of propionic acid or valeric acid to the growth media leads to the production of this copolymer.

Chemical synthesis of PHA

The ring opening polymerization reactions of 4 membered – lactones in the presence of a catalyst, can lead to chemical synthesis of PHA. High molecular weight P(HB) and P(HV) were synthesized from butyrolactone and valerolactone, respectively. Propionaldehyde and malonic acid were reacted first to form pentanoic acid via Knoevenager reaction followed by bromination and lactonization yielding valerolactone, which was polymerized by a suitable catalyst to get P(HV) (Sasikala and Ramana, 1996).

Metabolism

PHA is an intracellular material synthesized due to unbalanced nutrient condition during the stationary phase of growth when the cells become limited of essential nutrients but have an excess of carbon source (Dawes and Senior, 1973). During nutrient limiting

condition NADH accumulates in the cell and exerts feed back repression on enzymes of the TCA cycle. Acetyl coenzyme A (CoA) accumulates and acetyl CoA acetyl transferase is induced to initiate P(HB) synthesis (Page, 1989). Polymerization of acetyl co A consumes NADH and P(HB) synthesis acquires the role of an electron sink (Senior *et al*., 1972).

Enzymes involved in PHA biosynthesis

Generally the biosynthesis of PHA involves 3 enzymes; β-ketothiolase, acetoacyl CoA reductase (hydroxy acyl dehydrogenase) and PHA synthase (polymerase).

β-Ketothiolase

It is a homotetrameric enzyme with molecular mass of 160 – 190 kDa and is inhibited by co enzyme A. This enzyme is studied with respect to the C-C bond forming sequence and N – terminal amino acid sequence.

Acetoacetyl CoA reductase

This catalyzes oxidation-reduction reaction between hydroxy butyrate and acetoacetate and plays a key role in the PHA synthesis and degradation. This is a tetramer with identical subunits with molecular masses ranging from 85 – 140 kDa.

PHA synthase (polymerase)

This enzyme is associated with PHA granules isolated from different bacteria and helps in the polymerization reaction from hydroxyalkanoic acids as substrates. PHA polymerase may determine the type of monomer unit of PHAs.

PHA biosynthesis pathway

Mainly there are three naturally occurring PHA biosynthesis pathways in bacteria. Synthesis of PHA monomers from acetyl CoA is the common metabolic pathway found in many bacteria (Tsuge, 2002). This pathway consists of 3 enzymatic reactions catalysed by three different enzymes. Two acetyl CoA molecules combine to form acetoacetyl CoA in the presence of ketoacyl CoA thiolase (encoded by *phbA*) second

reaction is the reduction of acetoacetyl CoA to (R) 3-hydroxy butyryl CoA by an NADPH dependent acetoacetyl CoA reductase (encoded by *phbB*) lastly the (R)-3–hydroxy butyryl monomers are polymerized into P(HB) by polymerase (encoded by *phbC*) (Figs. 3 and 4; Huisman *et al.,* 1989).

Another pathway for mcl–PHA synthesis occurs through fatty acid β – oxidation intermediates (Fig. 5 and Table 1). The intermediates in these pathways are converted to (R)–3HA–CoA monomers by specific enzymes. Enoyl–CoA hydratase (*phaJ)* and (R)–3–hydroxy acyl ACP– CoA transferase (*phaG*) are capable of supplying (R)–3HA CoA from trans–2–enoyl–CoA and (R)–HA–ACP, respectively (Tsuge, 2002).

Third pathway for the synthesis of mcl-PHA is through fatty acid synthesis intermediates and β–oxidation. Constituents of fatty acid biosynthesis pathway not only use acyl–ACP but acyl-CoA as substrate and generate mcl–HA from β–oxidation in *E. coli.*

scl-PHA synthesis

The type of PHA synthesized is directly related to the structure of the carbon source used. Thus addition of 3–hydroxypropionate, 4–hydroxybutyrate, 4-hydroxy valerate or 5–hydroxyvalerate to the growth media leads to the synthesis of the corresponding CoA thioesters and incorporation into PHA by PHA synthase. Only a few bacteria can synthesize PHAs containing $C_3 - C_5$ monomers. Conversion of succinate to propionyl CoA through the action of methyl malonyl CoA mutase and oxaloacetate transcarboxylase has been tentatively proposed to lead to the synthesis of 3HV units (Poirier *et al.,* 1995)

mcl-PHA synthesis

The mcl-PHA is synthesized by *Pseudomonas oleovorans* when the bacterium is cultivated on various alkanes or fatty acids. This is linked with fatty acid *de novo* biosynthesis and fatty acid β-oxidation (De Smet *et al.,* 1983; Fiedler *et al*., 2002).

Metabolic pathways can be engineered *in vivo* and *in vitro*. In *vitro* PHA biosynthesis can be achieved using polymerizing enzymes and different substrates. PHA synthases catalyze polymerization under physiological conditions.

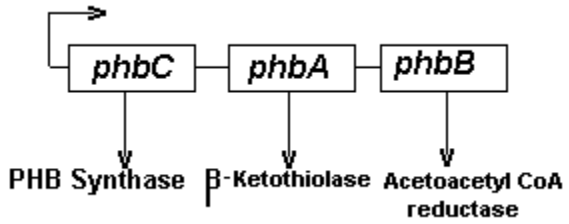


Figure 3: Genes involved in the production of PHA.

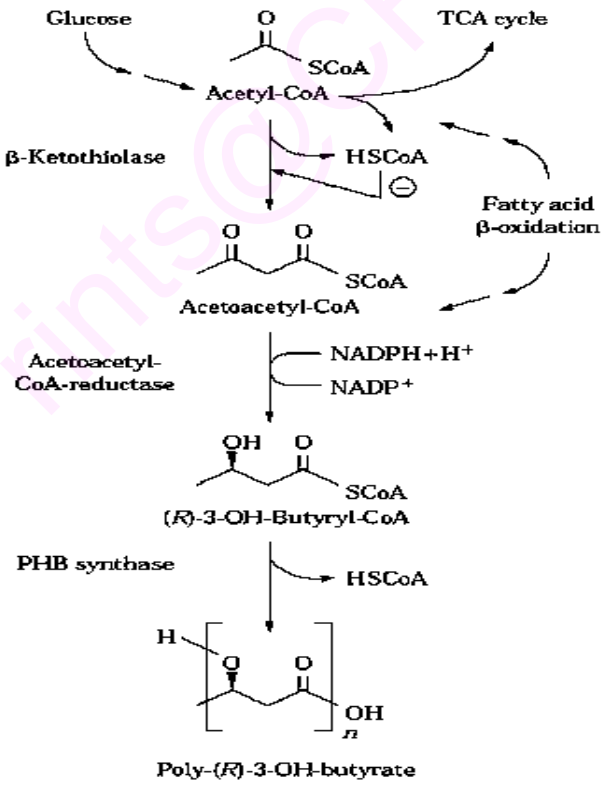


Figure 4: Synthesis of poly(hydroxybutyrate).

Source: Vander-Leij and Witholt (1995)

Table 1: Enzymes and corresponding genes involved in the biosynthesis of mcl-PHA.

Sl no	Enzyme	Gene in recombinant *E. coli*
1	Acyl-CoA synthase	*fadD*
2	Acyl-CoA dehydrogenase	*fadE*
3	Enoyl-CoA hydratase or crotonase	*fadB*
4	(S)-3-OH-Acyl-CoA dehydrogenase (NAD dependent)	*fadB*
5	3-Ketoacyl thiolase	*fadA*
6	3-Ketoacyl synthase: I, II, III	*fabB, F, H*
7	Ketoacyl ACP reductase	*fabG*
8	3-OH-Acyl ACP dehydratase	*
8a	3-OH-Decanoyl ACP dehydratase	*fabA*
9	Enoyl-ACP reductase	*
10	Fatty acid thioesterase: I,II	*tesA, B*
11	Ketoacyl-Co A reductase (NADPH dependent)	*
12	(R)-3-OH-Acyl-CoA dehydrogenase (NAD dependent)	*
13	3-OH-Acyl-CoA epimerase	*fadB*
14	Enoyl-CoA hydratase II	*
15	Enoyl-CoA isomerase	*fadB*
16	(R)-3-OH-Acyl-Co A (ACP- Co A) transferase	*
17	Several acyl transferases or acylases	*
18	Polymerase	None

Source: Vander-Leij and Witholt, (1995)

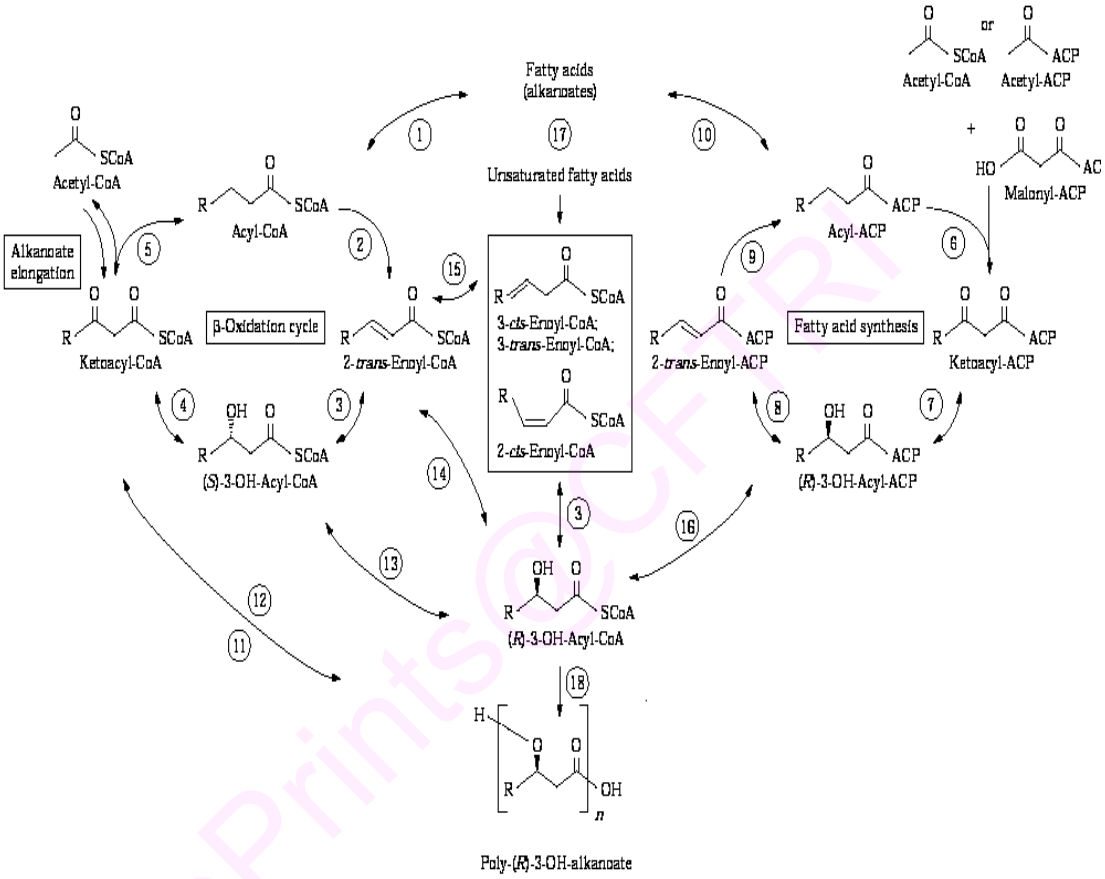


Figure 5: Synthesis of mcl-PHA in *Pseudomonas* sp. involving fatty acid synthesis, β-oxidation and elongation of PHA. Numbers in the pathway are mentioned in Table 1. (Source: Vander-Leij and Witholt, 1995).

PHA production in microorganisms

Growth conditions

Accumulation of PHA in bacteria proceeds under nitrogen or phosphorus limiting condition and in the presence of excess of carbon. It is difficult to compare PHA produced by different organisms due to the wide range of PHA, diversity in production, variety of substrates and different growth conditions. Optimization of growth conditions of microorganisms is very important to get maximum PHA yields. Grothe and coworkers (1999) investigated the effect of temperature, pH and ionic strength of the medium, type of nitrogen source, carbon and nitrogen ratio on PHA production. Optimum temperature for the growth of *Alcaligenes latus* and for the PHA synthesis appeared to be 33 ^{0}C. They also found that initial pH of 6.5 increases the growth rate of *A. latus* and it was 0.075gh^{-1}, maximum sucrose utilization was 0.38gh^{-1}, where 63% of P(HB) was accumulated (Grothe *et al.,* 1999). Optimization of nutrient limiting condition and PHA production by *Rhizobium meliloti* and its mutant strain has been reported using urea as nitrogen source to obtain 60% of PHA (Kshama *et al.,* 2004).

A. eutropus, has been used for optimal production of P(HB), a homopolymer which is accumulated under nitrogen limitation (Morinaga *et al.,* 1978). *Azotobacter beijerinckii* produces PHA under oxygen limitation compared to nitrogen or phosphorus limitation (Ward *et al.,* 1977). A wide variety of PHA copolymers are synthesized in *Bacillus* spp. from fermentation of different carbon sources (Valappil *et al.,* 2007; Anil Kumar *et al.,* 2007; Labuzek and Radecka 2001).

Besides the importance of nutrients and carbon and other physical growth parameters (pH, temperature), other survival mechanism of microorganism seems to play a significant role in the production of PHA. PHA supports the survival of microorganisms under stress conditions such as osmotic pressure or UV radiation (Brandl *et al.,* 1990). In general, cells containing PHA have a better survival rate than others and PHA also plays physiological function during the sporulation in *Bacillus* (Williamson and Wilkinson, 1958) and encystment in *Azotobacter* (Dawes, 1974).

Substrate and Precursor for PHA synthesis

PHA production is based on the substrate used and it has received attention as they grow on renewable resources where bacteria can utilize waste generated from food, agricultural and industrial process and fatty acids as carbon source. High carbon content substrates are used for the growth of the organism. Sugars such as glucose and sucrose are main carbon sources used in PHA production.

Carbon sources

Beet molasses, maltose, corn syrup, cane molasses, malt extract, palm oil mill effluents have been used as substrates for polymer production. Type of PHA produced depends on the organism and the substrate used.

PHA production from glucose and sucrose has been optimized. The development of technology by using cheaper carbon sources would be a key factor for further reducing the PHA cost (Tsuge, 2002). The cost of the carbon source contributes significantly to the overall production of PHA (Yamane, 1992). Use of cheaper carbon source can lower the production cost. When glucose was used at a concentration of $157gl^{-1}$, PHA content was 77%, with productivity of 3.2 $gl^{-1}h^{-1}$ and the cost of carbon source was 38% of the total production cost (Choi and Lee, 1999). When cornstarch was used as a substrate under the same conditions, production obtained was $1.19gl^{-1}$, and the cost of carbon source was 15% lower compared to glucose. Crude carbon substrates such as cane molasses, beet molasses, whey, plant oils; starch (corn and tapioca), cellulose and hemicelluloses can be used as excellent substrates for the growth and polymer production by bacteria. The copolymer production also depends on the substrate and the organism used. The co substrate acts as a precursor for the polymer production.

High Cell Density cultivation

High-density cultivation has been carried out to enhance PHA productivity with reduced culture time and increased cell concentration and the type of copolymer. This not only increases the cell concentration but also reduces cost of down stream processing (Yamane *et al.,* 1996). Fed batch culture has been the most popular culture system to

reach high cell density and P(HB) content (Kim *et al.,* 1994; Suzuki *et al.,* 1986b). In high cell density culture, optimal concentration of nutrients, pH, dissolved oxygen, antifoam, agitation, and aeration are maintained during fermentation (Kim *et al.,* 1992).

Yamane *et al.,* 1996 carried out fed batch culture of *A. latus* to achieve the high cell density to reduce the culture time, where in the sucrose solution and inorganic medium with the NH_3 solution, was fed periodically during fermentation. This resulted in 142 gl^{-1} of cell and 68.4 gl^{-1} of P(HB) in 18 h with initial cell concentration of 13.7gl^{-1}. Methanol has also been used as a sole carbon and energy source for the production of P(HB) by *Methylobacterium extorquens.* Methanol was added to the medium as a carbon source (0.01 gl^{-1}) and P(HB) obtained was 46% of the total biomass weight (Bourque *et al.,*1995).

Pseudomonas oleovorans was also grown to obtain mcl-PHA using n-octane as carbon source leading to maximum biomass concentration of 37 gl^{-1} by feeding nitrogen in combination with various metal ions. Final cell density achieved was 112 gl^{-1} but PHA content was low (Kellerhals *et al.,* 1999).

Efficient production of intracellular fermentation products requires both fast growth of the organism and accumulation of cells to high cell concentration and high yield. Recombinant *E. coli* containing *A. eutrophus* PHA biosynthesis gene has been employed for the production of P(HB) by fed-batch to get 77 gl^{-1} with the productivity of 2 gl^{-1} h^{-1} (Wang and Lee, 1998).

Visualization and detection of PHA

PHA exists in the cytoplasm as 0.2-0.5µm granules surrounded by a membrane (Sudesh *et al.,* 2000). PHA staining procedure for qualitative visualization of polymers by optical microscopy and with molecular tools for *insitu* identification are described (Serafim *et al.,* 2002). PHA inclusions are stained with lipophilic dyes and they appear as refractive granules in phase contrast microscopy. Sudan group of dyes has been used to observe lipids in the cell; this dye is more soluble in lipid material and imparts a black-blue colour to the PHA granule (Bartholomew, 1981; Murray *et al.,* 1994).

A more effective and specific visualization of PHA is carried out with Nile blue. The oxidized form of Nile blue (red or pink) is formed in aqueous solution because of

which PHA is visible as a bright fluorescence. Nile red is soluble in neutral lipid and they are liquid at 55^0 C and therefore it is adsorbed in PHA. The stained PHA granules are visible with wavelength of 460 nm the fluorescence of Nile blue increases with increase in PHA concentration (Ostle and Holt, 1982).

Analysis of PHA

Quantification of the intracellular polymer is performed by many techniques, after the extraction and hydrolysis to its monomer. Initially PHA was analysed by gravimetric method where lyophilized biomass was digested using hypochlorite and the sedimented material was held in chloroform followed by precipitation of clear solvent layer with diethyl ether or methanol (Williamson and Wilkinson, 1958).

Crotonic acid assay

Law and Slepecky (1996) developed this method where P(HB) is converted into crotonic acid by treating with sulfuric acid and the product produced is measured by UV spectroscopy at 235 nm.

IR spectroscopy (FTIR)

FT-IR has also been utilized to characterize the changes of macromolecules during melting and crystallization (Xu *et al.,* 2002). The bands at 980, 1230, 1278 and 1728cm^{-1} originate from crystalline phase. The bands at 1741 and 1724cm^{-1} are stretching vibration of the amorphous and crystalline carbonyl group, respectively. The IR spectroscopic analysis gives insights into the chemical structure without a previous hydrolysis of the polymer.

Gas Chromatography (GC)

GC analysis of PHA involves the extraction and methanolysis of PHA in the presence of acid and methanol to form methylester, which is then analyzed by GC (Braunegg *et al.,* 1978). PHA esterification can also be achieved by using propanolysis in HCl (Riis and Mani, 1988).

Gas Chromatography mass spectroscopy (GCMS)

Mass spectroscopy operates at reduced pressure to enable detection of the small amount of material that is routinely analyzed. Quantitative chemical ionization mass spectrometry afforded sensitivities for PHA constituents within the molecular range (Odham *et al.,* 1986).

The molecular masses of the isolated polymers are determined by the GCMS analysis (Eversloh *et al.,* 2001). The resolving power of mass spectrometry is adequate to deal with the size of repeating units and large oligomers differing in monomer composition can easily be identified. Fast atom bombardment mass spectrometry (FAB-MS) has been used to determine the repeating unit composition and sequencing (Ballistreri *et al.,* 1999).

Differential scanning calorimetry (DSC)

Differential scanning calorimetry equipment calibrated with indium standard and nitrogen atmosphere was used to determine the melting temperature (T_m) and the enthalpy of fusion (ΔH_f) from the endothermal peak on the DSC curves (Xu *et al.,* 2002).

PHA granules have been analyzed *insitu* by DSC without isolation from the cells and they revealed that polymer within the cells existed in an amorphous state but crystallized after dehydration or lyophilization (Song *et al.,* 1998).

Nuclear magnetic resonance (NMR) spectroscopy

When an atom is placed in a strong magnetic field it spins at a particular resonance that can be recorded and analyzed. Different atoms will have different frequency of resonance. Spectra of any nucleus possessing a magnetic moment can be obtained as probes. In biological studies ^{31}P, ^{13}C, ^{1}H, ^{15}N, ^{23}Na, ^{39}K and ^{19}F NMR are used to determine the intracellular products. NMR is used to characterize the composition and structure of PHA after extraction.

Recovery of PHA

After the growth, cells containing PHAs are separated from the broth by conventional procedures such as centrifugation, filtration or flocculation. The recovery of PHA involves following steps:

1. Cell wall disruption
2. Extraction
3. Purification

A number of methods have been developed for the recovery of intracellular PHA. Disruption of cell wall is an important step in the recovery of PHA. The intracellular products must be released into the medium before recovery and purification (Dunhill, 1972).

Cell wall can be disrupted by mechanical, chemical and biological or enzymatic methods. The cells can be genetically engineered to excrete the product, the cell wall can be made permeable and cell lyses may be induced (Dunhill, 1983).

Cell wall is a rigid and complex structure that protects the protoplasm from environment stress and also maintains osmotic regulation and cell shape. Microbial cell wall vary widely in composition and structure according to species. Method of cell lysis and extraction of PHA depends on the cell wall composition of the bacterial cell.

Bacterial cell wall

In 1884 Christian Gram developed cell wall staining technique for bacteria. Soon it became evident that bacteria could be divided into two major groups based on their response to the gram stain procedure. Gram-positive bacteria stained purple, where as gram-negative pink or red.

Gram-positive bacteria

Gram-positive cell wall consists of peptidoglycan layer of 20-80nm in thickness. This is a polymer layer containing many identical subunits. The polymer contains two sugar derivatives namely N–acetyl glucosamine and N–acetyl muramic acid and different amino acids, three of which are D–glutamic acid, D–alanine and meso–diaminopimelic

acid. The backbone of this polymer is made up of alternating of N–acetyl glucosamine and N–acetyl muramic acid. Peptide chains of four alternating D and L amino acid subunits are cross-linked with peptide. The carboxyl group of D–alanine is connected with amino group of diaminopimelic acid (Fig. 6). The nature of amino acids positions and degree of cross-linking differ among species. However gram-positive cell wall also contains large amount of teichoic acid, since it is negatively charged (Presscot *et al.,* 1990).

Gram-negative bacteria

Gram-negative cell wall is more complex in structure. It has a thin peptidoglycan layer of 7-8nm thicknesses, outer membrane lies next to the peptidoglycan layer, which contains lipoprotein and lipopolysaccharides. Lipopolysaccharides consist of three parts: 1. Lipid A. 2. Core polysaccharide. 3. 'O' side chain, which has toxic properties and is able to resist the chemical aggressions of the environment (Mayer *et al.,* 1985).

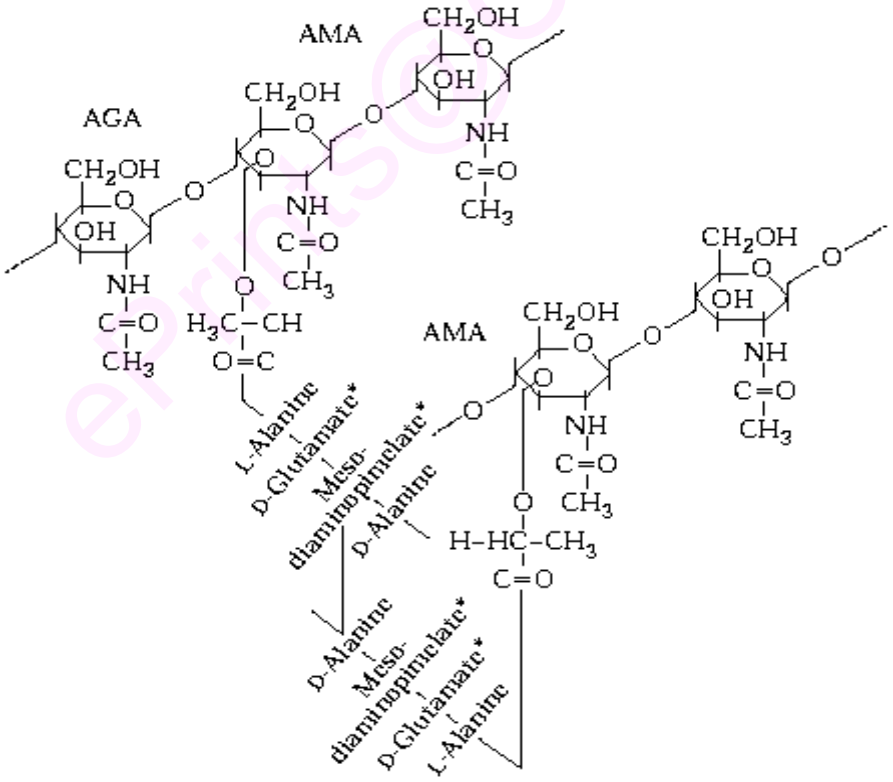


Figure 6: Structure of the building blocks of peptidoglycan in *E. coli.*
AGA - N-acetylglucosamine, AMA – N-acetylmuramicacid.
Source: Encyclopedia of microbiology

Effect of upstream conditions on cell lysis

The conditions that are maintained during the growth process affect the synthesis of cell wall constituents and will be reflected in the strength of cell wall. The medium used and the growth phase at which it is harvested, for instance *E coli* cells are weaker during the logarithermic phase and become stronger in later stages (Sauer *et al.,* 1989). Higher growth rate in continuous culture leads to weaker cell wall, where as lower growth rate as in batch culture leads to synthesis of stronger cell wall (Engler and Robinson 1981). It has been observed that it is easy to recover PHA from the cells having low DNA content such as the recombinant *E. coli* compared to parental strain of *A. eutrophus* (Hahn *et al.,* 1998). Addition of 0.1% peptone to *Azotobacter* sp. growth medium is known to favour easy lysis of cell wall. In such a medium cells were found to be fragile and could be lyzed easily in 1 N NH_3 (Page and Cornish, 1993). Recombinant cells which autolyse at particular harvesting time has been constructed so that the intracellular products can be recovered easily (Dunhill, 1983).

Cell disruption

Techniques for cell wall disruption have been evolved over the years and are well documented. Mechanical methods are based on shear forces that deform and rupture the cell wall and non-mechanical methods induce the cell lysis by chemical or enzymatic means (Belter *et al.,* 1998). The first step in the extraction of PHA is cell wall lysis to release the polymer, which is usually carried out using sodium hypochlorite, sodium dodecyl sulphate (SDS), alkali or acids. Mechanical disruption of the cell is achieved by ball milling, freeze-thaw step. Solvents such as chloroform, methanol, acetone etc., are used to solubilize and purify the PHA (Terada and Marchessault, 1999).

Mechanical methods

At present there are several conventional methods for disrupting cells which includes:

i. Grinding cells with glass beads i.e., cell homogenizer
ii. Shearing cells with a warring blender
iii. Disintegration by ultrasonication

High-pressure mechanical homogenizers

Homogenizers have been one of the most widely used devices for the disruption of microbial cells for large-scale recovery of intracellular byproducts. This device consists of a positive displacement pump capable of generating high pressure which forces the cell suspension through an adjustable discharge needle valve under high shear stress, which disrupt the cell.

Solid shear method

Ball mills or bead mills originally designed for non-biotechnological applications have also been used to disintegrate microorganisms. This device consists of agitator disks mounted concentrically with a motor – driven central shaft inside a grinding chamber. During operation, the chamber is filled with glass beads or steel beads. It has limitation because of high-energy consumption, high heat generation and erosion of the beads (Marffy and Kufa, 1974).

In another combination of physical and chemical method, cells were disrupted in a ball mill and also pretreated with salts, heat, or alkali to facilitate extraction (Tamer *et al.,* 1998a). Compared to high-pressure expansion of cells, ball mill has been found to be suitable for industrial use (Tamer *et al.,* 1998b).

Ultrasonics

Ultrasound has been used in the laboratory scale for extraction and purification of polymer. The mechanism of cell disruption is derived from the intensive shear induced by sonication and the suspension is subjected to sound frequencies above 20 kHz. A magneto restrictive converts the alternating current of an electric oscillator into mechanical waves that are transmitted to the suspension through a metallic probe usually made up of titanium. The sound waves create many microbubbles at various nucleation sites in the suspension, which collapse during the refraction period. This phenomenon produces intense local shock waves that cause the cell to deform beyond elasticity and rupture. The rate of microbial cell lysis is usually low but inclusion of small beads of glass or steel will increase the efficiency of disruption (Doulah, 1977; Kuboi *et al.,* 1995).

Mechanical methods are generally effective and efficient than the non-mechanical methods but there are also several disadvantages, they are energy intensive and generate high temperature and are nonspecific with respect to the organisms and products.

Non-mechanical methods

Non-mechanical methods can only increase the cell permeability of the cell wall without breaking it. Among the physical methods of lysing the cells osmotic shock and freeze thawing are two common methods.

In osmotic shock, cells are placed in either a hyper tonic or a hypotonic medium where cells shrink or expand respectively. In both the cases cells suffers with serious injuries. However, cells have osmoregulation mechanism but if suddenly placed in a hypotonic medium cells suffer osmotic shock, absorb water, swells and burst.

The release of intracellular product can also be induced by freeze thawing of the cells. The efficiency of this method depends on the rate of thawing. With a large mass of frozen suspension thawing rate alters.

In freeze-blast method the frozen cell suspension is rapidly blown at a high nitrogen gas flow (Omori *et al.,* 1989).

Chemical methods

A number of chemical methods exist for the recovery of PHA, which is intracellular (Berger *et al.,* 1989*;* Harison *et al.,* 1991; Ramsay *et al.,* 1990). Majority of PHA extraction process involves the chemicals and solvents (Hahn *et al.,* 1993). P(HB) can be extracted from bacterial cells with methylene chloride, propylene carbonate, dichloromethane and chloroform. Although the solvent extraction has been widely used for the recovery of PHA with a high purity, the operation becomes difficult in scale up process and a large amount of solvent is required. The solvents are hazardous and it also involves multiple treatments and the significantly high cost recovery, which make the process economically unfavorable. Another method based on differential digestion using sodium hypochlorite has been reported (Berger *et al.,* 1989). Though this method is simple and effective it causes degradation of P(HB) resulting in the low molecular weight.

Various acids (HCl, H_2SO_4), alkalies (NaOH, KOH and NH_4OH) and surfactants (diactylsulfosuccinate sodium salt, hexa dicyl trimethyl ammonium bromide, sodium dodecyl sulphate [SDS], poly oxy ethylene-p-tert oxytylphenol [Triton X–100], polyethylene (2) sorbitan monolaurate-Tween 20, have been used to digest non PHA cellular materials (Choi and Lee, 1999). Low temperature extraction of > 100^0 C with alcohol, ketone, dialkylether or monocarbonic acids have been attempted (Liddell, 1997). Attempts have been made to extract P(HB) from dried biomass with methylpyrrolidone (Schumann and Wendlandt, 2001). Extraction of PHA from biomass by diafiltration of aqueous slurry with solvents is also patented (Horowitz, 2002). PHA has also been extracted with acetone and heating (Anonymous, 1997). Extraction of the cells with methylene chloride or chloroform resulted in extraction of 25% P(HB) and refluxing increased this to 50% (Ramsay *et al.,* 1994). Alternatively P(HB) can be extracted with hot dioxane without degradation and precipitated with methanol (Mitsubishi, 1987). Extraction of P(HB) with solvents could be improved in the presence of a second solvent that is not soluble in water. P(HB) was precipitated in water after the evaporation of the solvent (Noda, 1997). Trials have been made to extract the polymer in the presence of oil as co-solvent (Noda and Schechtman, 1997).

It has been shown that it is relatively easy to extract PHA from halophilic bacteria, as they are lyzed using salt free water (Anonymus, 1995a). For economic downstream processing, cells were treated with NaOH instead of SDS/hypochlorite and it is reported that by this method the P(HB) can be obtained for $ 4/kg instead of $ 5/kg (Choi and Lee, 1999).

Hahn and his co-workers used dispersions of sodium hypochlorite solution and chloroform in 1:1 ratio, for the recovery of P(HB) and reported 91% recovery and >97% purity of high molecular weight polymer (Hahn *et al.,* 1994).

*Azotobacter chroococcum b*iomass was pretreated with freezing and sodium dodecyl sulfate (SDS, 10 g/l) for 15 min to effectively solubilize lipid and protein. Subsequently, digesting with 30% sodium hypochlorite for 3 min hydrolysed peptidoglycan and non-PHA biomass. Finally, 98% PHA was isolated by diluting and rinsing with water (Zhaolin and Xuenan, 2000*).* Lipids, which may interfere with extraction, have been extracted with supercritical CO_2 prior to further extraction

(Hampson and Ashby, 1999). Instead of dry heat, autoclaved biomass has been used for the treatment with SDS and hypochlorite. After precipitation with acid a homogeneous distribution of P(HB) was obtained. However this method resulted in reduced molecular weight of the polymer (Kim *et al.,* 2000). The polymer could be extracted using a mixture of heated biomass, acetic acid/acetic anhydride or acetic acid/butyrolactone (Lehmann, *et al*., 1993).

It has been reported that biomass obtained by high cell density fermentation and extracted with SDS gave good recovery of polymer without loss in molecular weight (Kim *et al.,* 2003). Purity and yield of the product could be enhanced by the addition of EDTA to detergents at pH 13 (Chen *et al.,* 1991; Chen *et al.,* 2001). Extraction of P(HB) in the presence of detergents could yield latex of low crystallinity (Anonymus 1995b). It is also suggested that a careful methodology can be followed to purify P(HB) in the presence of hypochlorite without degradation (Berger *et al*., 1989). Pretreatment with Triton enhanced the purity and quality of P(HB) that previously had been extracted by the hypochlorite method (Ramsay *et al.,* 1990). At ICI the polymer is isolated from microorganism with solvents, which were made leaky by spray drying. P(HB) is precipitated with methanol or acetone. In another method, 1,2-dichlorethane/water was added to the fermentation broth. At 72 °C dichlorethane evaporates with azeotrophic water. The material was then heated to 85 °C and filtered. The yield of the polymer was 55% (Solvay, 1984).

Enzymatic methods

Enzymatic methods of cell wall hydrolysis are becoming more attractive with increased selectivity, yield of product release and minimization of product damage. Enzymatic lysis requires a better understanding of chemical and physical structure of the cell wall because complete cell lysis is not possible by single enzyme. Peptidoglycan is the basic structural element in bacteria. For gram-positive bacteria single enzyme could lyse the cell because of the presence of only peptidoglycan layer and for gram-negative bacteria pretreatment with a surface-active agent to remove the outer membrane is required.

The main restrictions in using multi enzyme system are the availability of enzymes and associated high costs. Various organisms such as *Actinomycete* sp., *Bacillus* sp., have been utilized as sources of lytic enzymes. The selection of the lytic system depends on the nature of the wall to be lysed and the nature and location of the product inside the cell. Multi enzyme system determines the efficiency of the lytic process (Asenjo *et al.,* 1985).

Isolation of P(HB) by digesting heat-denatured cells with protease and phospholipase; adding detergents and H_2O_2 on proteolyzed cells, has been reported (Holmes and Lim, 1984; Liddell and Locke, 1994). Extraction of lipids by solvents in alkalized medium was followed by flocculation and separation of cells by lowering pH (Walker *et al*., 1981). Aluminum or Ferrous coagulants have been tried instead of centrifugation, which is used for sedimentation of cells from the broth to reduce energy consumption (Ryu *et al*., 2000). Application of microbial culture, which produces lytic enzyme extracellularly, was tested for isolation of PHA from *Rhizobium meliloti* cells (Kshama and Shamala, 2006).

An enzymatic digestion method of bacterial cell wall for the extraction of PHA has been developed by ZENECA (formerly ICI) in the United Kingdom. This process consists of thermal treatment of PHA containing biomass, enzymatic treatment and washing with surfactant to dissolve non-PHA cell material (Holmes and Lim, 1990). This method is found to be efficient and product obtained is also pure compared to other chemical methods.

Genetic engineering and cell lysis

Genetic engineering and developments in gene manipulation is now possible to manipulate cell lysis easily through recombinant technology or excrete the intracellular material or autolyse at desired stage. Physiology of the cell including wall strength may be adjusted or manipulated in favor of down stream process.

Adding certain substrates can also induce cell lysis. Addition of 0.1% or higher concentration of polyphosphate had a bactericidal effect on log-phase cells (Maier *et al.,* 1999). Mutants deficient in both glucose–6–phosphate dehydrogenase and phosphogluco isomerase lysed cells in 4-5 h after the growth in nutrient medium. Lysis was caused by

the intracellular accumulation of glucose–1–phosphate, which inhibited the 2 enzymes that convert glucosamine–6–phosphate to N–acetyl glucosamine, 6-phosphate (Prasad and Freese, 1994). Lytic enzyme was induced by bacteriophage in *Pseudomonas aeruginosa* and *Escherichia coli*, which hydrolysed–L–alanine–D–glutamic acid peptide bond in peptidoglycan layer of cell wall (Yanai *et al.,* 1976).

LeCorre and coworkers used a lytic enzyme system from *Cytophaga* sp. for lysis of the gram–positive bacteria namely *Bacillus* sp. and *Corynebacteria* (Le Corre *et al.,*1985). Phage endolysin from *Bacillus amyloliquefaciens* was used to enhance the permeability of *Pseudomonas aeruginosa* outer membrane. Glu 15 to His (E15H) and Thr–32 to Glu (T32t) substitutions were introduced into the *Bacillus* phage endolysin, where these two are considered to be the active center of the enzyme and which enhances the cell lysis (Orito *et al.,* 2004).

Autolysis of cell walls of *Bacillus subtilis* is possible by the release of N–terminal L–alanine without release of C terminal amino acids. The enzyme responsible for this is N–acylmuramyl–L–alanine amidase that is active during logarithmic phase. Autolysis follows first order kinetics and requires 9.2 Kcal/mole and results in the hydrolysis of 87% of the amide bonds between muramic acid and L–alanine.

Young and coworkers (1990) studied the cell death of *Bacillus subtilis* by surfactant palmityl trimethyl ammonium iodide. This caused the death at log phase in a chemically defined medium. They investigated the relationship between the induced death and lysis, by using an autolytic enzyme defective mutant and by exposure of cells to several treatments. They observed that the pretreatment of cells with erythromycin rendered the cells resistant to the surfactant, pretreatment with cerulenin caused the sensitization of cells and they concluded that the cell death caused by the surfactant at low concentration results from the induction of autolysis (Young *et al.,* 1990). Phage lytic gene that is under the control of xylose competes with glucose, lyses *Bacillus megaterium* when glucose is exhausted and thus releases P(HB) into the supernatant (Hori *et al.,* 2002).

Separation of PHA

Aqueous two-phase system (ATPS) is one of the methods used for the separation of biomolecules. It has many advantages as it reduces the process time, the energy consumed is low and the materials used are biocompatible with the biomolecule. ATP is formed by mixing two aqueous solutions of two hydrophilic polymers or polymer and a salt at certain threshold concentration (Tanuja *et al.,* 1997; Srinivas *et al.,* 1999). Hofsten and Baird (1962) developed ATPS to separate *Bacillus megaterium* cell constituent after cell lysis where they used dextran and PEG system. They found that separation of cell debris at the interphase and they also found that the addition of 0.2 M of NaCl would make the intracellular granule to settle at the bottom (Hofsten and Baird, 1962). Two phase separation of hypochlorite or enzymes treated cells using PEG/dextran has also been examined (Marchessault *et al*., 1990). Partition behavior of the protease in ATPE has been reported in many papers. Pancera *et al*. reported that PEG could influence the activity of enzyme by altering the structure of enzyme active site. Elimination of enzyme inhibitors from PEG phase during extraction is also reported to enhance enzyme activity (Pancera *et al.,* 2002).

Polymer properties

Inside the bacterial cell, PHA granules consist of a hydrophobic core of amorphous polymer that is surrounded by a membrane consisting of the PHA synthases, PHA depolymerase and phasin proteins. These proteins are embedded with a phospholipids monolayer.

Atomic force microscopy analysis of PHA isolated from *Ralstonia eutropha* cells revealed that they exhibit two types of surface structure and shape i.e., rough and ovoid or smooth and spherical (Dennis *et al.,* 2003).

The important property of PHAs is their complete biodegradability. Thermal and physical properties of P(HB) and P(HB-co-HV) are widely studied among PHA. P(HB) is 100% steriospecific with all of the asymmetric carbon atoms therefore it is highly crystalline. The glass transitions temperature is approximately 5 and melting temperature 177 ^{0}C. The molecular mass of PHA varies depending on the organism and the substrates used but generally the mass lies between 50,000 to 1,000,000 Da.

The mechanical properties of PHA is similar to polypropylene (PP), tensile strength of P(HB) is 43 Mpa where as that of PP is 38 Mpa. However, extension to break (5%) of P(HB), which is markedly lower than that of PP i.e., 400%. Wide variety of mechanical properties is exhibited by PHAs depending on the composition of monomer units (Table 2). mcl-PHAs are semi crystalline elastomers with a low melting point, low tensile strength and high elongation to break (Lee, 1996).

Table 2: Comparison of polymer properties of polyhydroxybutyrate and its copolymer with polypropylene.

Physical properties	P(3HB)	P(HB/HV) 90:10	P(HB/HV) 80:20	P(4HB)	PP
Melting point (^{0}C)	179	150	135	53	170
Tensile strength (Mpa)	43	35	20	104	38
Young's modulus (Gpa)	3.5	1.2	0.8	149	1.7
Elongation to break (%)	3.0	20	100	1000	400

Source: Lee, (1996)

Effect of surface morphology on the biocompatibility of PHA were studied by Kai *et al.*, where they observed that high degree of crystallinity generates protrusion on the P(HB) film surface but the presence of P(HB-co-HHx) reduces the crystallization of P(HB) and also gives rise to smooth surface which would, strongly improve the biocompatibility of P(HB) (Kai *et al.,* 2003).

Biodegradability

Biodegradability is the most attractive feature of PHA. Number of bacteria and fungi are known to degrade PHA. These microorganism present in soil, lake and activated sludge excrete extra cellular PHA depolymerases to degrade PHA into water soluble monomers and oligomers and use them as a carbon source (Lee, 1996).

The rate of biodegradation is dependent on the microbial population in the given environment, temperature and property of plastic to be degraded. PHA fits perfectly in the ecosystem due to its complete biodegradation where the end product of PHA

degradation in aerobic environment is CO_2 and H_2O while methane is produced in anaerobic condition.

Biodegradability of P(HB) was tested with an extra cellular P(HB) depolymerase from *Alcaligenes faecalis* T_2 and it has been noted that the rate of enzymatic degradation of P(HB) depended on crystallinity rather that on molecular weight (Kusaka *et al.*, 1999).

PHA blends having low and high molecular weight fractions have been examined for biodegradability. The results show that the presence of a second component, is sufficient to perturb the crystallization of P(HB-co-HV) and increase hydrolytic degradation. However the introduction of polar carboxylic groups in side chains increases the degradation rate where it promotes the water penetration into the polymer (Renard *et al.,* 2004).

Application of biodegradable polyesters

There is a great demand for the biodegradable polymers in the years to come with a view of having a clean environment. Potentiality of PHA has been shown for aerobic phosphorous uptake in enhanced biological phosphorous removal (Randall and Liu, 2002). PHAs are also used for denitrification of water and in wastewater treatment. This type of denitrification is termed as "solid-state denitrification" (Hirashi and Khan, 2003). Other applications of PHA (Chandra and Rustgi, 1998; Zinn *et al.,* 2001) are mentioned below:

- Packaging films, bags and containers.
- Carrier for long-term dosage of drugs, medicines, insecticides, herbicides.
- Disposable items such as utensils, diapers or feminine hygiene products.
- Starting material for chiral compounds.
- Surgical pins, sutures, staples and swabs.
- Wound dressing.
- Bone replacements.
- Blood vessel replacements.

The application of biodegradable polymers has been focused mainly on, medical, agricultural and packaging. Some of these have resulted in commercial products.

Medical applications

Biodegradable plastics have been developed as surgical implants in vascular and orthopedic surgery as implantable matrices for the controlled long-term release of drugs inside the body, as absorbable surgical sutures. It is also used as a bone fixation device, vascular grafts, adhesion prevention and artificial skin and also in drug delivery systems.

Agricultural applications

Plastic films are used in the agricultural field for greenhouse coverings, fumigation and mulching. Mulches permit growers to use plastic films to help plant growth and then photo degrade in the fields there by avoiding the bag removal. They are also used for controlled release of agricultural chemicals by which biologically active chemicals are made available to a target species at a specified rate and for a predetermined time

Packaging

Physical characteristics of packaging polymers are greatly influenced by the chemical structure. The challenge in the development of biodegradable packaging will be to combine polymers which are truly biodegradable into a laminate film or a film blend which has properties as good as those found in synthetic laminates. Several polysaccharide-based biopolymers are being used as possible coating materials or packaging films. Greater challenge lies in the development of biodegradable packaging especially for food packaging (Chandra and Rustgi, 1998).

Commercialization

Many companies around the world are developing PHA products. At present the ZENECA bioproducts is one of the producer of P(3HB) and P(3HB-co-3HV). About 1000 tones of polymer/annum is being produced under the trade name of BIOPOL. Some of the companies that are involved in PHA production are as follows:

1. Berlin Packaging Corp. (USA) - Marketing and distribution of ZENECA's BIOPOL.
2. Bioscience Ltd. (Finland) – Medical applications of PHA.
3. Bio Ventures Alberta Inc. (Canada) – Production of PHA by recombinant *E coli*
4. Metabolix, Inc. (USA) - Production of PHA by transgenic plants.
5. Monsanto (USA) - Production of PHA by transgenic plants (rapeseed and soybean)
6. Polyferm, Inc. (Canada) – Production of PHA from hemicellulose.
7. ZENECA Bio products (UK) – Production of P (3HB) and P (3HB-co-3HV) BIOPOL by a fed-batch culture of *A. eutrophus*
8. ZENECA seeds (UK) – Production of PHA by transgenic plants (rapeseed).

Current advances in fermentation, purification technology and metabolic engineering approaches can further lower the price of the polymer (Lee, 1996; Vander-Leij and Witholt, 1995; Poirier *et al.,* 1995).

Aims and scope of the thesis

The production of PHA from microorganisms includes selection of potent strain, optimized fermentation process, cell hydrolysis to release the intracellular polymer, extraction and purification of the polymer. Literature review presented above indicated that emphasis is on the optimum production and recovery of the PHA copolymer which leads to cost reduction. More acceptable methods of cell hydrolysis, non-organic solvent to isolate the PHA is required for economic production of the polymer. The methods and techniques used in the upstream or downstream process should not affect the quality of PHA.

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

- **Isolation and characterization of PHA producing bacterium and optimization of polymer yields.**
- **Comparison of physical, chemical and biological methods for the isolation of polymer from the cells.**

The objectives were worked out and data are presented in the following chapters.

Materials and Methods

(Common to Chapters)

In this chapter, materials and methods that are applicable commonly to several chapters are dealt with. Specific materials and methods are given in respective chapters.

MATERIALS

M. 1. Standards

Standards such as P(HB), P(3HB-co-3HV)-5mol%, P(3HB-co-3HV)-8mol%, were obtained from Sigma-Aldrich, USA.

M. 2. Microbiological media

Media and media ingredients were obtained from Himedia (Mumbai, India). This included: Nutrient agar, Nutrient broth, Luria Bertani broth, Luria Bertani agar, peptone, yeast extract, beef extract, tryptone and agar.

M. 3. Chemicals and solvents

All chemicals and solvents used were obtained from Merck, SRL, Qualigens, Nice Chemicals, Rankem or Himedia. The chemicals used were: Urea, ammonium nitrate, casein, ammonium acetate, ammonium chloride, sodium nitrate, ammonium citrate, citric acid, $(NH_4)_2SO_4$, Na_2HPO_4 $2H_2O$, KH_2PO_4, $MgSO_4$ $7H_2O$, KNO_3, NH_4NO_3, $(NH_4)_2HPO_4$, $NaNO_3$, sucrose, glucose, fructose, starch, rhamnose, lactose, inositol, dulcitol sorbitol, dextrin, mannitol, mannose, maltose, pectin, melibiose, raffinose, trehalose, sorbose, xylose, galactose, glucose, arabinose, fructose, butyric acid, valeric acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, benzoic acid; dinitrosalicylic acid, sodium potassium tartarate, NaOH, HCl, H_2SO_4, sodium hypochlorite.

Solvents used were: chloroform, acetone, ethanol, methanol, diethyl ether and hexane.

M. 4. Equipments used:

1. Air flow drier – Thermotech
2. Atomic absorption spectrophotometer – AA 6701F, Shimadzu, Singapore
3. Autoclave – Culture Instruments
4. Centrifuge – R 24 Remi instruments
5. Cooling Centrifuge – Cooling compufuge CPR 30, Remi
6. Deep freeze – Carrier-HF 300 CHP
7. Digital weighing balance – Anamed Mx72054 and Essac – DS 852J Teraoka Limited.
8. DSC – Perkin Elmer Thermal analysis, Switzerland
9. Elemental analyzer system - Elementar VarioEL, Germany
10. Electrophoresis system – GENEI, 0-300V/0-100mA
11. Electroporator machine – BIO-RAD Gene pulser Xcell
12. Fermentor –3 liter capacity, Bioflo 110, New Brunswick Scientific Co., USA
13. Fermentor 3 liter capacity, Bioferm LA1, Scigenics India Private Limited.
14. Film Thickness measuring machine –Testing machines inc New York, USA, model 549E
15. Fourier Transform Infrared Spectrophotometer – Thermo electron Inc., NICOLET 5700, FT – Raman Module, USA.
16. Gamma irradiation chamber – Gamma chamber 5000, BARC, India, with cobalt 60; model 444 TBQ, 12000 kCi, irradiation volume 5000 cc, diameter of sample chamber 20.5 cm.
17. Gas Chromatography – Fisons instruments GC – 8000 series, Italy.
18. Gas Chromatography Mass Spectroscopy – Perkin Elmer, Mass Spectrophotometer Model:Turbo mass gold, Switzerland
19. Gel documentation system – BIO-RAD
20. Heating mantles – Tempad India
21. Incubator – Serwell instruments INC.,
22. Incubator Shaker – C24, New Brunswick Scientific, USA
23. Laminar hood – Cleanair

24. Mettler balance – Mettler H18 Neo-Pharma Private Limited, Switzerland.
25. Nuclear Magnetic Resonance Spectrometer – BRUCKER AMX500 (Germany)
26. PCR machine – Primus, MWG-BIOTECH
27. pH meter – Control dynamics
28. Phase Contrast Microscope – OLYMPUS BX40, Japan
29. Refrigerators – Godrej ultra and Godrej Pentacool
30. Scanning Electron Microscope – LEO-435, Cambridge, UK
31. Spectrophotometer – Shimadzu, UV-160, Japan
32. Speed vacuum concentrator with Lyophilizer attachment– Biotron, Korea
33. Universal texture measuring system – LLOYD instruments LR5K, UK
34. Water-bath – Culture instruments

M.5. Cultivation and production

Maintenance and PHA production media used are given in Table 3. Details of culture methods generally used in various experiments are described below:

M.5.1. Sterilization of the media and glass wares

Media used for growth, production of PHA and for the maintenance were sterilized in flasks/tubes plugged with cotton, autoclaved at 121^0C and 15-lbs pressure for 20 min. Glass wares such as petri plates and pipettes were sterilized in copper container at 180 ^{o}C for 2 h.

M.5.2. Cultures

The bacterial cultures were isolated from the soil. Isolations were carried out after serial dilution of samples (1 g) in saline (9 ml). Diluted samples were streaked on to respective agar plates, and plates were incubated at 30 ^{0}C for 24-48 h. Colonies developed were isolated, purified by streaking and pure colony was transferred on to agar slant and allowed to grow for 18 h.

Table 3: Various media used for the maintenance and cultivation of *Bacillus* spp. and for the production of polyhydroxyalkanoate.

Maintenance Media Composition (g/l)	**Inoculum media Composition (g/l)**	**Production media Composition (g/l)**
Medium 1:	**Medium 1:**	**Medium 1:**
Nutrient Agar (pH 7)	Complex medium (pH 7)	PHA medium (pH 7)
Peptone 5.0	Peptone 5.0	Na_2HPO_4 2.2
NaCl 5.0	Beef extract 5.0	KH_2PO_4 1.5
Beef extract 3.0	Yeast extract 2.5	$(NH_4)_2SO_4$ 1.5
Agar 15	$(NH_4)_2SO_4$ 2.5	$MgSO_4.7H_2O$ 0.2
		Sucrose 20
Medium 2:	**Medium 2:**	**Medium 2:**
PHA agar (pH 7)	Nutrient Broth (pH 7)	KH_2PO_4 13.5
Na_2HPO_4 2.2	Peptone 5.0	Citric acid 1.7
KH_2PO_4 1.5	NaCl 5.0	$(NH_4)_2 HPO_4$ 4.0
$(NH_4)_2SO_4$ 1.5	Beef extract 3.0	$MgSO_4.7H_2O$ 1.2
$MgSO_4.7H_2O$ 0.2		Tryptone 2.0
Yeast extract 1.0		Sucrose 20
Sucrose 10		
Agar 15		
	Medium 3:	
	PHA medium (pH 7)	
	Na_2HPO_4 2.2	
	KH_2PO_4 1.5	
	$(NH_4)_2SO_4$ 1.5	
	$MgSO_4.7H_2O$ 0.2	
	Yeast extract 1.0	
	Sucrose 10	

M.5.3. Maintenance

The bacterial cultures were maintained on nutrient agar slants or PHA agar slants. Culture was incubated at 30 ^{0}C for 18 h and then refrigerated at 4 ^{0}C till further use. For long time storage, 18 h old cells were harvested from the broth and the pelleted cells were lyophilized in vials, sealed under vacuum and stored at 4 ^{0}C.

M.5.4. Inoculum

For inoculum development, a loopful of growth from 18 h old slant culture was transferred to 10 ml of sterile inoculum medium contained in test tubes and incubated at 250 rpm at 30 ^{0}C for 18 h and used for inoculating the production medium, usually at 10% v/v. Viable cell count was determined by plating the inoculum on to agar plates, incubating the plates at 30 ^{0}C up to 48 h and counting the number of colonies formed. Viable cells were represented as CFU/ml. The inoculum contained 2 x 10 ^{4} CFU/ml and it was used at 10% (v/v) level.

M.5.5. Shake flask cultivation

Shake flask cultivation was carried out by inoculating the production medium with 10% (v/v) of inoculum and the flasks were incubated on a shaker at 250 rpm for 48 to 72 h at 30 ^{0}C. All the experiments were carried out in triplicate.

M.5.6. Fermentor cultivation

Selected cultures were cultivated in 3l fermentor (New Brunswick Bioflo 110) or Scigenics fermentor at 30 ^{0}C and pH 7 above 40% of dissolved oxygen. pH was maintained automatically by using 10% (v/v) H_2SO_4 and NaOH solutions. The dissolved oxygen was maintained above 40% of the air saturation level by varying the agitation and airflow through cascading mode automatically. Fermentor cultivations were carried out in duplicate experimentation.

M.5.7. Statistical analysis

Statistical analysis of the results obtained for shake flask and fermentor experiments was carried out using computer based Microsoft excel programme with nonbiased or n-1 method.

M.5.8. Staining techniques

Gram staining:

Crystal violet:

Solution A: 2gms of crystal violet was dissolved in 20 ml of 95% ethyl alcohol.

Solution B: 80 ml of 1% ammonium oxalate solution.

Solution A and solution B were mixed and filtered.

Gram's Iodine: 1 gm of iodine was dissolved in 300 ml of potassium iodide (2g) solution Absolute alcohol

Safranin solution: 0.25 gm of safranin was dissolved in 10 ml of ethyl alcohol and made up to 100 ml by distilled water.

The staining was carried out as follows:

a) Heat fixed bacterial smear was stained with crystal violet and allowed for 30 seconds.

b) The smear was washed with distilled water and then Gram's iodine was flooded and left for 30 seconds.

c) Slide was washed with absolute alcohol to remove excess of stain.

d) Sample was counter stained with safranin (Prescott *et al.,* 1990).

M.5.9. Intracellular PHA staining

Sudan group of dyes has been used to observe lipids in the cell; this dye is more soluble in lipid material and imparts a black-blue color to the PHA granule (Bartholomew, 1981; Murray *et al.,* 1994). For PHA staining 0.3% of Sudan black dye was dissolved in poly ethylene glycol and filtered. Safranin solution (0.5%) was prepared in water. Cells were stained by applying sudan black stain on the heat fixed bacterial smear, allowed to react for 20 min, washed with distilled water and counter stained with safranin and observed under microscope.

M.5.10. Endospore staining

Reagents: 5% malachite green (aqueous), 0.5% saffranin (aqueous)

The heat fixed sample smear was first flooded with malachite green stain and steamed for 5 min. The slide was washed with water and counter stained with Gram's safranin for 1 min and observed under microscope.

M.6. Analysis and characterization

M.6.1. Biomass estimation

Biomass was measured by gravimetric method. Culture samples (20ml) were centrifuged at 7000 rpm for 15 min; the sedimented cells were washed with distilled water, dried in the oven at 70 ^{0}C to a constant weight (Kshama and Shamala, 2003).

M.6.2. PHA estimation

The polymer was estimated gravimetrically by the method of Williamson and Wilkinson (1958). The dried biomass was suspended in 5% sodium hypochlorite solution (1 ml for 20mg of biomass) and hydrolyzed at 37 ^{0}C for 1 h. The hydrolysate was centrifuged and the sediment was washed with distilled water, acetone, diethyl ether and absolute alcohol. The pelleted material, which contained PHA, was suspended in chloroform and the clear solvent layer was air-dried in a petri plate of known weight. The polymer was further dried at 70 ^{0}C to a constant weight (Williamson and Wilkinson, 1958; Berger *et al.,* 1989). PHA was also estimated by GC analysis using lyophilized cells. Details of the method are given under 2.6.6.

M.6.3. Scanning Electron Microscopy (SEM)

Bacterial cells were first subjected to primary fixation using 2.5% glutaraldehyde and subsequently washed with increasing concentration of acetone from 10 to 100% and vacuum dried in a desiccator. Then it was placed on a plate (anode) and coated with gold dust (cathode). The coated sample was exposed to a beam of electrons released from a tungsten filament under vacuum in LEO-435 VP scanning electron microscope. The electron dispersed from the material was captured as an image in the detector. The

detector used was secondary electron detector. The signal from the detector amplified and displayed on cathode ray tube screen at a magnification of 500 x and 700 x.

M.6.4. Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR was carried out according to reported method (Sun *et al.,* 1999) for PHA containing cells and isolated PHA as follows:

(a) Freeze dried cells were directly suspended in chloroform and placed on KBr window and spectrum was taken at 400-4000 cm^{-1} in FTIR-spectrometer (Perkin Elmer, USA)

(b) Standard P(HB), P(HB-co-HV) and purified sample PHA were dissolved in chloroform and layered on KBr window, allowed to dry and were subjected to FT-IR analysis.

(c) Alternatively, standard and sample PHA (5 mg) were mixed with 100 mg of FTIR grade KBr and pelletized. The FTIR spectrum was recorded at 400-4000 cm^{-1}.

M.6.5. Extraction of PHA for analysis

For GC, GC-MS, DSC and NMR analysis PHA was extracted from lyophilized cells with chloroform for 2-4 h at 45 ^{0}C and the clear filtrate was mixed with 2 volumes of hexane or 4 volumes of cold methanol to precipitate the polymer. The polymer was rinsed with 100% hexane or methanol and dried at 50 ^{0}C to a constant weight.

M.6.6. Gas chromatography

Freeze dried cells were directly subjected to methanolysis by heating at 100 ^{0}C for 140 min in the presence of sulfuric acid to get methyl ester of PHA, followed by GC (Brandl *et al.,* 1988). Methanolysis was carried out in sealed test tubes with 1 ml chloroform, 0.85 ml methanol and 0.15 ml concentrated H_2SO_4 . After cooling, seal was broken, deionized water was added, the contents were homogenized and the bottom phase was used for GC analysis. Methyl esters were analyzed in Fisons 2000 GC, with a flame ionization detector, in a 30 m DB-1 (fused silica gel-polymethyl siloxane) capillary column (ID 0.25 mm and film thickness 0.25 μm). N_2 1 ml/min was used as a carrier gas. The temperature of the injector and the detector were maintained at 220 and 250 ^{0}C,

respectively. The program used for analysis was; 55 ^{0}C for 7 min; rise to 4^0 C/min up to 100 ^{0}C; 10 ^{0}C/min rise up to 200 ^{0}C and hold at 200 ^{0}C for 10 min (Kshama and Shamala, 2003). P(HB) and P(HB-co-HV) were used as standards. Benzoic acid (Sigma, USA) was used as internal standard.

M.6.7. Gas chromatography-Mass spectrometry (GC-MS) analysis

GC-MS analysis was performed with Perkin Elmer Instrument, model Turbo Mass Gold (Switzerland), equipped with DB-1 column, the details of the column and conditions used are mentioned under 2.6.6. Spectra were obtained as electron impact (EI) spectra (50 eV).

M.6.8. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry was used to measure the melting temperature of the polymer. 5 mg of PHA samples were used for the analysis. The parameters used were: hold for 10 min at 40 ^{0}C, heat from 40 ^{0}C to 185 ^{0}C at 5 ^{0}C /min, cool from 185 ^{0}C to 40 ^{0}C at 5 ^{0}C /min. PHB and P (HB-co-HV) were used as standards.

M.6.9. Nuclear magnetic resonance (NMR) spectroscopy

Pure PHA 5 mg/ml was dissolved in 1 ml of $CDCl_3$ (deuterated chloroform) and subjected to ^{1}H with 500MHz on an AMX 500 (Burker 500) spectrophotometer. ^{1}H spectra were obtained at 27 ^{0}C, 45 ^{0} pulse angle, 10.5 ms pulse width, 10000 Hz spectral width, 3 s pulse repetitions, 32768 data points, 32 accumulations. ^{13}C NMR was carried out using 20 mg of sample. ^{13} C spectra were recorded at 27 ^{0}C with 8.5 ms pulse width (45 ^{0} pulse angle), 2 s pulse repetitions, 27100 Hz spectral width, 16384 data points, 22528 accumulations. Standards used were PHB and P (HB-co-HV).

M.6.10. Crotonic acid assay

Law and Slepecky (1996); Slepecky and Law (1960), developed a method where P(HB) is converted into crotonic acid by treating with sulfuric acid and the product produced is measured by UV spectroscopy at 235nm. 5 mg of PHA was dissolved in 1 ml of chloroform and 20 µl from this was mixed with 5 ml of concentrated H_2SO_4 and

boiled for 10 min and the colour formed was measured at 235 nm (Shimadzu, UV 160, Japan). Standard P(HB), P(HB-co-HV) up to 100 µg/test was used for construction of standard graph. Purity of the tested sample was obtained by comparison of OD value of sample with that of standard.

M.6.11. Sugar estimation

Reducing sugar estimation was carried out according to the method of Miller (Miller, 1959). Reducing sugar will react with dinitro salicylic acid in alkaline condition to give orange to dark brown color, which can be measured by spectroscopy at 540nm.
100 ml of reagent contained 1g of 3.5-Dinitrosalicylic acid, 20ml of 2N NaOH and 30g of Na-K-tartarate. Sample containing sucrose (5 ml) was inverted overnight at room temperature with a solution (1 ml) containing water and HCl in 1:1 proportion. The sample was neutralized and made up to a certain volume. To 1.5 ml sample, 2 ml of DNS was added and the sample was then boiled for 5 min. Sample was cooled and 20 ml of distilled water was added, contents were thoroughly mixed and absorbance was measured at 540nm against reagent blank in a spectrophotometer. Standard graph was prepared by using glucose in the range of 100 µg to 1000 µg.

Total sugar of the sample was also estimated by phenol sulphuric acid method.
Reagents: 5% Phenol and concentrated Sulphuric acid (Sawhney and Singh, 2006). Appropriate amount of the sample was made up to 0.5 ml by water. 300 µl of 5% phenol and 1.8 ml of concentrated sulphuric acid were added and the mixture was allowed to stand for 20-30 min and the colour developed was measured at 485 nm.

M. 6.12. Protein estimation

Protein content of the sample was estimated by Lowry's method (Lowry *et al.,* 1951).
Reagent: Alkaline copper sulphate reagent (ACR) and Folin Ciocalteu (FC) reagent.

To 0.5 ml of sample, 5 ml of ACR reagent was added and the mixture was incubated at room temperature for 10 min. 0.5 ml of 1:1 FC reagent was added to the mixture and the sample was allowed to stand for 20 min and the colour developed was

measured at 660nm. Standard graph was prepared by using bovine serum albumin up to at a concentration of 120 mcg/ml.

M.6.13. Molecular weight determination

The viscosity of PHA at different concentrations in chloroform was measured using a viscometer (Oswald viscometer) at 20 ^{0}C. Specific viscosity and reduced viscosity were calculated. The graph was plotted by considering reduced viscosity against different concentration of PHA in the solution. The point of intersection on reduced viscosity was taken as intrinsic viscosity and was substituted in the formula mentioned below.

Molecular weight was determined by viscosity-average molecular weight (Mv) using the Mark Houwink relationship, $[\eta] = KM^{\alpha}$ where $[\eta]$ is the intrinsic viscosity, M is the molecular weight, and K and α are constant for the particular solute-solvent-temperature combination (Barham *et al.,* 1984).

M.7. Molecular biology methods

M.7.1. Genomic DNA isolation

Bacterial DNA was isolated according to standard method (Sambrook *et al.,*1989).

Reagents: Lysis buffer (20mM Tris HCl, 2mM EDTA, Triton X 1%, lysozyme 20 mg/ml), SDS solution-20%, saturated phenol, chloroform, 3M-sodium acetate, 1X TE buffer pH 8 (Tris HCl 10mM, EDTA 1mM) 0.8% agarose gel.

Protocol:

- Overnight grown culture was taken in microcentrifuge tubes and was centrifuged at 8000 rpm for 10 min
- The pellet was washed with distilled water
- 200 µl of lysis buffer was added and incubated at 37 ^{0}C for 1 h in a shaker
- 50 µl of 20% SDS was added to the pellet
- Equal volume of cold saturated phenol was added and mixed thoroughly and centrifuged at 10,000rpm for 10 min

- Supernatant was transferred to fresh tube and equal volume of chloroform was added and centrifuged 10,000rpm for 10 min
- Supernatant was mixed with 30 µl of 3 M sodium acetate
- 300 µl of ice-cold absolute alcohol was added and the sample was centrifuged
- Pellet was washed with 70% ethyl alcohol and air-dried
- Dried pellet was dissolved in 20 µl of 1X TE buffer of pH 8 and placed on agarose gel.

M.7.2. Agarose gel electrophoresis

Reagents: 0.8% agarose gel, 50X and 1X TAE buffer (Tris base-242 g/l, Glacial acetic acid-57.1 ml/l, 0.5 M EDTA-100 ml/l for 50X), loading dye 6X (Xylene cyanol blue- 0.25% and Glycerol 30%), Ethidium bromide solution (0.5 mcg/ml).

Protocol

- 0.8% of agarose with 2% of 50X TAE was prepared and poured into the boat that was sealed with adhesive tape and fitted with comb
- Agarose was allowed to solidify, then comb and adhesive tape were removed
- Gel was placed in the electrophoresis tank containing 1X TAE buffer
- The samples were mixed with 2 µl of loading dye for 5-10 µl of sample
- Sample was run at 100 volts until the dye reached 75% of the gel area
- The gel was removed from the tank and placed in ethidium bromide solution for 5-10 min
- The gel was destained in distilled water, examined on UV transilluminator (Bio-Rad) and documented.

M.7.3. Polymerase chain reaction (PCR)

The PCR mixture (described below; primer details are given in respective chapters) was taken in a sterile PCR tubes and mixed well and placed in thermocycler (Primus, MWG-BIOTECH). Reaction was carried out by using an initial denaturation of 95 ^{0}C for 3 min followed by 35 cycles of denaturation at 94 ^{0}C for 40 sec, annealing temperature of 46 ^{0}C for 45 sec and elongation at 72 ^{0}C for 15 min. After the completion of reaction amplification was confirmed by gel electrophoresis.

PCR components used for 25 µl of reaction mixture were:

Template DNA	2.0 µl
Taq DNA polymerase	0.3 µl
Taq buffer 10X	2.5 µl
DNTPs	0.5 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Sterile pure water	18.7 µl
TOTAL	25 µl

Electrophoresis was carried out as mentioned earlier using 0.8% agarose gel.

M.7.4. PCR product purification

The PCR product was purified by using SIGMA – Gene Elute PCR clean – up kit as per the enclosed information.

M.7.5. Ligation of purified PCR product into 'T'- tailed vector

The purified PCR product was cloned to a vector by using Ins T/A clone PCR product cloning kit (MBI Fermentas).

Using the following reaction mixture ligation was carried out:

Vector	2 µl
Purified PCR product	5 µl
Ligase buffer	2 µl
T_4 DNA ligase	2 µl
PEG	2 µl
BSA	0.5 µl
Sterile water	6.5 µl
TOTAL	20 µl

M.7.6. Transformation

The cloned plasmid was transformed to target organism host by using instant cloning kit (Genei, Banglore).

M.7.7. Preparation of competent cells

Reagents: LB medium (Tryptone-1%, Yeast extract-0.5%, NaCl-1%), 0.1M $CaCl_2$, ampicillin

- Single colony was picked up from an overnight grown target organism and inoculated to a LB broth and incubated at 37 ^{0}C and 150 rpm till the OD of the culture reached 0.40-0.5.
- Culture was transferred aseptically to polypropylene tubes and stored in ice for 10 min
- The culture broth was centrifuged at 4000 rpm for 10 min at 4 ^{0}C to collect the cells
- The supernatant was discarded and pellet was suspended in ice cold 0.1M $CaCl_2$ solution
- The cells were collected by centrifugation at 4000 rpm for 10 min at 4 ^{0}C
- The supernatant was discarded and cells were again resuspended in 2 ml of ice cold 0.1 M $CaCl_2$ and stored at 4 ^{0}C up to 24 – 48 h

M.7.8. Transformation

- ✓ 100 µl of competent cells was added to about 50 µl of ligated product and mixed gently and kept in ice for 30 min
- ✓ The mixture was kept at 42 ^{0}C for 90 sec
- ✓ The mixture was immediately chilled by keeping it in ice for 1-2 min
- ✓ 800 µl of LB medium was added to the culture and incubated for 45 min at 37 ^{0}C at 150 rpm
- ✓ 100 µl of transformed mix was plated onto LB agar plate containing 100 mcg/ml of ampicillin and incubated to 37 ^{0}C for 24 h.

M.7.9. Plasmid isolation

Reagents: LB medium (Tryptone-1%, Yeast extract-0.5%, NaCl-1%), ampicillin, TE buffer

Solution I: 50mM glucose, 25 mM Tris-Cl, 10mM EDTA (pH 8)

Solution II: 0.2 N NaOH, 1% SDS

Solution III: 5mM Potassium acetate 60%, Glacial acetic acid 11.5 %, made up to 100 ml by distilled water

- A single colony from the plate was picked and inoculated into 2 ml of LB broth containing ampicillin (0.1mg/ml) and allowed to grow overnight at 37 ^{0}C and 180 rpm
- Overnight grown culture was then transferred to a micro centrifuge tube and centrifuged at 10,000 rpm for 2-3 min
- Solution I was added and vortexed vigorously until the pellet homogenized and then the sample was placed in ice for 5 min
- 200 µl of solution II was added and mixed gently by inverting the tubes were stored on ice
- 300 ml of chilled solution III was added and mixed gently and then centrifuged
- Supernatant was transferred in to a fresh tube and 600 µl of isopropyl alcohol was added and kept at –20^0 C for precipitation
- The sample was centrifuged and supernatant was discarded
- 150 µl of 70% ethyl alcohol was added and centrifuged
- The pellet was air dried and dissolved in 20 ml of TE buffer and analyzed by gel electrophoresis.

The recombinant plasmid obtained was then subjected to restriction digestion as given below:

M.7.10. Restriction digestion

Recombinant plasmid	5 µ l
TY Tango buffer 2X	6 µ l
Restriction enzyme	2 µ l
Restriction enzyme	2 µ l
Sterile pure water	10 µ l
TOTAL	20 µ l

The mixture was incubated overnight at 37 ^{0}C in a water bath and the insert release was confirmed by gel electrophoresis.

Chapter 1

Isolation, identification and characterization of polyhydroxyalkanoate producing Bacillus sp

1.1. Introduction

The genus *Bacillus* is widely distributed in nature and most commonly found in soil. It comprises of gram-positive, endospore forming, aerobic bacteria. Most of the *Bacillus* species are harmless to humans and animals; a few pathogens are identified, which includes *Bacillus anthraces* and *Bacillus cereus*.

Bacillus is a large and very diverse group of organisms. *Bacillus subtilis* was described as early as in 1835, as *Vibrio subtilis* by Ehrenberg. In 1864 Davaine gave the name *'bacteridium'* to the organism associated with anthrax. Cohn, in 1872 proposed the genus name "*Bacillus"*. The systematic of this genus has undergone massive changes. Division of the genus was started in 1991 by applying molecular techniques to bacterial systematic. 16SrRNA oligonucleotide cataloguing information showed the existence of phylogenetically distinct clusters, which provided the basis for the division of *Bacillus* into several phylogenetically distinct genera. Several groups of species have been separated from the core of *Bacillus* and described as new genera. In fact, there are actually still more species in *Bacillus* than in all other genera (Berkley *et al*., 2002).

Lemoigne (1926) isolated and characterized the P(HB) from *Bacillus megaterium*. Since this first report, PHA accumulation has been found in many microorganisms. Bacteria synthesize and accumulate PHA as carbon and energy source or as a sink of reducing power under nutrient limiting condition in the presence of excess of carbon. When the supply of limiting nutrients is restored, PHA can be degraded by intracellular depolymerases and subsequently metabolized as a carbon and energy source.

Many bacteria have been screened for the production of PHA. PHA synthases of *B. megaterium* is known to be distinctly different from all known PHA synthases (McCool and Cannon 1999) and hence it appeared feasible to isolate *Bacillus* sp. which is not reported so far as a PHA producer. The sequence of 16SrRNA gene was used for this purpose as it has been widely employed to estimate the relationship between the phylogeny and also it is most commonly used as a rapid tool for the identification of unknown bacterium up to the species level (Sacchi *et al*., 2002).

1.2. Materials and Methods

1.2.1. Isolation

Bacillus spp. were isolated from the soil collected locally from municipal waste disposal yard. Soil sample (1g) was preheated at 50 ^{0}C, 10 min to inactivate the vegetative bacterial cells, cooled and serially diluted using saline (9 ml) and plated on nutrient agar (Himedia, Mumbai, India). Colonies were streaked on nutrient agar plate for purification and purified isolates were screened for PHA production and the culture, which gave maximum yield of the polymer, was selected for further studies. Selected cultures were maintained on nutrient agar slants at 4 ^{0}C and sub cultured once in a month.

1.2.2. Screening of *Bacillus* spp. for PHA production

Bacillus spp. were grown up to 72 h, in shake flasks according to details given under materials and methods (common for chapters), sections M.5.3 to M.5.5, with medium 2 for inoculum and medium 1 for production (Table 3). The cells were stained by sudan black staining (M.5.9) to identify the PHA producers. Cells were obtained by centrifugation and PHA was estimated in the dry biomass by hypochlorite digestion and solubilisation in chloroform (M.6.1 and M.6.2).

1.2.3. Identification

Selected culture was identified by morphological characterization, biochemical method and 16SrRNA method.

1.2.3.1. Morphological characterization

Cells were initially stained by gram stain and endospore staining [Materials and methods (common for chapters), sections M.5.8 and M.5.10] and observed under microscope.

1.2.3.1.1. Cell size

Bacterial cell size was measured using ocular micrometer (one scale =1/10mm) and stage micrometer (1 scale division =1/100mm) in the microscope. The ocular

micrometer was calibrated by using the stage micrometer. At least 20 cells were considered for length and breadth measurements and average values were recoded for cell size.

1.2.3.2. Biochemical methods

Various biochemical tests were carried out according to reported methods (Reddy and Reddy, 2000).

1.2.3.2.1. Catalase test

Requirements: 24 h culture and H_2O_2

1ml of 3% H_2O_2 was poured on to agar slant culture and observed for evolution of bubbles. This test was also carried out using the fresh culture broth, which was placed on the slide containing a drop of hydrogen peroxide. Evolution of bubbles indicated a positive test for catalase.

1.2.3.2.2. Oxidase test

Requirements: 24 h culture, reagent tetramethyl-p-phenylenediamine-dihydrochloride (TPH).

2-3 drops of TPH was added to the culture tube. Oxidase positive culture was identified by the change in colour of the added reagent from pink to maroon.

1.2.3.2.3. Growth on different carbohydrates

Requirements: Different sugars, phenol red indicator (0.2 g of phenol red was dissolved in 500 ml ethyl alcohol (95%), 500 ml of distilled water)

The cultures were tested for growth and acid production on different carbon sources. Carbohydrates such as fructose, mannose, cellobiose, rhamnose, maltose, glucose, raffinose, galactose, sucrose, trehalose, dulcitol, adonitol, arabinose, sorbitol, xylose, mannitol, lactose, melibiose and inulin were used as carbon sources instead of sucrose in PHA medium [Materials and methods (common for chapters), Table 3: Production medium 1]. These carbon substrates were used at 2% level in the medium. The medium also contained phenol red solution (0.3%). The tubes were incubated at 30

^{0}C. The production of acids was detected by observation of change in colour of the dye from red to yellow.

1.2.3.2.4. Indole test

Requirements: Nutrient broth cultures and Kovac's reagent (para dimethyl amino benzaldehyde 5g, amyl alcohol 75ml, concentrated HCl 25ml).

Some bacteria oxidize tryptophan resulting in the formation of indole, pyruvic acid and ammonia. Indole thus formed reacts with Kovac's reagent resulting in the formation of cherry-red colored complex.

Young culture of the bacterium to be tested was mixed with about 10 drops of Kovac's reagent and formation of cherry red colour ring indicated positive test for indole.

1.2.3.2.5. Methyl red

Requirements: MR-VP broth tubes, (Peptone – 7g/l, K_2HPO_4 – 5 g/l, Dextrose – 5 g/l) methyl red and Barrit's reagent.

To the cultures grown in MR-VP broth, 1-2 drops of methyl red was added and mixed. Retention of methyl red colour indicated positive test.

1.2.3.2.6. Voges - Proskauer test

Requirements**:** MR-VP broth tubes and Barrit's reagent

To cultures grown in MR-VP broth, 1-2 drops of Barrit's reagent was added and contents were mixed. Appearance of crimson to ruby pink colour indicated positive result.

1.2.3.2.7. Citrate test

Requirements: Simmon's citrate agar medium (composition g/l: Ammonium dihydrogen phosphate 1 g, Dipotasium phosphate 1g, NaCl 5g, sodium citrate 2g, Magnesium sulphate 0.2g, Agar 15g and Bromo thymol blue 0.08).

The cultures were grown on Simmon's citrate agar medium at 37 ^{0}C. Change in the colour of agar purple to blue indicated positive result.

1.2.3.2.8. Starch hydrolysis

Requirement: Starch, iodine solution

The cultures were grown in the medium containing 1% of soluble starch for 24 h at 37 ^{0}C. At the end of the incubation period the plate was flooded with Gram's iodine solution. Excess of iodine was decanted to observe decolourized zones around the colonies where amylase produced from the organism degraded the starch to give clear zone. The zone was measured and expressed in mm.

1.2.3.2.9. Gelatin hydrolysis

Requirements: Gelatin containing nutrient broth medium and 24 h bacterial culture

Microorganisms are known to produce the enzyme gelatinase, which acts on gelatin to liberate amino acids. The organisms were inoculated to the medium-containing gelatin and incubated for 24 h at 37 ^{0}C. Observation of liquefaction of gelatin even at chilled temperature was considered as positive test for gelatinase.

1.2.3.2.10. Nitrate reduction test

Requirements: Solution A (Sulfanilic acid 8 g in 1 liter of 5 N acetic acid), Solution B (alpha-naphthylamine 5 g in 1 liter of 5 N acetic acid).

Some organisms possess the enzymatic capacity to convert nitrate to nitrite. This is determined by the addition of reagents. Solution A and B were added to 24 h culture broth and the appearance of red colour indicated positive test for nitrate reductase.

1.2.3.2.11. Urease test

Requirements: Urea agar medium (Casein hydrolysate 1 g/l, NaCl 5 g/l, potassium dihydrogen phosphate 2 g/l, glucose 1 g/l, urea 20 g/l, agar 20 g/l, phenol red 6 ml –of 0.2% conc.).

The cultures were grown on the urea agar medium containing phenol red. After the incubation period the plates were observed for the change in colour. The change in colour from red to deep pink indicated urease activity.

1.2.3.2.12. Growth at 60^0 C

The cultures were grown in the nutrient broth medium at 60 ^{0}C and increased turbidity in the medium indicated the growth of the organism.

1.2.3.2.13. Growth in different concentrations of salt

Different concentrations of salt i.e., NaCl at 0.5, 1, 3, 5 and 10% (g%, w/v) was used in nutrient broth and inoculated tubes were incubated at 37 ^{0}C. The turbidity in the medium indicated the growth of the organism.

1.2.3.2.14. Growth in 1% glucose

Glucose was added at 1% concentration level to the nutrient broth medium, tubes were inoculated and incubated at 37 ^{0}C. Increased turbidity in the medium indicated the growth of the organism.

1.2.3.2.15. Growth in the medium containing glucose and ammonium sulphate

Glucose at 1% (g%, w/v) and ammonium sulphate at 0.15% (g%, w/v) level were added to nutrient broth medium and inoculated culture tubes were incubated at 37 ^{0}C. Enhanced turbidity in the medium indicated the growth of the organism.

1.2.3.3. Identification by 16SrRNA method

16SrRNA gene sequence analysis has been widely used for the identification of the species. The sequence of 16SrRNA provides a measure of genomic similarity above the level of species allowing comparisons of relatedness across the genus, family etc.

In the present study the 1.2kb of entire 16SrRNA gene from the selected isolate was sequenced which involved, isolation and purification of chromosomal and plasmid DNA, PCR, preparation of competent cells, transformation of *E coli* and DNA cloning by standard procedures [Materials and methods (common for chapters), section M.7). The 16SrRNA gene was amplified by PCR using the DNA of the organism to be identified as a template. Two synthetic oligonucleotides primers used were:

5'CTCTAGAGCGATTACTAGCGATTCCGACTTCG3'
5'CGACGTCGGCTCAGGATGAACGCTGGCGGC3'.

The purified PCR product was cloned into pTZ57R/T vector by PCR product cloning kit (MBI Fermentas). Ligated products were transformed into *E. coli* (DH5α) host. Ligation was confirmed by agarose gel electrophoresis of the isolated plasmid from the transformed colonies. Isolated plasmids were subjected to double digestion using XbaI and BamHI for insert release and sequenced (Genei, Bangalore). The sequence was compared by using the BLAST program from the National Center for Biotechnological information (NCBI) gene bank for the identification of the species.

1.2.4. Optimization of cultural parameters for growth of selected isolate

To optimize cultural parameters initially, *B. flexus* which was identified, characterized and selected for PHA production was grown at different pH (2-11), temperature (10-60 ^{0}C) and NaCl concentrations (0.5 – 10%, w/v). Medium and shake flask culture conditions used have been mentioned earlier [Materials and methods (common for chapters), Table 3: medium 1 under inoculum and production media; sections M.5.4 and M.5.5). At the end of 72 h of cultivation period, optical density of the cultures were measured at 620 nm.

1.2.5. PHA production by standard isolates

In order to find out the capacity of isolated strain of *B. flexus* to produce PHA, its growth and PHA production were compared with that of standard *Bacillius* spp. such as *Bacillus subtilis* 168 (ATCC 23857), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* 4810 (Central Public health lab, London, UK), *Bacillus flexus* (MTCC 2909, Chandigarh, India), *Bacillus megaterium* (MTCC 6544, Chandigarh, India). These were cultivated in PHA production medium [Materials and methods (common for chapters), Table 3: medium 1 under inoculum and production media; sections M.5.4 and M.5.5) and PHA was quantified as mentioned [Materials and methods (common for chapters), Sections M.6.1, M.6.2 and M.6.6].

1.3. Results and Discussion

1.3.1. Screening of *Bacillus* spp. for PHA production

Twenty-five purified *Bacillus* spp. cultures were isolated from the municipal waste soil. Ability of these to synthesize PHA was analyzed. Cell dry weights and PHA content of the tested strains are shown in Table 1.1. One of the cultures gave maximum biomass of 3.1 g/l and PHA yield of 1.9 g/l (Strain no 9) and this was selected for further studies.

1.3.2. Taxonomy of the selected strain

Bacteria that produce heat-resistant endospores are classified in several genera under the family *Bacillaceae,* with the exception of the anaerobic endospore-forming bacteria. Genus *Bacillus* is the largest and best-known member of this family. Since endospore-formation is a universal feature of this group, spore morphology has traditionally been assigned considerable significance in their classification and identification. However several species have been allocated to *Bacillus* even though they are described as non-spore-formers such as *B. thermoamylovorans* (Combet-Blanc *et al.* 1995).

The selected *Bacillus* sp. isolate no 9, was observed to be a Gram positive, motile, endospore forming, rod shaped, aerobic bacterium (Table 1.2 and Fig. 1.1). This is classified as Group-1 i.e., Morphological groups of aerobic, endospore-forming bacteria, cells rod-shaped, sporangia not swollen and cell diameter less than 1μm according to *Bergey's manual of systematics* (1986). The bacterium grew well at pH 7, 30 ^{0}C and up to 5% salt concentration (Fig. 1.2).

The newly acquired polyhydroxyalkanoate producing *Bacillus* sp was identified as a strain of *Bacillus flexus* using a wide range of physiological and molecular techniques. 16SrRNA gene analysis was carried out by amplifying the gene by PCR and cloned in to pTZ57R/T vector and transformed to *E. coli.* The plasmid was isolated and subjected for restriction digestion for the confirmation of 16SrRNA fragment (Fig. 1.3). Gene sequence analysis of the plasmid was conducted (Figs. 1.4 and 1.5).

Table 1.1: PHA production by different *Bacillus* spp. isolated from soil.

Culture No.	Biomass (g/l)	PHA (g/l)	% PHA (of dry biomass)
1	0.30	0.20	66
2	1.20	0.30	25
3	1.20	0.50	41
4	2.00	-	-
5	2.00	0.50	25
6	2.30	0.80	34
7	1.20	0.20	16
8	0.90	0.20	22
9	**3.10**	**1.90**	**61**
10	0.70	0.20	28
11	1.10	0.30	27
12	2.00	0.40	20
13	2.30	-	-
14	0.90	0.30	33
15	1.20	0.60	50
16	0.80	0.20	25
17	1.40	0.10	7.1
18	1.20	0.20	16
19	2.80	0.10	3.5
20	4.00	0.10	2.5
21	1.10	0.20	18
22	2.40	0.50	20
23	0.90	-	-
24	3.50	0.90	25
25	2.70	0.60	22

The sequence of 16SrRNA gene is highly conserved in all organisms. Ribosomal RNA molecules are functionally constant, universally distributed and moderately well conserved across broad phylogenetic distances. The regions in the rRNA gene evolve at slightly different rate due to their involvement in the protein biosynthesis. 16SrRNA gene sequence are specific and occur in all organism which provides the degree of similarity among the group of the organism indicating evolutionary relatedness, which helps to place the organism in proper group.

The 16SrRNA gene sequences of other well known *Bacillus* standards *B. flexus AB021185, B. flexus AJ55046, B. megaterium, B. simplex, B. fumiriola, B. cereus, B. anthracis, B. thuringiensis, B. subtilis, B. fusiformis, B. licheniformis* was downloaded from www.pubmed.res.in and multiple sequence alignment was done using clustalW programme. Based on this, phylogram was constructed using the software available in the internet www.ebi.ac.uk (Fig. 1.6). The position of the isolated strain within the same group of species based on 16SrRNA gene was analysed by the tree constructed using maximum homology obtained from NCBI blast tool (Fig. 1.7).

The sequence similarity within the species was 99%, other closely related species of *B. flexus* was found to be *B. megaterium* having a homology of 97% and it shared the same cluster with *B. simplex*. Other *Bacillus* spp., which appeared lower in the homology, formed a distinct group. Though both the groups radiated from the same point they had a wide variation in the homology.

Based on numerical phenotypic and molecular genetic data of classification, *Bacillus* strains are distributed among 31 major (4 or more strains), 18 minor (2 or 3 strains) and 30 single member clusters (Priest *et al.,* 1988). Clusters are regarded as taxo-species. *B. flexus* forms cluster 23 which is closely related to *B. megaterium*. It has certain differences such as: cells are smaller, aesculin is not hydrolysed, acid is not formed from pentoses. It degrades casein, elastin, gelatin, pullulan, and starch. The bacterium shows urease positive reaction, Proskauer negative reaction and does not reduce nitrate to nitrite.

The bacterium is mainly isolated from soil. On agar plate culture colony developed is flat, smooth, opaque, with a diameter >0.9 um. The cells are motile, gram variable, ends of cells are round, spores are oval and centrally placed, sporulation occurs

after 72 h of growth. The isolate degraded arbutin, casein, elastin, gelatin, pectin, starch and was found resistant to erythromycin, rifamycin and tetracycline. It produced acid from fructose, galactose, glucose, glycerol, lactose, maltose, mannitol, raffinose, sucrose, trehalose, and found to utilize acetate, citrate, formate, succinate. *B. megaterium* has been reported to produce PHA, however *B. flexus* which is phylogenetically close to *B. megaterium* so far has not been reported to produce PHA and hence utilization of this bacterium for the present study appeared significant.

Table 1.2: Morphological characters of *Bacillus* sp isolate 9

Morphological characters	
Gram's Staining	+
Endospore staining	+
Size	1.5 x 0.2 μm
Shape	Rod
Sudan black staining for PHA	+
Motility	Motile
Features of the colonies	
Shape	Circular
Colour	Off-white
Surface	Smooth

Table 1.3: Physiological characters of *Bacillus* sp. 9.

Physiological characters	
Catalase	+
Urease	+
Oxidase	+
Citrate	-
Tripal sugar iron	+
Nitrate reduction	-
Indole production	+
Methyl red	+
Vogues proskauer	-
Starch hydrolysis	+
Gelatin hydrolysis	+
3% NaCl	+
1% Glucose	+
$(NH_4)_2SO_4$ + Glucose	+
Growth at 60 ^{0}C	-
Anaerobic condition with 1% glucose	+
Acid production from sugars	
Lactose	+
Manitol	+
Arabinose	+
Xylose	+
Rhamnose	+
Cellobiose	+

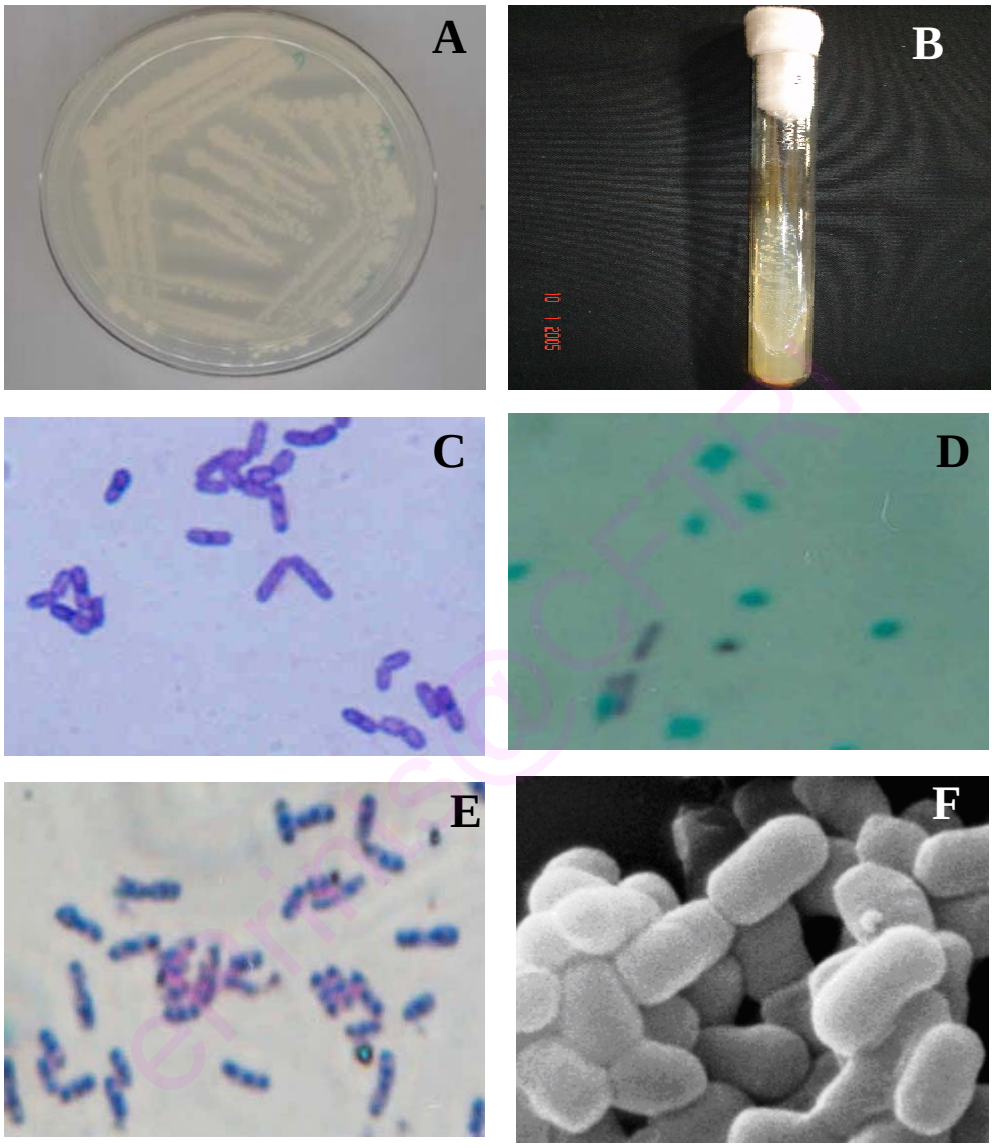


Figure 1.1: *B. flexus* cells characteristics as observed visually, by phase contrast and scanning electron microscopy. A – *B. flexus* (*Bacillus* sp strain 9) grown on agar plate, B – On agar slant, C –Gram staining (100x), D – Endospore staining (100x), E – Sudan black staining (100x) and F - Scanning electron microscope image (6Kx).

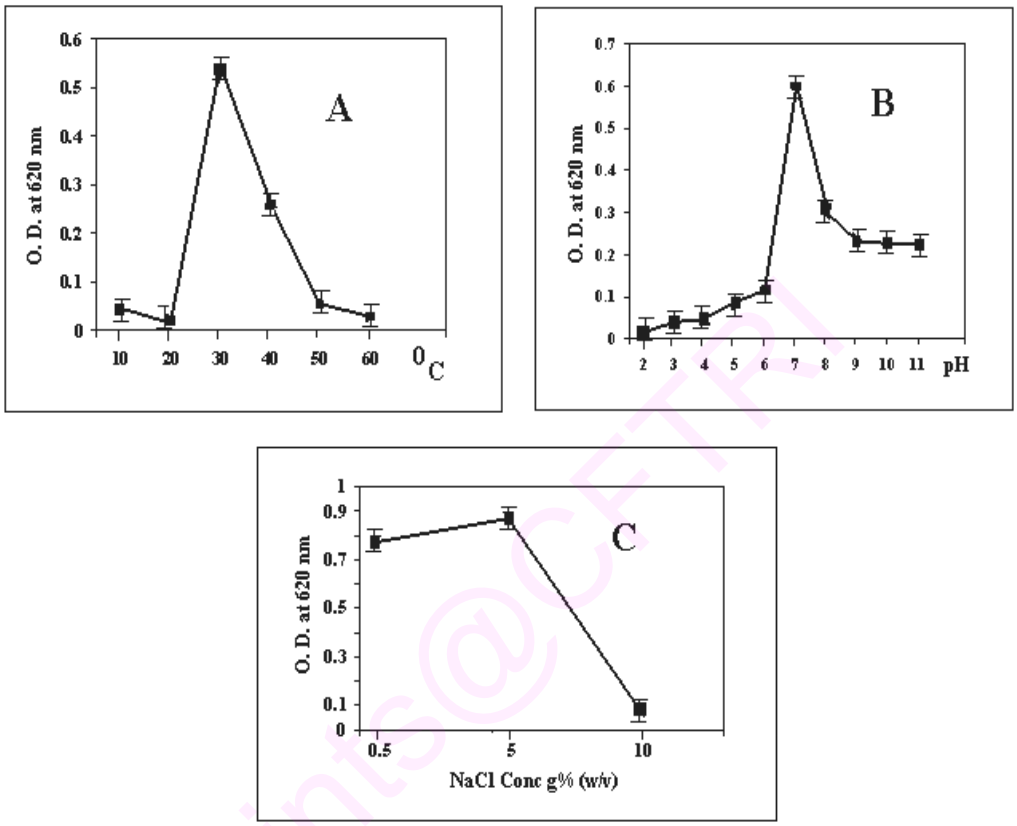


Figure 1.2: Optimization of the growth parameters for cultivation of *B. flexus.*

A – Temperature, B – pH, C – NaCl concentration.

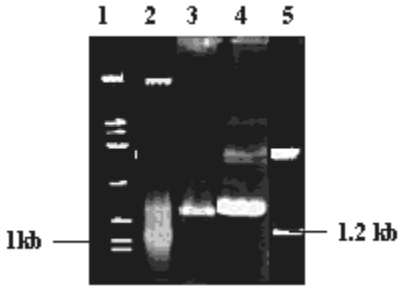


Figure 1.3: Agarose gel electrophoresis of 16SrRNA analysis.
1=Marker; 2=Chromosomal DNA; 3=Amplified DNA;
4=Recombinant plasmid and 5=Restriction digestion.

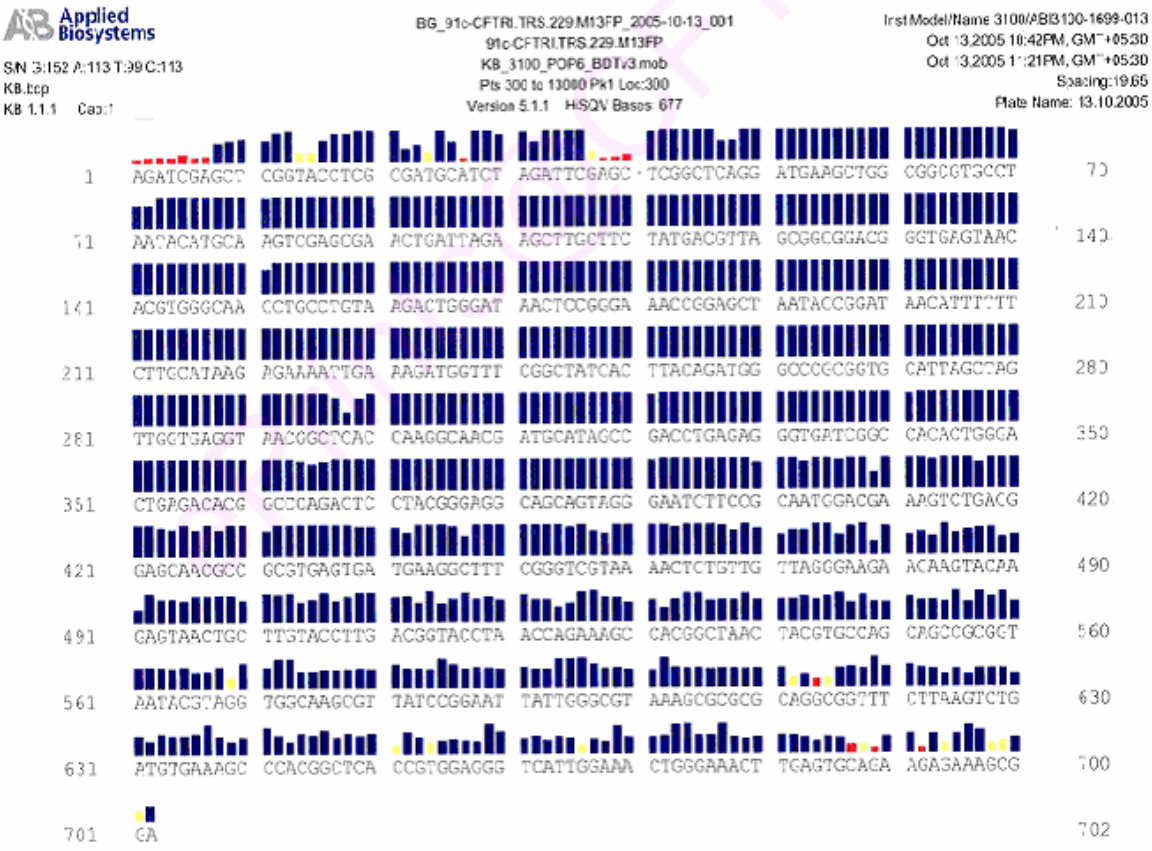


Figure 1.4: Sequence of 16SrRNA gene isolated from *Bacillus* sp. (isolate 9).

Figure 1.5: Electropherogram of the 16SrRNA gene.

Table 1.4: Blast results of 16SrRNA gene sequence of *B. flexus.*

NCBI	BLAST results		
Accession	**Description**	**Max score**	**Max ident**
DQ333292.1	Bacillus flexus isolate LLH 16S ribosomal RNA gene, partial	1199	99%
AJ550461.1	Bacillus flexus partial 16S rRNA gene	1188	99%
EF522790.1	Bacillus sp. 093903 16S ribosomal RNA gene, partial sequence	1158	99%
EF157300.1	Bacillus flexus strain XJU-3 16S ribosomal RNA gene, partial	1157	99%
DQ359936.1	Bacillus flexus 16S ribosomal RNA gene, partial sequence	1146	99%
EF127831.1	Bacillus flexus strain LF-3 16S ribosomal RNA gene, partial	1146	99%
DQ993322.1	Bacillus sp. MHS030 16S ribosomal RNA gene, partial sequence	1146	99%
DQ270722.1	Bacillus sp. B-1013 16S ribosomal RNA gene, partial sequence	1146	99%
DQ376024.1	Bacillus flexus 3xWMARB-5 16S ribosomal RNA gene, partial	1136	99%
AJ784845.1	Bacillus sp. Con a/4 partial 16S rRNA gene, isolate Con a/4	1194	99%
AF217809.1	Bacillus sp. KYJ963 16S ribosomal RNA gene, partial sequence	1182	99%
DQ270752.1	Bacillus sp. B-2036 16S ribosomal RNA gene, partial sequence	1175	99%
DQ339687.1	Bacillus flexus BRL02-65 16S ribosomal RNA gene, partial	1171	99%
AY647285.1	Bacillus flexus strain MSU1610 16S ribosomal RNA gene, partial	1168	99%
DQ837542.1	Bacillus flexus strain XJU-1 16S ribosomal RNA gene, partial	1138	99%
DQ412062.1	Bacillus sp. LOB-3 16S ribosomal RNA gene, partial sequence	1129	99%
EF101733.1	Bacillus flexus strain HU37 16S ribosomal RNA (rrnE) gene,	1120	99%
DQ514312.1	Bacillus flexus strain P27-25 16S ribosomal RNA gene, partialnce	1188	99%
AJ563600.1	Bacillus flexus partial 16S rRNA gene, strain B14	1177	99%
DQ365587.1	Bacillus flexus strain GS11 16S ribosomal RNA gene, partial	1170	99%
DQ339688.1	Bacillus flexus isolate BRL02-66 16S ribosomal RNA gene,	1151	98%
AY422986.1	Bacillus flexus 16S ribosomal RNA gene, partial sequence	1151	98%
DQ339689.1	Bacillus flexus isolate BRL02-67 16S ribosomal RNA gene,	1134	98%
DQ129520.1	Uncultured bacterium clone AKIW848 16S ribosomal RNA gene,	1125	97%
AY030338.1	Bacillus megaterium strain KL-197 16S ribosomal RNA gene,	1136	97%
EF428248.1	Bacillus megaterium strain HDYM-24 16S ribosomal RNA gene,	1133	97%
DQ532282.1	Uncultured bacterium clone KSC2-10 16S ribosomal RNA gene, e	1133	97%
DQ298266.1	Uncultured bacterium clone SR9 16S ribosomal RNA gene,	1129	97%
AY030336.1	Bacillus megaterium strain KL-181 16S ribosomal RNA gene,	1127	97%
AB271751.1	Bacillus megaterium gene for 16S rRNA, partial sequence	1120	97%
AY505510.1	Bacillus megaterium strain GSP10 16S ribosomal RNA gene,	1120	97%
AJ315065.1	Bacillus sp. 19497 16S rRNA gene	1120	97%
AM237398.1	Bacillus megaterium partial 16S rRNA gene, isolate OS-223.b	1118	97%
DQ872156.1	Bacillus megaterium strain TBA-R1-001 16S rRNA gene, partial	1127	97%

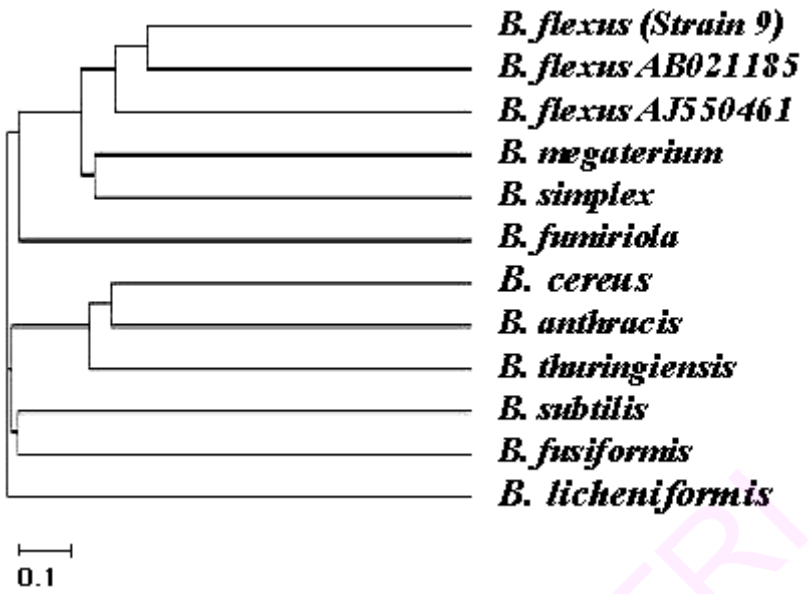


Figure 1.6: Phylogenetic tree showing the position of the selected *B. flexus* compared to other known *Bacillus* spp.

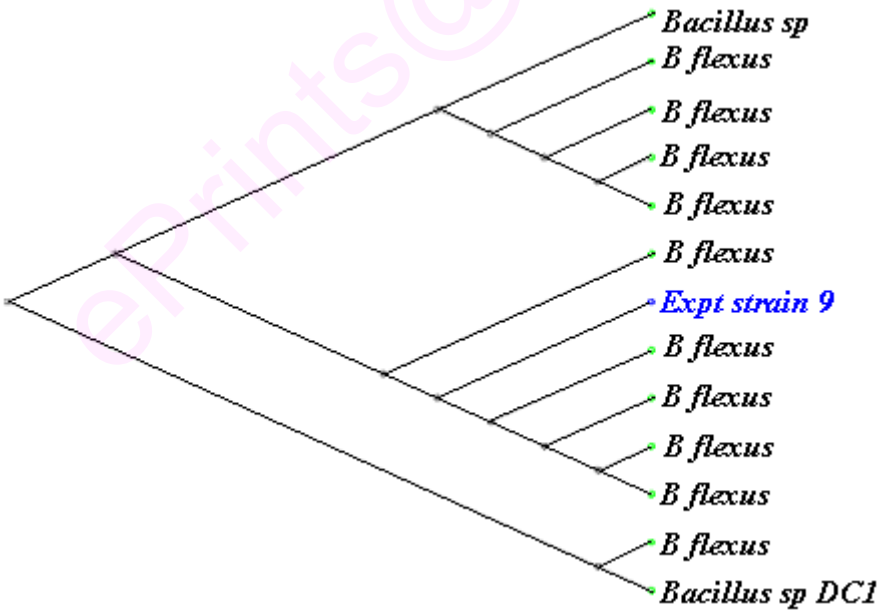


Figure 1.7: Phylogenetic tree of *B. flexus* within the species.

1.3.3. Importance of *Bacillus* spp.

The newly isolated strain of *B. flexus* was compared with other standard *Bacillus* strains for PHA production. The strain was found to produce maximum PHA compared to other standard strains and it gave 61% polymer yield on biomass weight (Table 1.5). The genus *Bacillus* has the potential to produce novel PHA with a range of monomer composition. *B. cereus* is known to produce P(HB) with 2-4% of hydroxyhexanoate (HHx) and terpolymer containing hydroxyoctanoate (HO) units (Caballero *et.al.,* 1995). Also *B. cereus* UW85 when fed with capralactone produced tercopolymer with 3HB, 3HV and 3HHx (Labuzek and Radecka, 2001). *Bacillus* sp. INT005 is reported to produce P(3HB), P(3HB-co-3HV), P(3HB-co-3HHx) and P(3HB-co-6HHx-co-3HHx) from different carbon source (Tajima *et.al.,* 2003). In *B. cereus* SPV degradation of PHA did not occur (Valappil *et al.,* 2007). Thus the capacity of the acquired new strain to produce PHA copolymers, which is so far not identified in the genus, or species and down stream processing was investigated further and are detailed in the forth coming chapters.

Table 1.5: PHA production by standard cultures.

Standard cultures	Biomass (g/l)	PHA (g/l)	% PHA (dry wt of the biomass)
Bacillus subtilis 168 (ATCC 23857)	2.38	0.00	-
Bacillus subtilis (ATCC 6633)	1.78	0.00	-
Bacillus cereus (4810, CPHL, UK)	2.26	0.20	8.8%
Bacillus megaterium (MTCC 6544)	0.98	0.16	15.0
Bacillus flexus (MTCC 2909)	3.20	0.12	3.75
Bacillus flexus (present strain 9)	**3.10**	**1.90**	**61.3**

In 1872, Ferdinand Cohn, named the Gram-positive bacterium which was capable of growth in the presence of oxygen, and formed endospore as *Bacillus subtilis.* This formed one of the representative species of a large and diverse genus of bacteria named *Bacillus*, which was placed under the Family *Bacillaceae.* Endospore formation is known to be a strategy for survival in the soil environment, wherein these bacteria predominate.

Owing to the resistance of endospores to environmental stress, long-term survival under adverse conditions, the genus is ubiquitous and can be isolated from a wide variety of sources. Additionally there is physiological and phylogenetic diversity. The phylogenetic approach to *Bacillus* taxonomy has been accomplished largely by analysis of 16SrRNA molecules by oligonucleotide sequencing (Berkley *et al.,* 2002). *Bacillus* spp. are able to degrade most of the substrates derived from plant and animal sources. This includes proteins, starch, pectin, cellulose, hydrocarbons etc. and they are assumed to play a significant role in the biological cycles of carbon and nitrogen. In the soil environment the bacteria actively multiply when suitable substrates for their growth are available, and presumably they form spores when their nutrients become exhausted. In association with sporulation process antibiotics are also produced. Antibiotics produced by *Bacillus* spp. show broad range of antimicrobial activity: difficidin shows broad spectrum of activity; antibiotics such as bacitracin, pumulin, laterosporin, gramicidin are effective against Gram-positive bacteria; whereas as colistin and polymyxin are anti-Gram-negative; anti fungal antibiotics such as mycobacillin and zwittermicin are also produced by this genus. They form several ecophysiological groups, as they are involved in antibiotic production, denitrification, nitrogen fixation and nitrification. The genus occurs as lithotrophs, autotrophs and they also show acidophily, alkaliphily, psychrophily, thermophily, and parasitism (Priest, 1993). Many of them are versatile chemoheterotrophs capable of utilizing a variety of simple organic compounds such as sugars, organic acids, amino acids etc. Some of them are capable of fermenting carbohydrates to yield glycerol and butanediol. They can grow in the presence or absence of organic growth factors. Majority of species are mesophiles and grow at temperature of 30^0 to 45 ^{0}C, but some are thermophiles and they can survive and grow at temperature as high as 65 ^{0}C. True psychrophiles are able to grow and sporulate at 0 ^{0}C. *Bacillus* spp. can grow over a range of pH from 2 to 11.

The ubiquity and diversity of the genus, formation of endospores which are resistant to chemical and physical agents, production of antibiotics and enzymes of commercial importance, toxicity of their spores and protein crystals for many insects, have attracted vast research interest regarding this group of microorganism. Based on ecological diversities, multiplicity of substrate degradation and production of variety of

industrially important molecules, the genus may be called as "potential biocell". Bacillus enzymes represent nearly 60% of the global industrial-enzyme market. This includes: alkaline proteases (detergent and leather industry); neutral proteases (for leather and baking industry); amylases (for high fructose syrup manufacture, textile and paper industries); glucanase (brewing industry); xylanase (paper industry). However the potentiality and exploitation of *Bacillus* spp. for production of other important molecules is yet to be worked out. Very few *Bacillus* spp. are known to be harmful to humans and animals and most of them have GRAS (Generally Regarded As Safe) status, and they are major sources of amylase and protease enzymes for food use. The above mentioned points along with ease with which the bacterium can be cultivated and their established safety record, make them prime candidates as "potential biocells". Polyhydroxyalkanote, which is an intracellular polymer, can be co produced along with enzyme of industrial importance, which can lead to economic production of polymer and enzyme, simultaneously. Hence research into the mechanisms and control of biosynthesis pathways will directly enhance and diversify the industrial productivity and sustenance of *Bacillus* spp. as "potential biocells".

Conclusion

Today cellular morphology is often judged insufficiently therefore it is considered to be of little importance for the identification of the bacteria. Gram staining reaction will not always be the same, however a Gram-positive cell wall may become Gram-negative under certain conditions. In case of spore formation and sporangium shape it needs to be induced by providing such an environment, for example through some kind of deficiency i.e., nutrient limiting or sub optimal condition. But cells may lyse than to form spores and the conclusion 'non spore formers' is easily drawn. Poor microscopic methodology can also lead to incorrect conclusions. Brill and Wiegel described a PCR method based on certain sporulation genes to distinguish between bacteria containing sporulation genes. These problems can be rectified up to certain level by physiological properties. For many physiological tests several media have been described and these will yield differing results. A number of commercial identification kits are available for the rapid and standardized identification of *Bacillus* but again results obtained with one kit are only

comparable with those obtained with the same material. A combination of morphological, physiological and molecular methods appears essential for identification of the species.

In conclusion this study has led to a finding of a new unexplored *B. flexus* strain, which has the ability to produce PHA up to 60% PHA in the biomass. Optimization of culture conditions, characterization of polymer synthesized may lead to a possible turning point for the use of *Bacillus* sp. as a commercial PHA producer.

Chapter 2

Production of polyhydroxyalkanoate by Bacillus flexus using different substrates, optimization of growth and characterization of the polymer

2.1. Introduction

Many bacteria are capable of accumulating P(HB) within the cell during stationary phase, when essential nutrient is limiting, but carbon concentration is in excess. During this imbalance, reduced nicotine adenine dinucleotide (NADH) accumulates in the cell and exerts feed-back repression on enzymes of the TCA cycle, acetyl coenzyme A (CoA) accumulates and acetyl-CoA acetyltransferase is induced to initiate P(HB) synthesis, polymerization of acetyl-CoA units consumes NADH and P(HB) synthesis assumes the role of an electron sink (Dawes and Senior, 1973).

PHAs are produced from renewable resources such as glucose, sucrose and vegetable oil derivatives. In 1990, two industrial processes were developed for the production of P(3-HB-co-3HV) from a mixture of glucose and propionic acid by ZENECA byproducts at Bellingham (UK) (Byrom, 1992) and from sucrose by Chemie Lenz GmbH (Austria). However the cost of the polymer was relatively high compared synthetic plastics. There seems to be a challenging future for economic production of microbial polymers especially those with copolymers. This may be solved if a carbon source and a bacterial strain are properly selected in conjunction with development of an efficient fermentation technology.

Much research is being conducted pertaining to the efficient production of PHA using inexpensive carbon sources because production cost is still high in comparison with those of synthetic petrochemical based plastics (Haywood *et al.,* 1991; Page and Knosp, 1989). Using sucrose as inexpensive carbon source high productivity has been achieved in an optimized fermentation process (Lee and Choi, 1998).

In the present work, in order to obtain high yield and quality of PHA, various carbon sources, nitrogen sources and economic substrates were assessed as media supplements. Media components were optimized based on response surface methodology (RSM). The growth kinetics of *B. flexus* under nutrient limitation was studied by using a simple model giving mathematical description of kinetics of microbial growth, substrate consumption and product formation.

2.2. Materials and Methods

2.2.1. Shake flask cultivation of *B. flexus*

B. flexus was cultivated in media containing different salts of nitrogen, amino acids, carbon sources, plant oils, free fatty acids, economic media components such as palm oil effluent, molasses, corn starch, whey, rice and wheat bran extracts as sources of carbon/nitrogen/nutrients to enhance growth and PHA copolymer synthesis. Media (50 ml), details of which are given below, were prepared in 250 ml capacity Erlenmeyer flasks in duplicate, inoculated with 18 h old *B. flexus* inoculum (10%, v/v), having 2 x 10^4 viable cells/ml. Flasks were incubated at 30 ^{0}C and 250 rpm up to 72 h. At the end of cultivation period, cells were harvested; biomass and PHA were estimated as explained earlier [Materials and methods (common for chapters), section M.6). Characterization of PHA was carried by FTIR/GC/GC-MS/NMR spectroscopy/DSC, wherever applicable, as mentioned earlier [Materials and methods (common for chapters), section M.6).

Different media were prepared for the above experiments as follows:

2.2.1.1. Effect of nitrogen sources on PHA production

To find out the effect of nitrogen sources on growth and PHA production, *B. flexus* was cultivated in media containing (g/l) 2.2 Na_2HPO_4, 1.5 KH_2PO_4, 0.2 Mg SO_4 $7H_2O$ and 20 sucrose. Nitrogen sources such as urea, ammonium nitrate, ammonium phosphate, ammonium acetate, ammonium chloride, sodium nitrate, ammonium sulphate, ammonium citrate and potassium nitrate were used at nitrogen concentration of 0.32 g/l. Various amino acids such as aspartic acid, lysine, proline, threonine, alanine, metheionine, valine, tryptophan, serine, isoleucine, cysteine, arginine and tyrosine were also tested as nitrogen sources (nitrogen concentration of 0.32 g/l) in the above mentioned basal medium.

2.2.1.2. Effect of carbon sources on PHA production

In addition to nitrogen sources, carbon sources also have profound effect on bacterial growth and PHA production. Basal medium containing (g/l) 2.2 Na_2HPO_4, 1.5 KH_2PO_4, 0.2 Mg SO_4 $7H_2O$ and 1.5 $(NH_4)_2SO_4$ were supplied with glucose, sucrose, arabinose, dextrin, dulcitol, fructose, galactose, inositol, lactose, mannose, mannitol, maltose, raffinose, rhamnose, sorbitol, sorbose, trehalose, mellibiose, at a concentration of 8.42 g carbon/l.

2.2.1.3. Utilization of economic substrates for PHA production

For economic production of PHA, various agro industrial residues were utilized as nutrient sources. The materials used were: molasses, corn steep liquor, rice (*Oryza sativa*) bran (extract), wheat (*Triticum* sp) bran (extract) and milk whey. Cornstarch and mahuva (*Madhuca indica*) flower (extract) were also used as cheaper alternative carbon source to sucrose. Composition of these materials is given below:

a) Corn steep liquor (CSL; Anil starch product, Ahamadabad, India) contained (g%): moisture, 77; protein, 11; ash, 9.6 and reducing sugars 1.6.
b) Milk whey (g%): moisture 93, protein 0.4, fat 0.5, lactose 5, and ash 0.4.
c) Wheat (*Triticum* sp) bran procured from the local market had (g%): moisture, 12.8; protein, 16.9; ash, 6; fiber, 9; fat, 3.8; and carbohydrates, 34.
d) Mahua flower (*Madhuca* sp): Sun dried mahua flowers (succulent corollas) obtained from the forest area of Gujarat, India, contained (g%): 17 Moisture; 50 reducing sugar; 7.3 non-reducing sugar; 4 protein; 3.5 ash and 1.3 acidity.
e) Molasses procured from the local cane sugar industry contained (g%): Total solids, 75; sugars, 49; ash, 8 and protein 3.

Media prepared using these substrates were:

1. Corn steep liquor was used at 10 g/l as nitrogen source and other components used were (g/l): 2.2 Na_2HPO_4, 1.5 KH_2PO_4, 0.2 Mg SO_4 and sucrose 20.
2. Milk whey was used as source of carbon at 40 ml% in the medium replacing sucrose.
3. Molasses was used as carbon source at 4.08 g% to replace 2% sucrose.
4. Starch present in wheat bran was hydrolysed by amylase and amyloglucosidase prior to its use as carbon substrate. For this, 100 g of wheat bran was suspended

in water (500 ml) of pH 6, 0.1% α-amylase (Anil starch products, Ahmadabad, India) was added and the mixture was incubated at 80 ^{0}C for 30 min. After incubation, pH was adjusted to 4.5, amyloglucosidase (Anil starch products, Ahmadabad, India) was added at 0.2% level and the sample was incubated at 55 ^{0}C overnight, filtered, centrifuged and used for media preparation. 500ml of this filtrate was mixed with 500 ml basal medium containing 2.2 Na_2HPO_4, 1.5 KH_2PO_4, 0.2 Mg SO_4 and 0.5 g $(NH_4)_2SO_4$.

5. Medium was prepared by using cornstarch as carbon source. The medium contained (g/l): Corn starch 20, 2.2 Na_2HPO_4, 1.5 KH_2PO_4, 0.2 Mg SO_4 and 1.5 g $(NH_4)_2SO_4$.
6. 50 g portion of the dried mahua flower was suspended directly without shredding in 100 ml of water and boiled for 5 minutes. The suspension was passed through filter cloth and the residue was resuspended in 100 ml of boiling water twice for 5 min and filtered. Total volume of the supernatant collected was 200-ml. Dinitrosalicylic acid method (Miller, 1959) was used to estimate reducing sugar in the extract before and after inversion with HCl. 140 ml of mahua flower extract was used in medium to impart 20 g/l of sugar and other nutrients added were (g/l): Na_2HPO_4 2 H_2O, 2.2; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.5; $MgSO_4$ 7 H_2O, 0.2.
7. Control experiment was carried out for the above by using sucrose as a carbon source in the medium.

2.2.2. Optimization of growth by Response surface methodology (RSM):

Experimental design

Development of fermentation strategies can lead to economic production of the polymer. When many factors affect desired response of fermentation, response surface methodology (RSM) can be used effectively for optimizing the fermentation process parameters. In the present work effect of KH_2PO_4; $(NH_4)_2HPO_4$; $MgSO_4$ 7H_2O; citric acid and sucrose on growth and PHA production by *B. flexus* were determined by RSM by modulating the variables according to five factor, five level CCRD (Table 2.1). Experiments were carried out in duplicate. Six replicates (27-32) were included for estimation of a pure error of sum of squares. *B. flexus* inoculum was added to the media

(10%, v/v) and the flasks were incubated at 250 rpm, 30 ^{0}C up to 72 h. Treatment schedule used for experimentation are tabulated in Table 2. 2.

2.2.2.1. Statistical analysis

A second order polynomial equation was used to fit the experimental data given in table 4. 2. The model proposed for the responses Y_1. Y_2, Y_3, Y_4 and Y_5 was $Y_i = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{14}x_1x_4 + a_{15}x_1x_5 + a_{23}x_2x_3 + a_{24}x_2x_4 + a_{25}x_2x_5 + a_{34}x_3x_4 + a_{35}x_3x_5 + a_{45}x_4x_5 + \varepsilon$, Where Y_i (I = 1-5) is the predicted response for biomass and PHA yield. a_0 is the value of the fitted response at the center point of design, a_i, a_{ii}, a_{ij} the linear, quadratic an cross-product terms, respectively. A non-linear mathematical optimization procedure of the Quattro Pro software package (Quattro, Pro, version 4.0, Borland International Inc., USA) (Saxena and Rao, 1996) was used. Further in order to deduce workable optimum conditions, a graphical technique was used for the optimization of the fitted polynomial for biomass and PHA yield (Floros and Chinnan, 1988). The optimum condition was verified by conducting experiments under these conditions.

Responses were monitored and results were compared with model predictions. The fitted polynomial equation was expressed as surface and contour plots in order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimum conditions.

Table 2.1: Variables and their levels for CCRD.

	Symbols	-2	-1	0	1	2	Mean	Standard Deviation
KH_2PO_4	X_1	1.50	4.88	8.25	11.63	15.00	8.25	3.38
$MgSO_4$	X_2	0.20	0.65	1.10	1.55	2.00	1.10	0.45
Citric acid	X_3	0.00	0.75	1.50	2.25	3.00	1.50	0.75
$(NH_4)_2HPO_4$	X_4	1.00	2.25	3.50	4.75	6.00	3.50	1.25
Sucrose	X_5	5.00	13.75	22.50	31.25	40.00	22.50	8.75

2.2.3. Measurement of nutrient uptake and PHA production

To find out the nutrient limiting condition that is required for PHA synthesis, *B. flexus* cells were grown under shake flask condition in medium optimized by RSM. The

medium contained (g/l): KH_2PO_4 13.5; citric acid 1.7; $(NH_4)_2 HPO_4$ 4.0; $MgSO_4.7H_2O$ 1.2; tryptone 2.0; sucrose 20. Uptake of carbon was calculated based on estimation of residual sugar in the medium [Materials and methods (common for chapters), section M.6.11].

Table 2.2: Treatment Schedule for five-factor CCRD and response in terms of biomass and PHA yield.

Exp No.	KH_2PO_4	$MgSO_4$	Citric acid	$(NH_4)_2HPO_4$	Sucrose	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	3.325	3.357	1.225	1.257
2	1	-1	-1	-1	-1	2.800	2.841	1.025	1.135
3	-1	1	-1	-1	-1	3.625	3.689	0.925	1.019
4	1	1	-1	-1	1	3.725	4.193	0.750	0.897
5	-1	-1	1	-1	-1	2.800	2.503	0.525	0.593
6	1	-1	1	-1	1	2.650	2.759	1.125	1.243
7	-1	1	1	-1	1	3.875	4.007	0.975	1.079
8	1	1	1	-1	-1	3.300	3.443	1.225	1.409
9	-1	-1	-1	1	-1	3.575	3.447	1.475	1.415
10	1	-1	-1	1	1	3.73	4.00	2.300	2.289
11	-1	1	-1	1	1	5.100	5.399	0.525	0.501
12	1	1	-1	1	-1	3.65	3.96	1.250	1.303
13	-1	-1	1	1	1	5.775	5.713	1.475	1.423
14	1	-1	1	1	-1	2.100	2.049	0.850	0.877
15	-1	1	1	1	-1	3.450	3.421	0.700	0.713
16	1	1	1	1	1	7.000	7.377	2.125	2.191
17	-2	0	0	0	0	4.150	4.353	0.725	0.751
18	2	0	0	0	0	4.750	4.125	1.825	1.587
19	0	-2	0	0	0	2.975	3.225	1.525	1.521
20	0	2	0	0	0	6.100	5.429	1.450	1.241
21	0	0	-2	0	0	4.500	4.031	2.075	2.017
22	0	0	2	0	0	4.075	4.127	2.100	1.945
23	0	0	0	-2	0	3.675	3.543	1.100	0.781
24	0	0	0	2	0	5.975	5.687	1.200	1.301
25	0	0	0	0	-2	1.750	1.935	0.550	0.415
26	0	0	0	0	2	5.400	4.799	1.100	1.019
27	0	0	0	0	0	4.715	5.087	1.250	1.453
28	0	0	0	0	0	5.455	5.087	1.425	1.453
29	0	0	0	0	0	4.755	5.087	1.200	1.453
30	0	0	0	0	0	5.125	5.087	1.550	1.453
31	0	0	0	0	0	4.725	5.087	1.350	1.453
32	0	0	0	0	0	5.325	5.087	1.725	1.453

Nitrogen was estimated by Nesseler's reagent (Srienc *et al*., 1984). Residual sodium, potassium and magnesium were measured in Atomic absorption

spectrophotometer – AA 6701F, Shimadzu, Singapore. The supernatant of the fermented broth was diluted with deionized water to appropriate concentration. Concentration of the sample was determined by measuring the absorbance by a standard concentration of the element by following relationship.

Concentration = Concentration of the standard x 0.0044/Absorbance of the sample.

2.2.4. Elemental analysis of PHA containing cells

B. flexus cultivated in nutrient limiting conditions was analysed for C:N:S:H by elemental analysis using Elementar Analysen system, GMBH, VarioEL model (Germany). About 3 mgs of lyophilized cells was used for analysis. Sulfanilic acid was used as a standard.

2.2.5. Kinetic studies on growth and PHA synthesis in *B. flexus*

A simple kinetic model was developed which can provide an adequate description of PHA production process involving bacterial fermentation. In this study, a modification of the logistic equation has also been attempted for describing the growth of the bacterium. The microbial growth was described by means of a simplification of Monod's model for low substrate concentration. A simplified Luedeking-Pirat type model represented the product kinetics. The kinetic constants were evaluated based on non-linear regression and the differential equations were solved using Runge-Kutta algorithm using MATLAB software.

2.2.5.1. Numerical calculations

For optimal estimation of model parameters, a non-linear regression technique using MATLAB 7.0 was used, which minimizes the deviations between the model predictions and the experimental batch results. For the calculation of the model predictions, the system of differential equations of the model was solved using an integration program based on the Runge Kutta Method of 4th order. The program was developed in MATLAB 7.0 based on the following algorithm. In this algorithm, the

integration interval from 0 to the global residence time (t) was divided into N subintervals with h = t/N. The set of equations used in this method was as follows:

$$k_1 = f(t_i, x_i);\ k_2 = f\left(t_i + \frac{h}{2}, x_i + h\frac{k_1}{2}\right);\ k_3 = f\left(t_i + \frac{h}{2}, x_i + h\frac{k_2}{2}\right);$$

$$k_4 = f(t_i + h, x_i + hk_3);\ x_{i+1} = x_i + \frac{h}{6}(k_1 + 2k_2 + 2k_3 + k_4)$$

$$i = 0, \ldots.. N-1$$

where t (= n h), k_1, k_2, k_3, k_4 are the internal parameters defined in the Runge–Kutta algorithm; x_i and x_{i+1} were the calculated concentrations at t_i and t_{i+1}, respectively.

2.2.6. Effect of organic acids on PHA synthesis

To synthesize PHA copolymers, various fatty acids have been used in bacterial fermentations as co carbon substrates at 2 g/l concentration. In this study sucrose was used as main carbon source and other organic acids as co carbon source to synthesize PHA in *B. flexus*. Various organic acids including butyric, valeric, hexanoic, heptanoic, octanoic and decanoic acids (from Merck) as well as linoleic, oleic, stearic, succinic, acetic, palmitic, propionic, and malic acids were used at 2 g/l concentration as co substrates. The bacterium was cultivated (pH 7, 30 ^{0}C, 250 rpm) in medium containing (g/l): 2.2 Na_2HPO_4, 1.5 KH_2PO_4, 0.2 Mg SO_4 $7H_2O$, 1.5 $(NH_4)_2SO_4$ and 20 g/l sucrose. After 24 h of growth above mentioned organic acids were added at 0.2% concentration in 10 ml of sterile water and incubation was continued up to 72 h. Medium without organic acid served as control. Harvested cells were washed with hexane and methanol prior to drying and estimation of PHA.

2.2.7. Utilization of palm oil effluent for PHA copolymer synthesis

Palm fruit from which the palm oil effluent (POE) was obtained, was composed of 80% mesocarp and 20% nut and had total oil content of 43%. It also had crude protein (3), fiber (3), ether extractable (2-3) and ash (2-3) components. The palm oil effluent obtained after the extraction of oil-contained residual oil and suspended solids amounting

to 1.9 g% and 0.3 g% of ether extractable. Composition of fatty acid was (%), myristic 1, palmitic 45, stearic 4, oleic 40 and linoleic 10.

Inoculum was prepared in 100 ml sterile medium contained in 500 ml capacity Erlenmeyer flask. The composition of the medium used was (g/l): Na_2HPO_4 $2H_2O$, 2.2; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.5; $MgSO_4$ $7H_2O$, 0.2; Sucrose, 10; yeast extract, 1 and pH of medium was set at 7.0. Medium was inoculated with growth from agar slant and flask was incubated at 30 ^{0}C and 250 rpm for 18-24 h. The inoculum contained 3 x 10^4 CFU/ml.

PHA production was carried out in triplicate in 500 ml Erlenmeyer flasks using above-mentioned medium, with 20 g sucrose/l. Inoculum was added at 10 % level (v/v) and flasks in triplicate were incubated on rotary shaker at 30 ^{0}C, 250-rpm for 72 h. Fermentation was carried out by a) using sucrose as main carbon source b) using palm oil effluent (POE) as basal medium instead of water, ranging from 20-100% (v/v) for preparation of medium. Palm oil was used for comparison.

2.2.8. Utilization of plant oils as co carbon substrates for PHA-copolymer synthesis

Several plant oils were tested as co carbon substrates for growth and PHA synthesis by *B. flexus.* This has significance for PHA synthesis as a few of them are not edible and edible oils that have turned rancid can also be utilized optimally as a medium component for PHA copolymer synthesis. Composition of medium, addition of oils and biomass handling were same as mentioned under 2.2.1.3 of current chapter. Types of oil used and their proximate composition are given below:

a) Castor (*Ricinus communis*) oil: Ricinoleic acid 90%, linoleic acid 4%, oleic acid 3%, stearic acid 1% and palmitic acid 1%.
b) Coconut (*Cocos nucifera*) oil: Lauric 44, myristic 17, palmitic 8, caprylic 8%, capric 6, stearic 1, oleic 5, linoleic 1 and arachidic 0.5.
c) Ground nut (*Arachis hypogea*) oil: Palmitic 6, steraic 3, oleic 52, linoleic 13, arachidic 2, behenic 1 and lignoceric 1 .

d) Mustard (*Brassica* sp) oil: Oleic 22, linoleic 14.2, palmitic 1.5, stearic 0.4, linolenic 6.8, erusic 47 and behenic 2.
e) Neem (*Azadiracta indica*) oil: palmitic acid 19.4, stearic acid 21.1, oleic acid 42, linoleic acid 14.9 and arachidic acid 1.4.
f) Palm (*Elaeis guineensis*) oil: Myristic acid 0.2, palmitic acid 15, stearic acid 2, oleic acid 43, linoleic acid 39 and linolenic acid 1.
g) Pongemia (*Pongemia glabra*) oil: Oleic acid 71.3% and linoleic acid 10.8%
h) Rice (*Oryza sativa*) bran oil mainly contained: Palmitic acid 15%, oleic acid 43%, linoleic acid 39%, palmitoleic 0.4, myristic 0.4 and stearic 3.
i) Sesame (*Sesamum* sp) oil: Palmitic 7, palmitoleic 0.5, stearic 6, oleic 50, linoleic 35, linoleic 1 and eicosenoic 1.
j) Sun flower (*Helianthus annus*) oil: Palmitic 4, stearic 7, oleic 40 and linoleic 48.

In another experiment saponified oils were used as co substrates. Saponification was carried out by refluxing 30 ml of the oil with 400 ml of methanol and 4.95 g of NaOH. At the end of 30 min, sample was cooled and transferred to chilled water. Saturated NaCl was added to the chilled sample and the precipitate was collected and dried.

2.2.9. Pathway for PHA synthesis in *B. flexus*

To find out the pathway through which PHA is synthesized following media were used: a) Control medium with sucrose b) Medium containing sucrose and rice bran oil (2g/l), wherein oil was added in 10 ml sterile water after 18 h of growth. The samples were fermented at 30 ^{0}C, 250 rpm, for 72 h with and without 0.1mg/ml of acrylic acid that was added after 18 h of growth.

2.2.10. Fermentor cultivation of *B. flexus*

PHA production was carried out in a jar fermentor (Bioflo 110, New Brunswick Scientific Co. USA) of 3 l capacity (Fig. 2.1), containing 1.8 l medium mentioned below and 200 ml inoculum [Materials and methods (common for chapters), section M.5.6). Fermentation was carried out at 30 ^{0}C, up to 48 h, in duplicate in medium containing (g/l): 1) 11.6 KH_2PO_4, 1.5 Mg SO_4 7 H_2O; 4.7 g $(NH_4)_2HPO_4$ and 31.2 sucrose 2) 2.2

Na_2HPO_4; 1.5 KH_2PO_4; 0.2 Mg SO_4 7 H_2O; 1.5 g $(NH_4)_2SO_4$ with 20 sucrose as main carbon source/ by addition of rice bran oil 2 g/l (in 10 ml water) as co carbon source to sucrose containing medium, after 18 h of fermentation.

2.2.11. Analytical Methods

Analytical methods used for estimation of biomass, PHA extraction and quantification, estimation of residual reducing sugar in the broth, GC, FTIR, GC-MS and NMR analysis of PHA are mentioned in [Materials and methods (common for chapters), section M.6).

2.2.12. Nitrogen estimation

Nessler's reagent was used for determination of nitrogen present in the medium and the colour developed was measured at 423nm (Srienc *et al*., 1984). Standard graph was prepared by using ammonium sulphate.

2.3. Results and Discussion

2.3.1. PHA production using various N sources

Different nitrogen sources such as urea, ammonium nitrate, casein, ammonium phosphate, ammonium acetate, peptone, ammonium chloride, tryptone, sodium nitrate, ammonium sulphate, ammonium citrate, potassium nitrate and amino acids were used in the medium based on equal concentrations of nitrogen 0.32 g/l. Cells were harvested by centrifugation and PHA was extracted by using sodium hypochlorite solution. Higher concentrations of biomass of 2.5, 2 and 1.95 g/l were obtained with ammonium acetate, ammonium sulphate and ammonium phosphate as N-sources, respectively (Fig. 2.2). Corresponding PHA yields obtained were 1.8, 1.1 and 1.0 g/l. Enhancement of growth and PHA production using acetate has been reported for *Rhodospirullum* (Khatipov *et al.,* 1998; Liebergesell *et al.,* 1991). In biosynthesis of PHA, HB monomer is produced through acetyl CoA (Hassan *et al.,* 1997). Utilization of acetate is activated by the enzyme acetate-CoA ligase (EC 6.2.1.1), which is the initial step in its metabolism.

Figure 2.1: Cultivation of *B. flexus* in a fermentor.

In the present experiment it was observed that acetate utilization resulted in better cell growth as well as PHA synthesis. Initially the substrate served as a carbon source for growth, and when the nitrogen concentration in the medium depleted then it was channelised towards PHA synthesis. Due to this dual activity higher growth and PHA synthesis may be expected when ammonium acetate is used as a nitrogen source in the medium.

Amino acids other than aspartic acid and isoleucine were utilized for growth and PHA production (Fig. 2.3). The amino acids may be utilized for growth as well as formation of cell components. Amino acid is utilized in the exponential growth phase of the organism as a source of nitrogen and with decrease in its concentration oxidation of NADH fail to occur, which exerts feed back repression on the enzymes of TCA cycle which leads to the accumulation of actyl CoA. The acetyl transferase gets induced and polymerization of acetyl CoA units consumes NADH to initiate P(HB) synthesis, which assumes the role of an electron sink. The ammonium deficiency in the medium whether in the form of ammonium salts or amino acids appeared not to allow the synthesis of certain amino acids such as valine, leucine and isoleucine, which enhance the production of 2-keto-3-methylbutyric acid, 2-keto-4-methylvaleric acid and 2-keto-3-methylvaleric acid. These methyl-branched 2-ketoalkanoates are oxdatively decarboxylated to the corresponding CoA thioesters by the branched chain 2-ketoacid dehydrogenase complex, and are further degraded via various other CoA thioesters to intermediate of the central metabolism with propionyl-CoA as the intermediate of the valine and isoleucine catabolic pathways. Therefore, it is obvious that propionyl-CoA is synthesized by the cells themselves from intermediates of amino acid metabolism. These intermediates also are formed directly from corresponding amino acids with other nutrient depletion conditions such as phosphorous deficiency. The precursor intermediates formed by the branched chain amino acid are degraded to propionyl-CoA. β-ketothiolase combines with propionyl-CoA and acetyl-CoA to form 3-keto-valeryl-CoA; later it is reduced by acetoacetyl-CoA reductase to 3-hydroxyvaleryl-CoA, which is a substrate for the PHA synthase (Steinbuchel and Pieper, 1992).

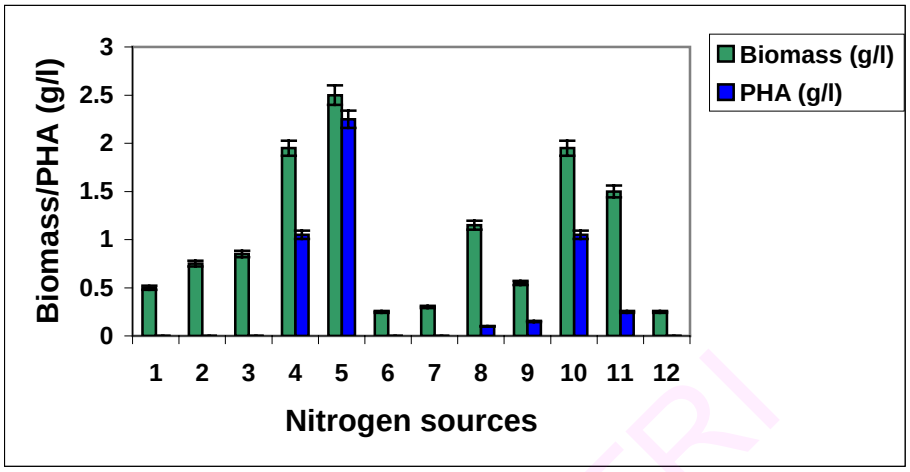


Figure 2.2: Effect of different nitrogen sources on growth and PHA production in *B. flexus.* 1-Urea; 2-Ammonium nitrate; 3-Casein hydrolysate; 4-Ammonium phosphate; 5-Ammonium acetate; 6-Peptone; 7-Ammonium chloride; 8-Tryptone; 9-Sodium nitrate; 10-Ammonium sulphate; 11-Ammonium citrate and 12-Potassium nitrate.

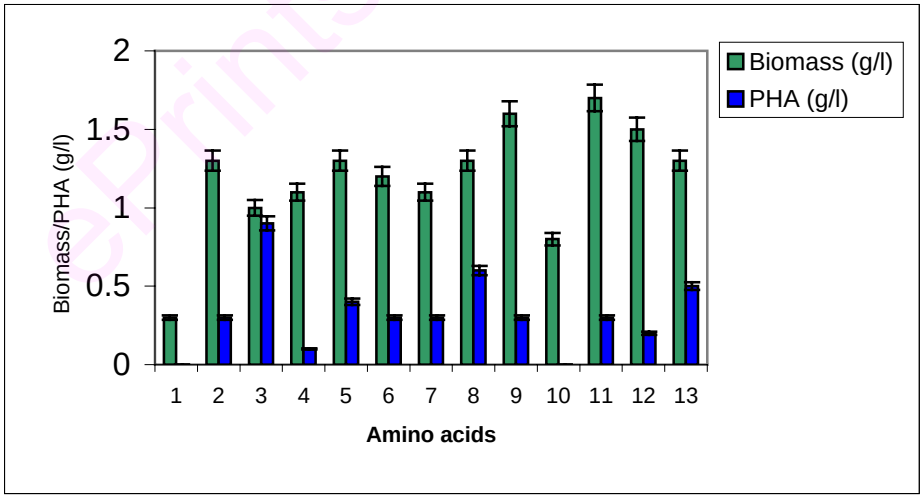


Figure 2.3: Effect of different amino acids on growth and PHA production in *B. flexus.* 1-Aspartic acid; 2-Lycine; 3-Proline; 4 - Threonine; 5-Alanine; 6-Metheionine; 7-Valine; 8-Tryptophan; 9-Serine; 10-Isoleucine; 11-Cysteine; 12-Arginine; 13-Tyrosine.

2.3.2. PHA production using various C sources

Carbon source has profound influence on the PHA content of the cell. PHA accumulation begins within the cell in the presence of excess of carbon in the medium, while other nutrients are depleting. Carbon sources such as sucrose, arabinose, dextrin, dulcitol, fructose, glucose, galactose, inositol, lactose, mannose, mannitol, maltose, raffinose, rhamnose, sorbitol, sorbose, trehalose and mellibiose were used at 8.42 g carbon/l level in the medium. PHA production varied from 3% to 80% and a maximum of 80 % PHA in the biomass was obtained in the presence of galactose (Fig. 2. 4). However biomass production was low (0.6 g/l) in the presence of this substrate. Optimal amount of PHA (1g/l; 50% of biomass weight) as well as biomass (2 g/l) were obtained in medium containing sucrose.

2.3.3. Utilization of economic substrates for PHA production

One of the limiting factors for the commercialization of PHA is the high cost of the carbon substrates used for fermentation. Hence the production of the polymer from unrefined and cost effective substrates rich in different carbon components appeared feasible for PHA production. There are several agro industrial by products and cheaper raw materials, which are rich in carbon, nitrogen, minerals, vitamins, amino acids etc. that can be used as nutrient source for bacterial growth and polymer production. Cheaper raw materials such as whey, wastewater from olive mills, molasses, corn steep liquor, starchy wastewater, palm oil mill effluent, have been used as nutrient supplements for PHA production by various genera and species of bacteria (Page, 1989; Hassan *et al.,* 1997; Gouda *et al.,* 2001; Yu, 2001; Lapointe *et al.,* 2002; Marangoni *et al.,* 2002; Pozo *et al.,* 2002). In the present study, molasses, whey, mahua flower extract and wheat bran extracts were used as carbon substrates to replace sucrose. All the substrates other than whey gave promising results with respect to growth and PHA production (Fig. 2.5).

Whey is composed of mainly lactose, lactate and proteins. Hydrolysis of lactose is essential for its up take and metabolism to form acetyl CoA. Lactate is metabolized through propionyl-CoA and malonyl-CoA. In the absence of appropriate enzyme system the bacterium will not be able to metabolize substrate such as whey. Data (Fig. 2.5)

indicate that *B. flexus* could grow to certain extent (biomass 1.8 g/l) due to presence of organic growth factors however it could not utilize lactose for higher growth or PHA production.

The bacterium was able to utilize sucrose, glucose and fructose for growth and PHA production (Fig. 2.4). Hence any substrate that is rich in these carbon sources can be used in production medium. Molasses is one of the cheapest agro industrial residues, which is rich in sugars. Earlier the term molasses was referred to by product of sugar industry, which included cane and beet as raw material. Currently any effluent rich in sugars (40% or more) are termed as molasses. Therefore different types of molasses that are available depending on the raw material employed for main product formation are: cane molasses, beet molasses, citrus molasses, hemicellulose extract and starch molasses. Cane and beet molasses are extensively used in ethanol fermentation by yeasts. Beet molasses has been used for PHA production in *Alcaligenes eutrophus* and recombinant *E. coli* (Liu *et al.,* 1998). As the recombinant strain lacked invertase it could not utilize sucrose directly and hydrolysed molasses was used as carbon source. In our study we have employed cane molasses that is available in large quantity in our country (about 1.5 million tones/annum) and it was found that *B. flexus* could use sucrose and the monomers such as glucose and fructose for growth and PHA synthesis. Biomass of 3.2 g/l with 46% PHA was obtained on this substrate.

B. flexus was able to use starch also directly for growth and polymer synthesis (3.9 g/l of biomass with 45% PHA). It is known that *Bacillus* spp. posses amylases and hence they can hydrolyse the starches present in the medium and metabolize glucose further. Starches are relatively cheaper than pure forms of sugars such as sucrose, glucose or fructose. Wheat bran, which is obtained as a by-product in wheat milling is rich in starch and can be utilized as a cheaper source of sugar. However, starch present in wheat bran was hydrolysed and clarified prior to its utilization in the medium, otherwise the insoluble materials would interfere with collection of biomass and extraction of PHA. This substrate also supported good growth (2 g biomass/l) and PHA production (46% of Biomass).

Corn steep liquor was used as a nitrogen source. Maximum growth (biomass 4 g/l) was observed in medium containing corn steep liquor with 1.8 g/l of PHA.

Mahua flower extract also supported good growth (3.5 g biomass/l) and PHA production (2.4 g/l; 69% PHA of biomass weight). Dry mahua flower is available in India at about 0.3$per kg. The carbohydrates identified in the flowers are mainly sucrose, glucose and fructose. Other components present are amino acids, proteins, various vitamins and organic acids (The Wealth of India, 1962). Due to richness of carbohydrates and organic acids this substrate was examined for the purpose of PHA production. *B. flexus* is able to utilize all the above mentioned carbohydrates for growth and PHA production. In the present work the observed increase of biomass and quantitative yields of PHA by *B. flexus* in shake flask cultivation with flower extract medium compared to sucrose may be due to the presence various types of utilizable carbon compounds and growth promoting nutrients such as amino acids and proteins present in this natural substrate.

Overall data indicated that several economic substrates could be utilized for growth and PHA production by *B. flexus*.

2.3.4. Optimization of growth and PHA production by RSM

PHA is an intracellular polymer, which is accumulated by bacteria under nutrient depletion conditions. The regulation of PHA synthesis differs amongst various bacterial genera. In *Azotobacter beijerinckii* oxygen limitation initiated the accumulation of the polymer, relaxation of an oxygen limitation led to an immediate decrease in the PHA content (Senior and Dawes, 1973). *Alcaligenes eutropus*, has been used for optimal production of P(HB), a homopolymer which is accumulated under nitrogen limitation (Morinaga *et al.,* 1978). In this study based on the treatment schedule and response obtained it was possible to assess the nutrients that were required for growth enhancement and PHA accumulation (Table 2.2). Maximum growth and PHA production was found in expt. 16 (7 g/l of biomass, 2.1 g/l PHA or 28% PHA of biomass weight). Maximum PHA synthesis in the cells was observed in expt. no. 10 (3.7 g/l of biomass, 2.3 g/l of PHA or 57% PHA of biomass weight). Amongst the nutrients tested, citric acid did not show effect either on growth or PHA accumulation (Expt. No. 21 and 22), because there was no change in growth or PHA production when this was used at maximum level or not included in the fermentation medium. Considering that citric acid

had no effect on *B. flexus*, it was observed from expt. no. 10 and 16 that $MgSO_4$ 7 H_2O had profound effect on cell growth as the biomass increased from 4 g/l to 7 g/l (maximum obtained from overall treatment schedule), by increased addition of the salt from 0.65 g/l to 1.55 g/l. Increased addition of this salt however did not enhance PHA yields indicating that it did not take part in the PHA synthesis process.

From the results it was observed that KH_2PO_4 enhanced PHA production (from expt. 11, 17 and 18) and *B. flexus* growth was not inhibited even at 15-g/l concentration of this nutrient. Ammonium phosphate and sucrose enhanced growth and PHA production (Expt. no. 23 and 24 & 25 and 26, respectively). Nitrogen concentration used in the experiment varied from 0.21 to 1.27 g/l, P from 0.22 to 3.4 g/l and C from 2.1-16.6 g/l. In a preliminary experiment it was observed that *B. flexus* produced PHA under N limitation conditions with excess carbon source. The optimal ratio of N:P:C for growth and PHA synthesis appeared to 1:3.6:13 (Expt. 10 and 16), in which P contributed from $(NH_4)_2HPO_4$ is also accounted. Elemental analysis of PHA containing cells showed N:C in 8.1:41.6 (1:5). Reduction of carbon to 9g/l (Expt. no. 21) also supported 50% PHA synthesis in the cells. Such a concentration would be sufficient for optimal polymer synthesis and to maintain excess amount of carbon in the medium during polymer production.

2.3.4.1. Diagnostic checking of the model

Biomass (Y_1) and PHA (Y_2) yields were measured in the experiments as responses. The coefficients for the actual functional relatives for predicting Y_1 and Y_2 are presented in Table 2.3. The insignificant terms were omitted based on student's t-ratio (Triveni *et al*., 2001). The two responses under different combination as defined in the design (Table 2.1 and 2.2) was analysed using the analysis of variance (ANOVA) appropriate to the experimental design. The ANOVA for the data obtained using CCRD is presented in Table 2.4. It is evident from the data presented that the first and second order terms were found to be significant and lack of fit was not significant. The lack of fit measures the failure of the model to represent data in experimental domain at points, which are not included in the regression.

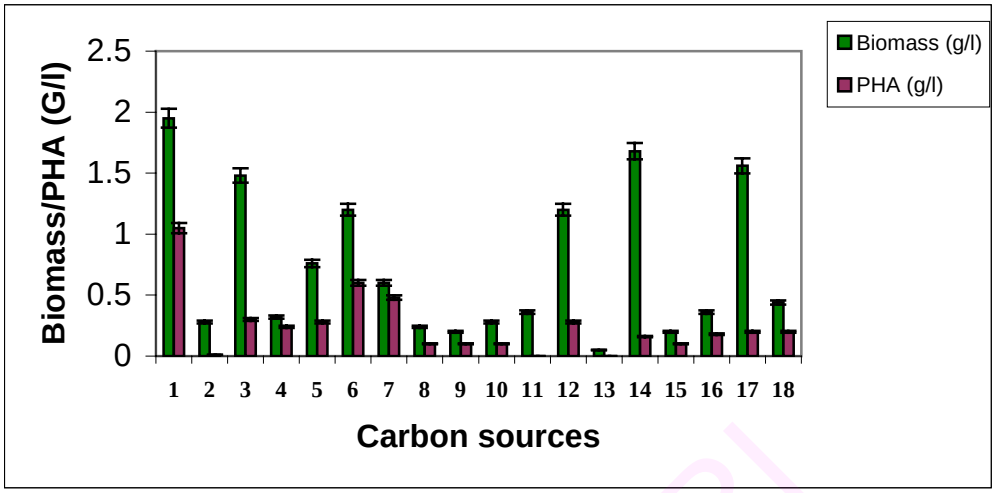


Figure 2.4: Use of various carbon sources for growth and PHA production by *B. flexus.* 1-Sucrose; 2-Arabinose; 3- Dextrin; 4-Dulcitol; 5-Fructose; 6-Glucose; 7-Galactose; 8-Inositol; 9-Lactose; 10-Mannose; 11-Manitol; 12-Maltose; 13-Raffinose; 14-Rhamnose; 15-Sorbitol; 16-Sorbose; 17-Trehalose; 18-Mellibiose.

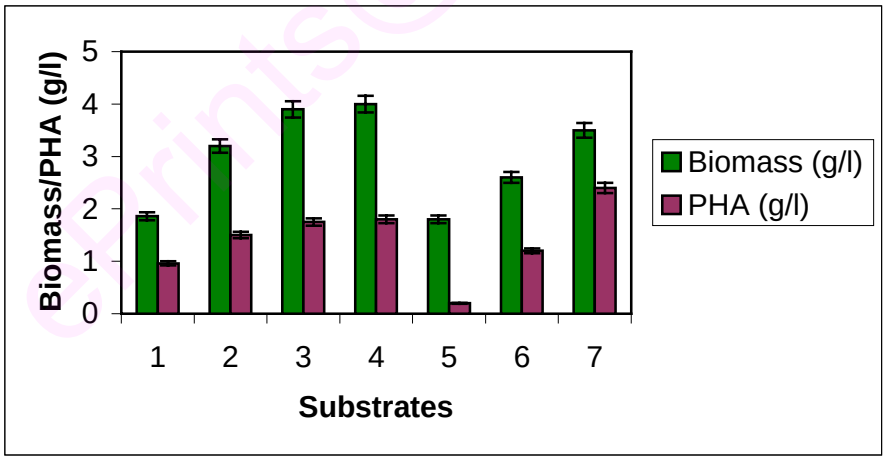


Figure 2.5: Utilization of various economic substrates as carbon sources for growth and PHA production in *B. flexus* :1 Control; 2 Molasses; 3 Corn starch; 4 Corn steep liquor (nitrogen source); 5 Whey ; 6 Wheat bran extract and 7 Mahua flower extract.

Table 2.3 : 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	5.087**	0.209	24.340	1.453**	0.092	15.793
a_1	-0.057	0.107	-0.533	0.209**	0.047	4.447
a_2	0.551**	0.107	5.150	-0.070	0.047	-1.489
a_3	0.024	0.107	0.224	-0.018	0.047	-0.383
a_4	0.536**	0.107	5.009	0.130**	0.047	2.766
a_5	0.716**	0.107	6.692	0.151**	0.047	3.213
a_{11}	-0.212*	0.097	-2.186	-0.071	0.043	-1.651
a_{22}	-0.190*	0.097	-1.959	-0.018	0.043	-0.419
a_{33}	-0.252*	0.097	-2.598	0.132**	0.043	3.070
a_{44}	-0.118	0.097	-1.216	-0.103*	0.043	-2.395
a_{55}	-0.430**	0.097	-4.433	-0.184**	0.043	-4.279
a_{12}	0.364**	0.131	2.779	0.102*	0.058	1.790
a_{13}	0.055	0.131	0.420	0.030	0.058	0.517
a_{14}	-0.017	0.131	-0.130	0.117*	0.058	2.017
a_{15}	0.039	0.131	0.298	0.086	0.058	1.483
a_{23}	0.102	0.131	0.779	0.227**	0.058	3.914
a_{24}	0.067	0.131	0.511	-0.092	0.058	-1.586
a_{25}	0.092	0.131	0.702	-0.123*	0.058	-2.121
a_{34}	0.195	0.131	1.489	-0.020	0.058	-0.345
a_{35}	0.339*	0.131	2.588	0.142*	0.058	2.448
a_{45}	0.486**	0.131	3.710	0.111*	0.058	1.914

*Significant at 5% level, ** Significant at 1% level
Table value of $t_{0.05, 11} = 1.796$ and $t_{0.01, 11} = 2.178$.

The predicted optimum and experimental values are shown in Table 2.5. In order to deduce workable optimum conditions graphical optimization technique was adopted. This technique drastically reduces the amount of time effort required for investigation of multifactor, multiresponse systems. The specifications necessary for each response were first set and these also served as constraints in optimization.

The suitability of the model equation for predicting the optimum response values was tested using the recommended optimum conditions. This set of conditions was determined to be optimum by a RSM optimization approach, which was also used to validate experimentally and predict the value of the responses using model equations. The experimental values were found to be in agreement with the predicted ones.

Table 2.4: 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*
Regression							
First order terms	5	26.580	5.320	46.370*	2.129	0.426	10.644*
Second order terms	15	16.950	1.130	9.860*	4.295	0.286	7.159*
Total	20	43.520		6.424			
Residual							
Lack of fit	6	2.410	0.400	3.510^{ns}	0.367	0.061	1.529^{ns}
Pure error	5	0.570	0.110		0.200	0.040	
Total error	11	2.990			0.567		
Grand Total	31	46.510			6.991		
Coefficient of Determination (R2)			0.936			0.919	

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

Table 2.5: Predicted and experimental value of response at optimum condition for *B. flexus* cultivation.

	KH_2PO_4	$MgSO_4$	Citric acid	$(NH_4)_2HPO_4$	Sucrose
	x_1	x_2	x_3	x_4	x_5
Coded Value	1	1	1	1	1
Uncoded	11.63	1.55	2.25	4.75	31.25
	Experimental Value		Predicted Value		
Biomass Yield (Y1)	7.0		7.3		
PHA Yield (Y2)	2.1		2.2		

2.3.4.2. Response surface plotting

Effect of potassium phosphate, sucrose, citric acid, magnesium sulphate and ammonium phosphate on responses such as biomass yield and PHA yield are reported (Table 2.3) by the coefficient of second order polynomials. In the response surfaces based on coefficient with 3 variables, one of them was kept optimum level and the other two were varied within the experimental range. In general exploration of the response surfaces indicated a complex interaction between the variable.

The response surfaces obtained based on coefficients is shown in Fig. 2.6 in which two nutrients were varied within the experimental range and other nutrients were kept at optimum levels. Varying of $(NH_4)_2HPO_4$ and KH_2PO_4 did not show any effect on biomass production (Fig. 2.6, 1A). Interaction of sucrose and $MgSO_4$ showed that $MgSO_4$ was essential for biomass production and sucrose was essential for both biomass and PHA production (Fig. 2.6, 2A, 2a). At maximum level of these nutrients (2g/l and 40 g/l) biomass produced was 7g/l with 2.3 g/l of PHA. Sucrose and $(NH_4)_2HPO_4$ had positive effect on growth as well as PHA synthesis (Fig 2.6, 3A, 3a). Biomass of 10 g/l and 2.3 g/l PHA were obtained at 40g/l of sucrose and 6 g/l of $(NH_4)_2HPO_4$.

RSM studies have been used to compare and assess nutrient limitation conditions favorable for PHA production in *Rhizobium meliloti* and to compare nutritional performance of parent mutant strain (Kshama *et al*., 2004). It has also been used for to optimize PHA production by *Bacillus* sp. to study the effect of corn steep liquor on biomass and PHA production (Vijayenrda *et al.,* 2007).

The suitability of the model equation for predicting the optimum response values were tested using the recommended optimum conditions. This set of conditions was determined to be optimum by a RSM optimization approach, which was also used to validate experimentally and predict the value of the responses using model equations. The experimental values were found to be in agreement with the predicted ones.

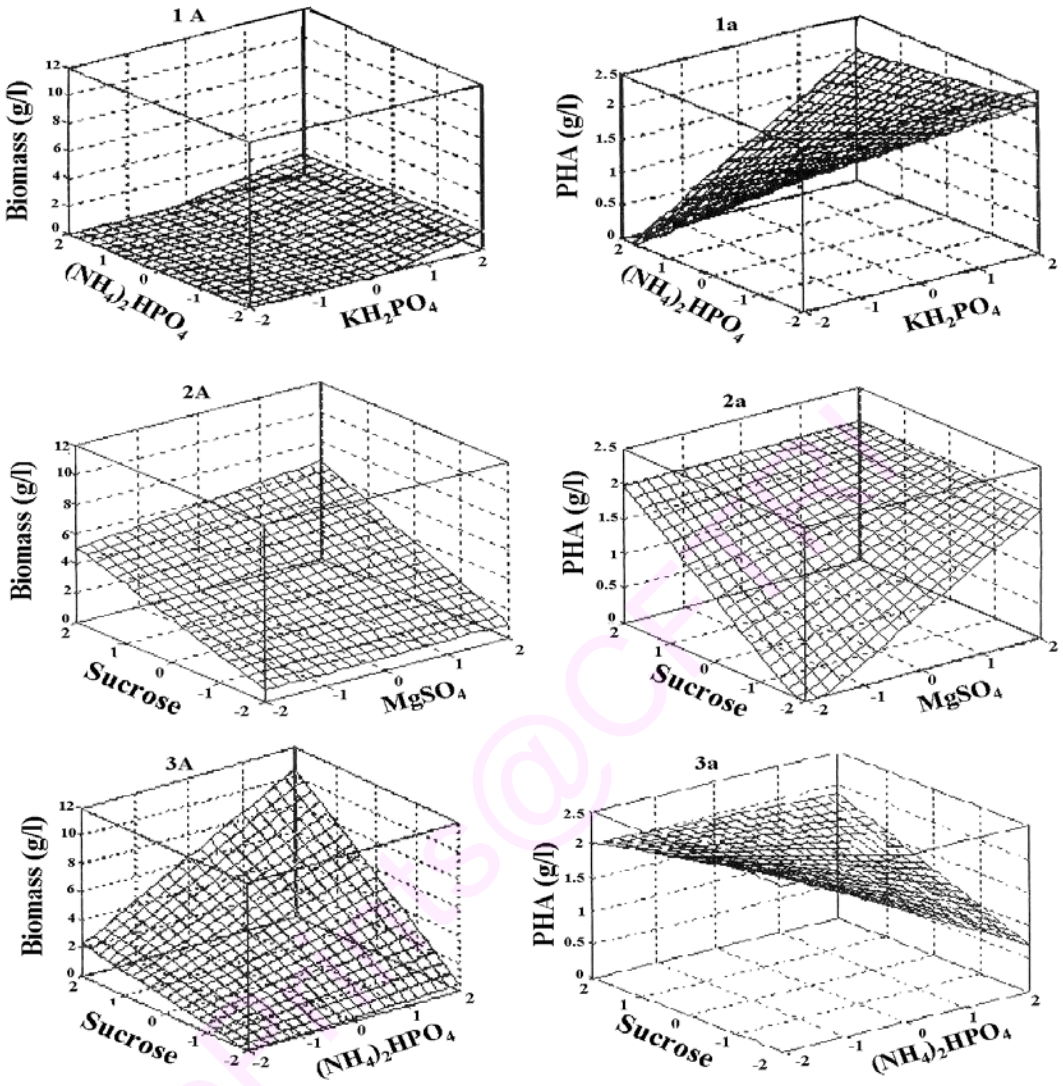


Figure 2.6: Response surface plots showing the effect of nutrients: $(NH_4)_2HPO_4$ and KH_2PO_4 (1A, 1a); Sucrose and $MgSO_4$ (2A, 2a) & sucrose and $(NH_4)_2HPO_4$ (3A and 3a), on biomass (g/l) and PHA (g/l) yields in *B. flexus.* Levels –2 to 2 are given in Table 4.1.

2.3.4. Effect of nutrient uptake on PHA synthesis

Growth and PHA synthesis were measured in optimized medium and it was observed that *B. flexus* required nitrogen limitation for PHA production. Concentration of PHA increased as N became limiting (Table 2.6).

Table 2.6: Uptake of nutrients by *B. flexus* during growth and PHA production.

	Minerals uptake (g/l) by *B. flexus* during growth				
Nutrients	**Initial conc. (g/l)**	**24 h**	**48 h**	**72 h**	**Total uptake (%)**
Carbon g/l	8.42	5.47	3.36	1.26	85.1
Nitrogen g/l	1.16	0.60	0.30	0.10	91.4
Phosphorous g/l	3.70	2.90	2.10	1.30	64.9
Potassium g/l	3.80	0.86	0.84	0.80	76.7
Magnesium g/l	0.14	0.11	0.10	0.10	28.6
Biomass and PHA production					
Biomass g/l	0.1	2.6	3.6	3.2	-
PHA g/l	Nil	0.75	1.75	1.5	-
PHA % of biomass	NIl	28	58	50	-

Analysis of the medium revealed that maximum growth was obtained with 1:9 ratio of N and C, respectively. At this concentration, carbon conversion to PHA was 40% of total carbon supplied and the efficiency lowered to 20% in medium containing 1:4 of N:C. Excess of carbon resulted in enhancement of biomass production. Decrease in C content (N:C= 1:4) resulted in decreased yields. PHA accumulation was initiated with depletion of N. Phosphorous was utilized rapidly after the depletion of nitrogen. Magnesium and potassium were essential for growth rather than for PHA synthesis.

Elemental analysis of PHA containing cells showed that it contained N:C:S:H in 11:43:0.5:7 ratio. Data indicated that the cells had accumulated more carbon in the form of PHA under limiting nutrient conditions.

2.3.4. Cultivation of *B. flexus* in a fermentor using optimized conditions

B. flexus was cultivated in 3 l fermentor in medium containing 11.6 KH_2PO_4, 1.5 Mg SO_4 7 H_2O; 4.7 g $(NH_4)_2HPO_4$ and 31.2 sucrose as mentioned under section 2.2.10 of current chapter. Rapid growth and higher PHA yields were obtained (Fig. 2.7) compared to shake flask conditions (Table 2.5; Biomass, 7 g/l and PHA, 2.1 g/l). Concentration of biomass and PHA obtained after 48 h of fermentation were 10 and 5.1 g/l, respectively. Higher yield obtained was due to maintenance of required pH and aeration for the growth and polymer production by the bacterium.

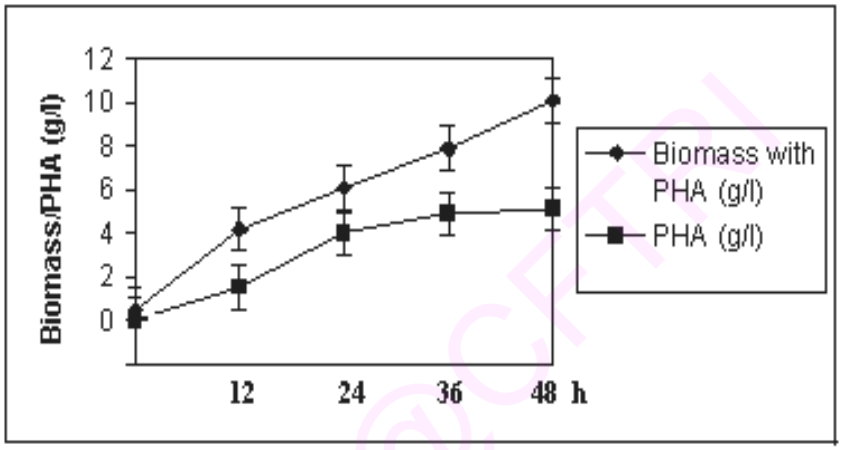


Figure 2.7: Fermentor cultivation of *B. flexus* in RSM optimized medium.

2.3.5. Mathematical modeling

The Luedeking-Piret model is often used to describe the rate of microbial metabolic product without depending on the substrate concentration. This model allows a metabolic product to be accumulated even after the substrate has been depleted (Luong *et al.,* 1988). The kinetic constants were evaluated based on non-linear regression and the differential equations were solved using Runge-Kutta algorithm using MATLAB software. The Runge-Kutta algorithm was used for fitting together all experimental data. The kinetic results obtained for the production of PHA were close to those of other kinetic parameter determination method. Runge-Kutta algorithm was validated for experimental data analysis in order to get, in a simplest way. This algorithm was a more efficient calculation method for kinetic analysis because of possibility of fitting together

all experimental data obtained at same kinetic parameter (Mateos *et al.,* 2005). In the present study, based on the experimental data, a batch fermentation kinetic model was developed to present the PHA biosynthesis in *B. flexus.* Data is presented in Figs. 2.8 - 2.12.

2.3.5.1. Rate of cell growth

Growth of *B. flexus* was described by means of a simplification of Monod's model for low substrate concentrations, which expresses the growth rate as a function of substrate and biomass concentrations (Tobajas *et al*., 2007).

$$\frac{dX}{dt} = k_x SX$$

Where k_x represents the kinetic constant for growth rate (g/l h) and S and X are the substrate and biomass concentrations (g/l), respectively.

The cell components of *B. flexus* consists of two main parts i.e. PHA (X_P) and residual biomass (Total biomass-PHA; X_R). This residual biomass is the catalytic component and it is responsible for the metabolic activity of the cell.

The specific growth rate of the microorganism (μ_m) is expressed in terms of the equation below:

$$\frac{dX_R}{dt} = \mu_m X_R \left[1 - \frac{X_R}{X_m}\right]$$

Fig 2.9 shows that the experimental values of biomass concentration correlate well with that of the model values, to provide adequate representation of growth and fermentation kinetics of *B. flexus.* The predicted values from the proposed model were then compared with those of monod and logistic equations. In this case model describing the biomass growth rate together with the kinetics of fermentations. As shown in Fig. 2.9 simulation results of the model compared favorably with the experimental data. The model was able to predict the biomass concentration data for the duration of fermentation.

The log phase and the stationary phase are separated by an extremely short transition period, which is almost comparable to the action of a switching mechanism. In this period nitrogen source drastically falls down, with increased PHA concentration. The

rate of residual biomass production stopped and remained at zero from this stage till further fermentation period.

2.3.5.2. Rate of PHA production

Production of PHA continued in *B. flexus* in growth as well as stationary phases. The non-growth and growth associated synthesis can be represented by Luedeking-Piret type model (Luedeking and Piret, 1959), which allows a correlation between cell mass and product concentration.

The non-growth and growth associated synthesis was found to be very useful to describe the kinetic data from many fermentation processes, including biopolymers (Weiss and Ollis, 1980; Klimek and Ollis, 1980).

The data shows a clearly diminishing value for dX_P/dt throughout this phase, however, eventually reaching $dX_P/dt = 0$ at t = 72 h. This allows for a decreasing product rate over time. The growth and synthesis of PHA can be explained by the equation below (Mulchandani *et al*., 1989).

$$\frac{dX_P}{dt} = n' X_m \left(1 - m' t\right)$$

Where n' and m' are the constants and t is measured from the onset of stationary phase.

The utilization of this equation in growth forms shows straightforward analytical representation for the production of PHA. The Luedeking – Piret model has been used to describe the rate at which *B. flexus* synthesize PHA in batch culture. The model indicates that the product formation rate is linearly proportional to the biomass growth rate i.e., growth-associated and the instantaneous biomass concentration (non-growth associated). It can be observed that the bacterium grew up to 30 h but PHA synthesis continued till 60 h and became constant thereafter (Fig. 2.10). It also indicates that the PHA production is both growth and non-growth associated. Approximately 50% of the total PHA was synthesized during the exponential growth phase. This model thus showed that PHA accumulated after nitrogen substrate depletion.

The rate of PHA production X_P is linearly correlated with the rate of residual biomass X_R during the exponential phase and storage phase. It is of course logical that the maximum PHA synthesis will depend on the type of bacterium and growth conditions.

2.3.5.3. Rate of sucrose utilization

Sucrose is used as a carbon source in the PHA production process. The rate of consumption of sucrose is the proportional to the rate of growth of residual biomass (dX_R/dt), rate of formation of PHA (dX_P/dt) and sucrose required for the maintenance (which is proportional to residual biomass, X_R).

The kinetics of microbial growth, substrate consumption and product formation are routinely formulated in terms of equation that lead to coupling between the associated rates. Mulchandani *et al*., (1988) proposed a generalized form of the logistic equation for describing the batch kinetics of microbial growth for the biopolymer synthesis.

$$\frac{dX_S}{dt} = -\beta_m X_R \left[1 - \frac{X_R}{\alpha_m}\right]$$

Batch microbial growth is characterized by two regions, that of exponential growth where $dX/dt = \mu_{max} .X$ (μ = constant) and of stationary growth by $dX/dt = 0$ it is common for the transition region between these two kinetic regions of substrate dependent growth rates.

The model was used to predict that the biomass concentration is also applicable for representing the substrate concentration profile with some difference in the actual value. In order to account for the availability of the substrate a combination of Monod's and logistic or modified logistic has been proposed. To certain extent model was applicable when the cell grew at low substrate concentrations i.e., substrate is not limiting for the metabolic activity. About 50% of the sucrose was utilized during the first 24 h of the exponential growth period of the organism. The experimental values of sugar utilized fitted well with the predicted values of the model (Fig. 2.11).

Substrate inhibition is not found in the PHA formation, where it a usual problem in other microbial fermentations. The Luedeking-Piret model however, neither explicitly nor implicitly depends on substrate concentration. A linear relationship between the specific growth rate and the product formation could be considered as a specific case and it may not be valid for all the microorganisms.

The results of a number of independent experiments each done under identical conditions are compiled in Fig. 2.8, indicating residual biomass, product PHA formation, substrate concentration and utilization of nitrogen. After a lag phase of 6 h, the biomass concentration rapidly increased to a maximum of 3 g/l. *B. flexus* can accumulate up to

40% of PHA in the late storage phase. From Fig. 2.8 the time courses of residual biomass concentration and PHA have the same slope in a semi logarithmic plot. This indicates that PHA synthesis is fully associated with cell growth even when there is no substrate limitation. The parameter values are given below:

Constants	Values
Biomass equation	
X_m (g/l)	1.470
μ_m (h^{-1})	0.217
X_o (g/l)	0.120
Product equation	
n' (g-p/g * h)	0.048
m' (h^{-1})	0.017
Substrate equation	
α_m (g/l)	11.066
β_m (h^{-1})	0.021

2.3.5.4. Rate of nitrogen utilization

Nitrogen was the growth-limiting substrate in the growth of *B. flexus* in the medium. The maximum biomass concentration will be obtained at a time at which the limiting substrate is completely consumed.

Effect of nitrogen concentration on the microbial activity of *B. flexus* was studied. Nitrogen was also found depleting as the biomass increased (Fig. 2.12). An agreement between the theoretical and experimental yields thus allowed confidence in further analysis of experimental data. These values were then used to simulate the profiles of biomass, PHA production and substrate concentration with one limiting nutrient during the growth. The phosphorous utilization profile was not taken into consideration, for the comparison between the theoretical and experimental yields since it is also a limiting nutrient. However, during the storage phase when the nitrogen source is depleted, the increase of cell dry weight is only due to the accumulation of PHA as can be seen from the time course of the change in residual biomass when linearly plotted as in Fig. 2.8. When nitrogen concentration decreased, the rate of PHA synthesis was maximum and then decreased.

In the very beginning of the storage phase the PHA production rate (dX_P/dt) is equal to the rate of cell dry weight (dX/dt).

$$\frac{dX_N}{dt} = -\frac{1}{Y_{RN}}\frac{dX_R}{dt}$$

The PHA content approaches a maximum value as shown in Fig. 2.8. Therefore one can conclude that the total yield of PHA is indirectly a function of the nitrogen concentration in a nitrogen-limited culture. As shown in figures simulation results using the model compared favorably with the experimental data. The model was able to predict the biomass concentration data for the duration of fermentation with some difference in the actual value. The adequacy of this model in representing PHA fermentation suggested that the process is limited by the nutrient addition (Suzuki *et al.,* 1986a). In the present case the production of PHA continued during growth as well as stationary phase ($dX_R/dt = 0$). However, the rate of PHA production (dX_P/dt) was found to continuously decrease with time (Ollis, 1983).

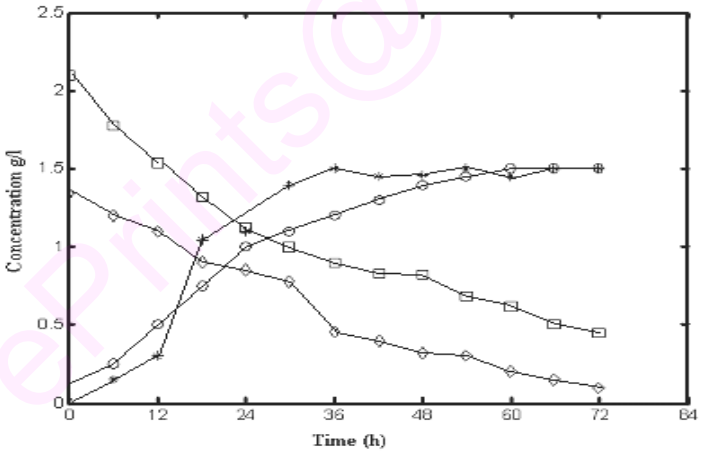


Figure 2.8: Kinetics of PHA production by *B. flexus* by actual experimentation in shake flask at 30^0 C, 250 rpm for 72 h. X_R Residual biomass (biomass-PHA; g/l); X_P PHA; X_S Residual sucrose (g%); X_N Residual nitrogen (g/l).

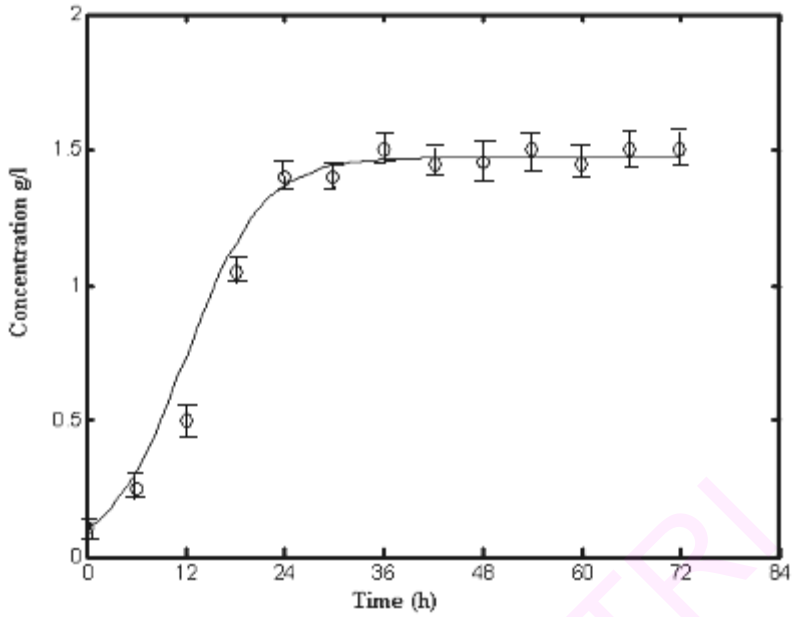


Figure 2.9: Variation of residual biomass (total biomass-PHA) with time indicating experimental values (o) and model fitting (—).

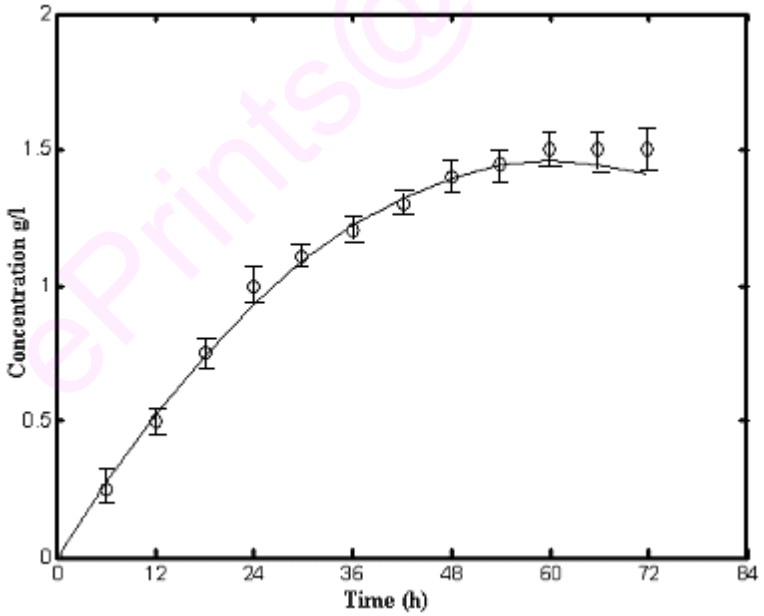


Figure 2.10: Variation of product (PHA) concentration with time indicating experimental values (o) and model fitting (—).

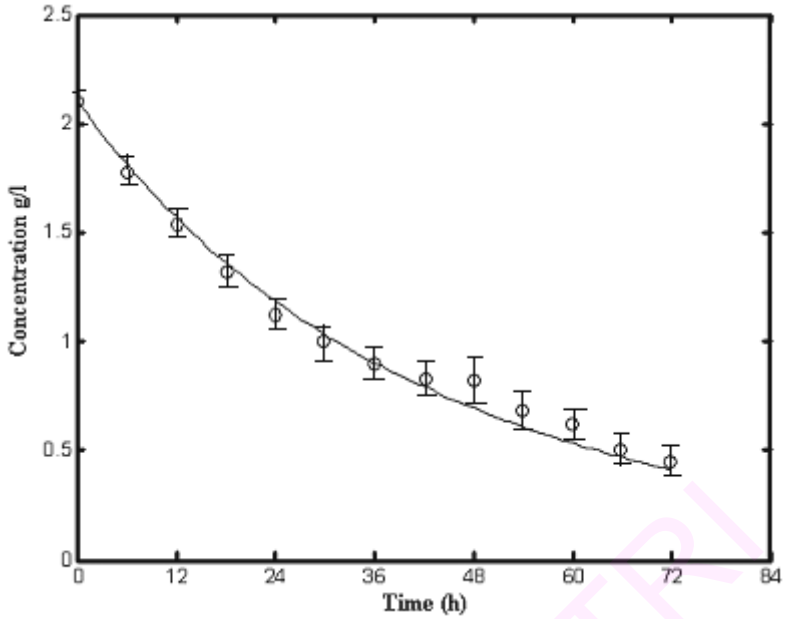


Figure 2.11: Variation of residual carbon concentration with time indicating experimental values (o) and model fitting (—).

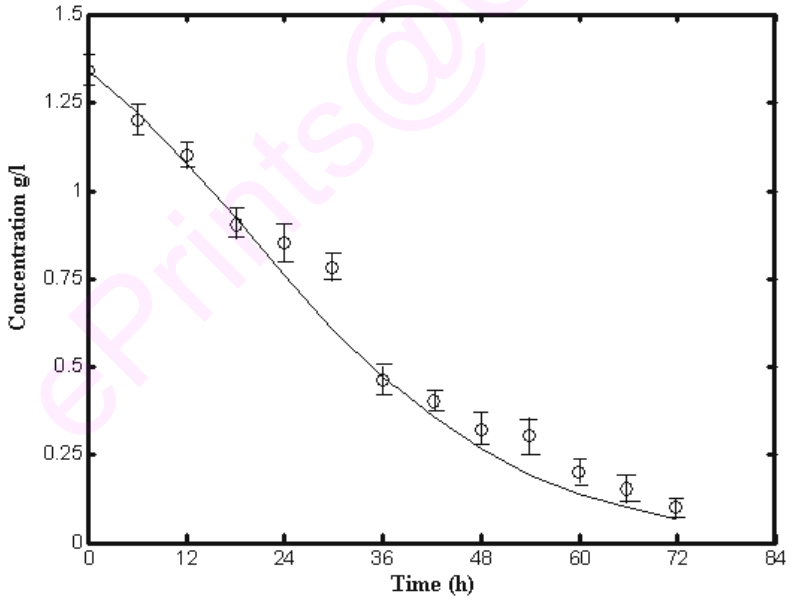


Figure 2.12: Variation of residual nitrogen concentration with time indicating experimental values (0) and model fitting (—).

2.3.6. Effect of organic acids, fatty acids on PHA copolymer synthesis

Hydroxyalkanoate monomers which form PHA are broadly classified into short chain length PHA (scl-PHA) composed of 3-5 carbons monomers and medium chain length PHA (mcl- PHA) consisting of 6 to 14 carbons. Several alkanoic acid monomers are known to be components of PHA. Homopolymer such as P(HB), an scl PHA, is the most common type of polymer produced by bacteria (Anil Kumar, 2008). Sugars such as glucose and sucrose are the most common substrates used for PHA production and they are metabolized to synthesize P(HB). Although P(HB) is most commonly synthesized to higher concentrations in the cell, the material is brittle and has poor elastic properties. In contrast to this mcl-PHAs are thermo elastomers and recent advances in PHA production have shown that copolymers of scl-PHA [Ex: P(HB-co-HV)] or scl-co-mcl PHA have better thermo mechanical properties and hence are feasible for various practical applications (Anil Kumar *et al.,* 2007; Reeta *et al.,* 2008).

Various bacteria are known to synthesize copolymers of P(HB) with other hydroxyalkanoates of C3 to C14 depending on the intrinsic PHA biosynthesis pathways and carbon sources used for fermentation (Tsuge, 2002). Supplementation of co carbon substrates such as fatty acids in the medium leads to the biosynthesis of PHA copolymers (Choi and Lee, 2000). In the present work, various fatty acids such as butyric, valeric, hexanoic, heptanoic, octanoic, nonanoic and decanoic acids were used in the medium as co substrates for PHA copolymer synthesis. Other fatty acids and organic acids used were: linoleic acid, oleic acid, stearic acid, succinic acid, acetic acid, palmetic acid, propionic acid and malic acid. Enhancement of growth (Fig. 2.13) was observed with butyric, valeric and hexanoic acids (3.1, 3.2 and 3 g biomass/l, respectively compared to control (1.9 g/l). There was concomitant increase in PHA concentration in the biomass by 1.3, 1.4 and 1.3 g/l compared to control (1.0 g/l). PHA formed was a copolymer P(HB-co-HV) of 97:3 mol% in valeric and hexanoic acid medium. Amongst other fatty acids and organic acids tested acetic and propionic acid supported enhanced PHA production (1.2 and 1.5 g/l) compared to respective control (0.7 g/l) (Fig. 2.14). Acetate metabolism is important for growth and PHA production as formation of acetyl CoA moieties is significant from the point of TCA cycle, fatty acid path way as well as PHA synthesis. Based on this higher biomass and PHA concentration can be expected from acetate

metabolism. However, PHA produced was a homopolymer of P(HB) in the presence of acetic acid. Propionic uptake resulted in the formation of P(HB-co-HV) of 97:3 mol%. Precursor substrates such as valeric acid and propionic acids have been used as carbon sources during the growth of *Ralstonia eutropha* to obtain P(HB-co-HV) (Doi *et al.,* 1990). For the synthesis of P(HV), 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 polymerizes this to the growing chain of PHA. Propionyl CoA is synthesized under certain physiological conditions from propionic acid, via propionyl CoA synthase enzyme or from fatty acid β-oxidation of odd chain fatty acids or from various aromatic amino acids via trans carboxylation reactions or from succinyl CoA- methylmalonyl CoA pathway (Steinbuchel and Eversloh, 2003). In the present study supplementation of malic acid to the medium resulted in maximum biomass production of 4 g/l compared to control (2.2 g/l) or other organic acids (Fig. 2.14). This may be expected as malic acid may be utilized as a carbon source for growth through TCA cycle.

2.3.7. Production of PHA copolymers using palm oil effluent

Palm oil effluent (POE) contains significant amounts of oil and fatty acids, which contribute to high chemical and biological oxygen demand of the effluent (Alias and Tan, 2005). POE has been anaerobically treated to obtain organic acids and later the acids were converted to PHA by *Rhodobacter sphaeroides* (Hassan *et al.,* 1997). Bacteria that can utilize palm oil has been isolated from POE so that the bacterium can be directly cultivated on the effluent for PHA synthesis (Alias and Tan, 2005). In the present work it was found that the biomass and PHA production were maximum when the effluent was used at 100% level instead of water for medium preparation (Fig. 2.15). Biomass and PHA yield ranged from 4.8-8 g/l and 1.5 to 4 g/l, respectively. PHA isolated from 20% POE medium was composed of poly(hydroxybutyrate-co-valeratate-co-hydroxyoctanoate) P(HB-co-HV-HOc) of 97:2:1. HV concentration increased to 3 mol% when the effluent was used at 100% concentration in medium preparation. ^{1}H NMR obtained for isolated polymer is shown in Fig. 2.16.

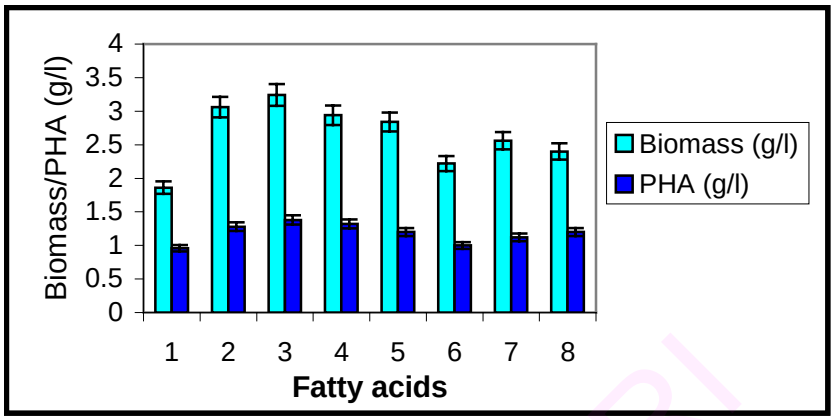


Figure 2.13: Utilization of different fatty acids for growth and PHA production by *B. flexus*: 1-Control; 2-Butyric acid; 3-Valeric acid; 4-Hexanoic acid; 5-Heptanoic acid; 6-Octanoic acid; 7-Nonanoic acid; 8-Decanoic acid.

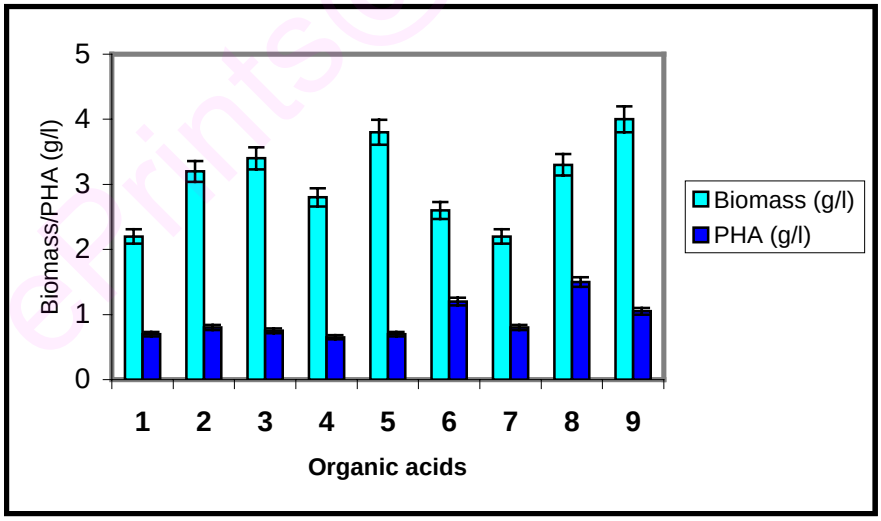


Figure 2.14: Production of PHA in various organic/fatty acids by *B. flexus*: 1- Control; 2-Linoleic acid; 3-Oleic acid; 4-Stearic acid; 5-Succinic acid; 6-Acetic acid; 7-Palmetic acid; 8-Propionic acid; 9-Malic acid.

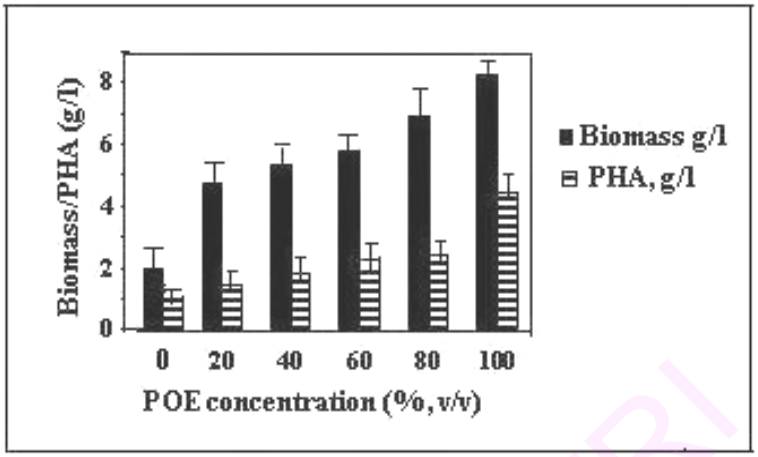


Figure 2.15: Growth and production of PHA in *B. flexus* at different concentrations of palm oil effluent (POE; v/v).

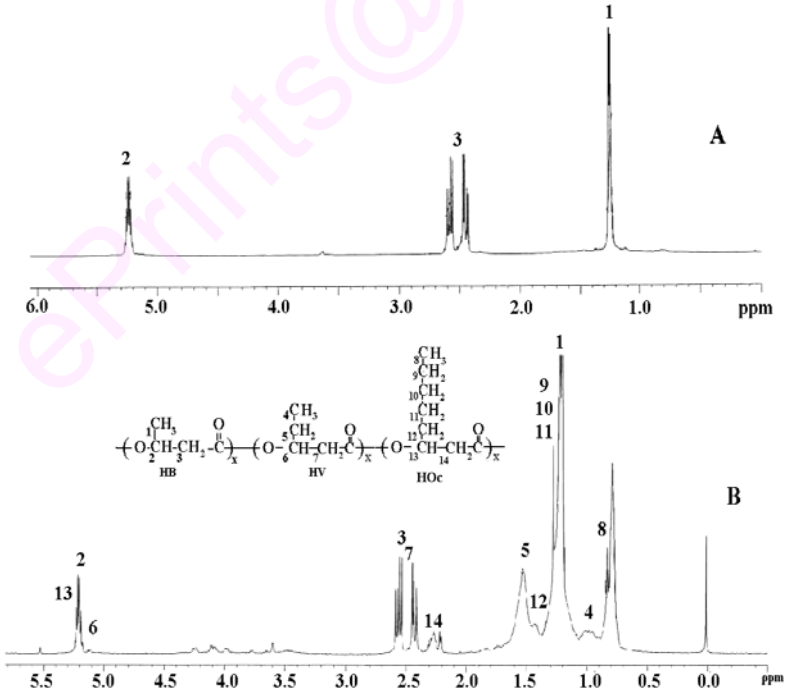


Figure 2.16: ^{1}H NMR spectrum of PHA. A: Standard PHB; B: PHA isolated from *B. flexus* cultivated on palm oil effluent and sucrose

^{1}H NMR spectrum of purified PHA from POE medium showed signals characteristic of P(HB) and HV which are dealt in detail under section detailed below (2.3.8.1). Additionally mcl mononer of hydroxyhexanoate was observed as indicated in Fig. 2.16. The NMR spectrum obtained is in accordance with data reported in the literature (Anil Kumar *et al.,* 2007). Data indicated that POE, which causes pollution problem due to high BOD, could be efficiently used for bacterial fermentation to enhance cell growth and as a co carbon source to synthesize PHA copolymers.

2.3.8. Effect of plant oils on PHA copolymer synthesis

Plant oils or fatty acids can be good carbon sources for PHA production. Instead of fatty acids, fatty acid rich substrates such as rice bran oil, pongemia, castor oil, palm oil, sunflower oil, coconut oil, sesame oil, neem oil, groundnut oil and mustard oil, were saponified and substituted as co carbon substrates to the medium. Most of the oils that contained oleic and linoleic acids supported good growth (1-1.6 g biomass/l) and PHA production (0.5 to 1 g/l). Ground nut oil was not preferred as carbon source for growth or PHA production. Amongst all the oils, castor oil, palm oil and rice bran oil were found to be better suited for PHA production by *B. flexus* (Fig. 2.17). Biomass obtained was 1.6, 1.4 and 1.5 g/l with PHA concentration of 1, 0.6 and 1.1 g/l, respectively. PHA concentration in the biomass was 62, 43 and 73%, respectively. Unsaponified oils of these were tested as co substrates and data indicated that rice bran oil gave maximum biomass (3.8 g/l) and PHA yields (2.2 g/l) compared to other two substrates (Table 2.7). Supplementation of saponified oil resulted in decrease in biomass. 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 is related to final decline in microbial growth and activity (Lawford and Rousseau, 1993).

2.3.8.1. Characterization of PHA

FTIR, GC, GC-MS, ^{1}H and ^{13} C NMR were used for qualitative, quantitative and structural analysis of purified PHA samples.

Fourier transform infrared spectroscopy has been demonstrated to be a powerful technique to detect PHA and to study the cell components in intact form (Helm *et al.,* 1991; Serafim *et al.,* 2002). FTIR spectra reflect the proportions of functional groups present in the sample as a fingerprint. The energy exchange effectively modulates a portion of the monochromatic light with a Raman-shifted spectrum (Berkley *et al.,* 2002). Organic substances show characteristic group frequencies in infrared region. When the temperature of the test material is increased it begins to emit radiant energy. Depending on emissiveness the emitted radiation forms a curve as a functional wavelength or frequency. The intensity of the absorption bands is related to the concentration of the substance that absorbs the incident radiation. Certain wavelengths are associated with changes in the structures of the absorbing molecules. The resulting curves are distinguished from one another by the presence or the absence of particular bands of energy. Thus the absorption bands in an infrared spectrum are at frequencies of corresponding to the frequencies of vibration of the molecule concerned. Infrared spectra can thus provide a total simultaneous chemical analysis. Fig. 2.18 shows intense absorption bands around 1724 cm^{-1} which represents the stretching vibration of C=O groups, which is one of the characteristic bands for PHA. Other accompanying bands located near 1280 and 1165 are attributed to C-O-C groups. The CH stretching bonds is assigned to the bands located in the spectral region around 2900 cm^{-1}. Sun and his co workers identified P(HB) and also mcl-PHA by FT-IR technique (Sun *et al.,* 1999; Shamala *et al*., 2008) where purified P(HB), mcl-PHA and P(HB+mcl-HA) showed their strongest band at 1728 cm^{-1}, 1740 cm^{-1} and 1732 cm^{-1} respectively. The methylene C-H vibration near 2928cm^{-1} had the strongest band in mcl-PHA spectra. Results obtained in our experiments confirmed that the polymer isolated was PHA and the polymer obtained by the cultivation of bacterium in the presence of rice bran oil gave FTIR spectrum which had strong vibration in the region of 2933-2975 cm^{-1}, which indicated the presence of PHA copolymer.

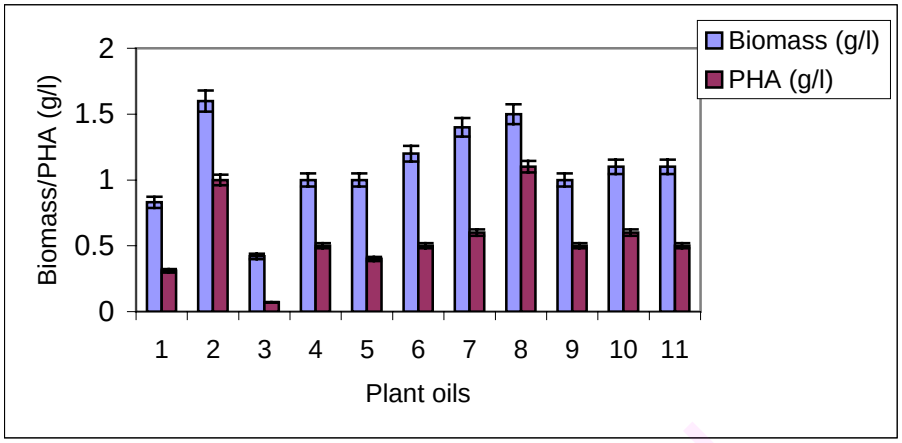


Figure 2.17: Production of PHA by *B. flexus* using saponified plant oils as co-substrate.1-Control; 2-Castor oil; 3-Coconut oil; 4-Groundnut oil; 5-Mustard oil; 6-Neem oil; 7-Palm oil; 8-Rice bran oil; 9-Sesame oil; 10-Sunflower oil; 11-Pongemia oil.

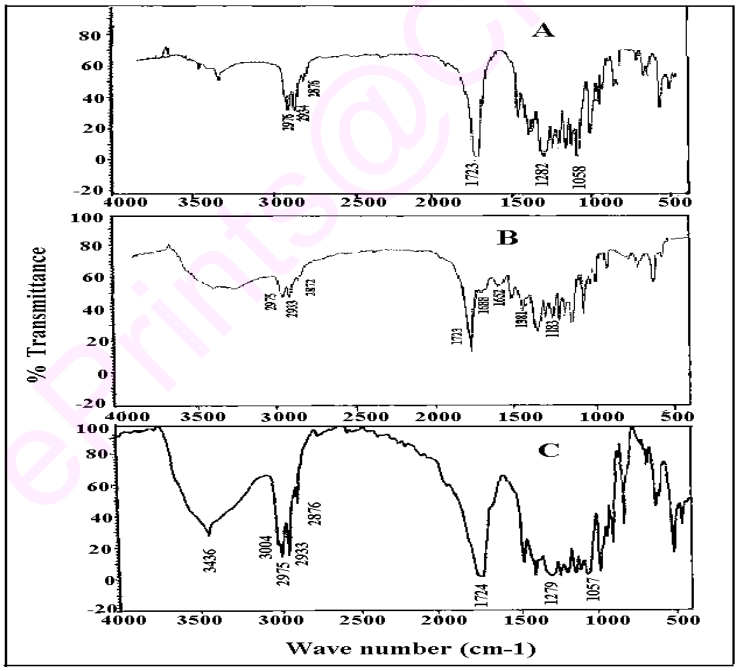


Figure 2.18: FTIR spectra of the PHA: A) Standard containing P(HB-co-HV); B) PHA isolated from *B. flexus* grown in the presence of sucrose as carbon source; C) PHA isolated from *B. flexus* grown in the presence of sucrose as main carbon source and rice bran oil as co carbon source.

Gas chromatography is a common method used for qualitative and quantitative analysis of PHA. PHA isolated from the bacterium was converted to fatty acid methyl ester and analyzed. Chromatogram obtained for standard P(HB-co-HV), polymer isolated from bacterial cells grown on sucrose alone and sucrose + rice bran oil is shown in Fig. 2.19.

Table 2.7: Use of selected unsaponified oils as co-substrate for the production of PHA in *B. flexus*.

Oil as co substrate	Biomass (g/l)	PHA (g/l)	PHA (% of biomass)	Mol% HB:HV:HOc:HDec
Control	1.8±0.2	0.9±0.1	50	100:0:0:0
Castor oil	2.0±0.5	1.1±0.2	55	98:0:1:1
Palm oil	2.2±0.6	1.1±0.4	50	97:2:1:0
Rice bran oil	3.8±0.2	2.2±0.1	58	97:0:2:1

GC-MS was used for characterization of PHA isolated from cells grown on sucrose and in the presence of plant oils. Fig 2.20 shows the total ion current chromatogram. The base peak of m/e 103, which is formed by cleavage at the a- position of the hydroxy functional group and the characteristic peak of m/e 74, which results from the Mclafferty rearrangement, indicated the 3-hydroxy functional structure. The molecular fragments due to the 3-hydroxy functional group were: m/e 103 ($C_4H_7O_3$+), m/e74 ($C_3H_6O_2$+), m/e71($C_3H_3O_2$+), m/e 61 ($C_2H_3O_2$+) and m/e 43 (C_2H_3O). The peaks obtained were matched with MS library. Peak retention for hydroxybutyrate, hydroxyoctanoate and decanoate were at 6.8, 16.8 and 23.5 min. Retention of hydroxyvalerate was 7.7 min, which was obtained in palm effluent as substrate. Typical fragmentation pattern of PHA obtained were: m/z of 43, 59, 74, 87 and 103/101 which are in agreement with those reported in the literature (Valappil *et al.,* 2007). Presence HB, HV, HOc in PHA obtained from cultivation on palm oil effluent and HB, HOc and Dec in cells cultivated on rice bran oil as co carbon source was confirmed by GC-MS.

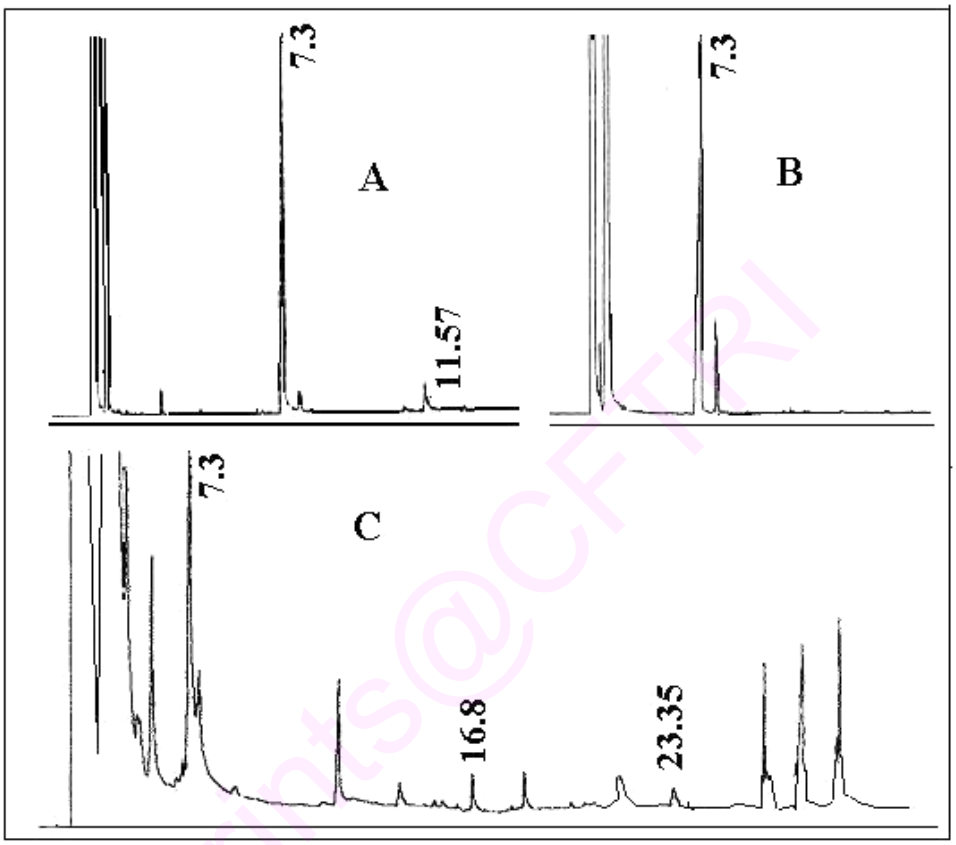


Figure 2.19: Gas chromatogram of PHA. A) Standard P(HB-co-HV); B) PHA isolated from *B. flexus* grown in the presence of sucrose as main carbon source and C) PHA isolated from *B. flexus* grown in the presence of sucrose as main carbon source and rice bran oil as co carbon source. 7.3 min=hydroxybutyrate; 11.57 min=hydroxyvalerate; 16.8 min=hydroxyoctanoate and 23.5 min= hydroxydecanoate**.**

Nuclear magnetic resonance analysis was carried out using PHA isolated from *B. flexus* cells grown on sucrose as main carbon source and also with rice bran oil as co carbon source. NMR spectroscopic analysis is useful to determine the polymer monomer structure and composition. In ^{1}H NMR protons of different types of functional groups –OH, =CH–, CH_2 and -CH_3 have characteristic magnetic resonances, and changes in the local environment of such groups can be detected easily. ^{1}H NMR spectrum of PHA produced on sucrose and rice bran oil substrates are shown in Fig. 2.21. Presence of 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, confirmed the presence of P(HB) in the polymer. A triplet at 0.9 ppm and a methylene resonance at 1.59 and methyne resonance at 5.15 indicated the presence of valerate in the polymer. The peaks assigned to mcl-PHA of octanoate and decanoate are indicated in Fig. 2.21. Only P(HB) was found in polymer obtained from sucrose fed cells. ^{13}C NMR of the polymer shows the chemical shifts assigned to hydroxybutyrate, ocatnoate and decanoate. In addition to major amount of P(HB), C8 and C10 monomers were found in polymer isolated from rice bran oil fed cultures (Fig. 2.22). Carbon chemical shift data is summarized and presented in Table 2.8. Assignments of chemical shifts for HB and mcl PHA are in agreement with literature reported data (Hujiberts *et al.,* 1994; Fukui *et al.,* 1998; Pedro, 2003).

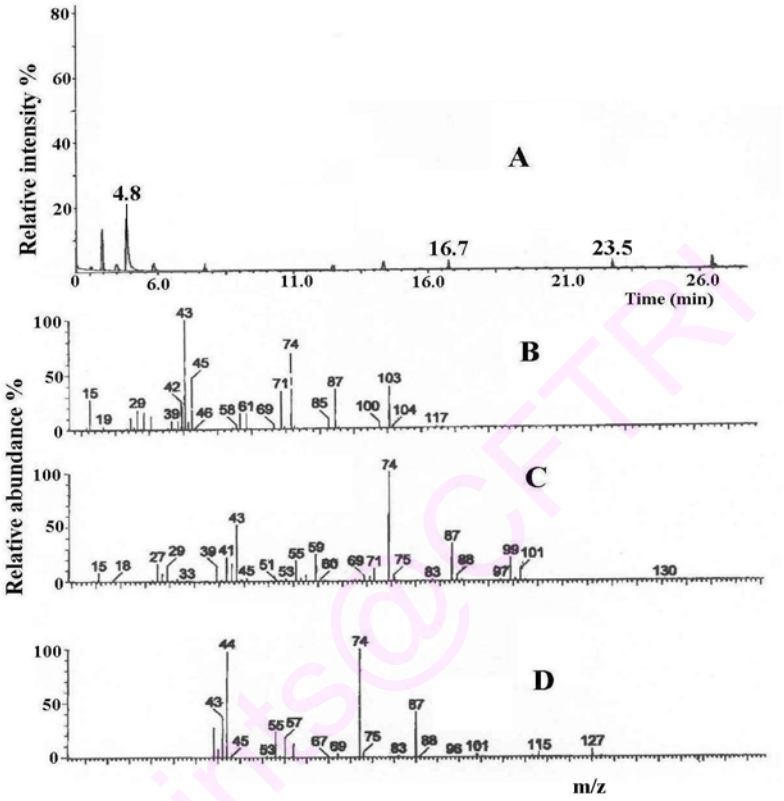


Figure 2.20: Total ion current chromatogram (A) of the methanolysis product of *B. flexus* cultivated in rice bran oil, mass spectrum of methylesters of 3-hydroxybutyre (B), octanoate (C) and decanoate (D).

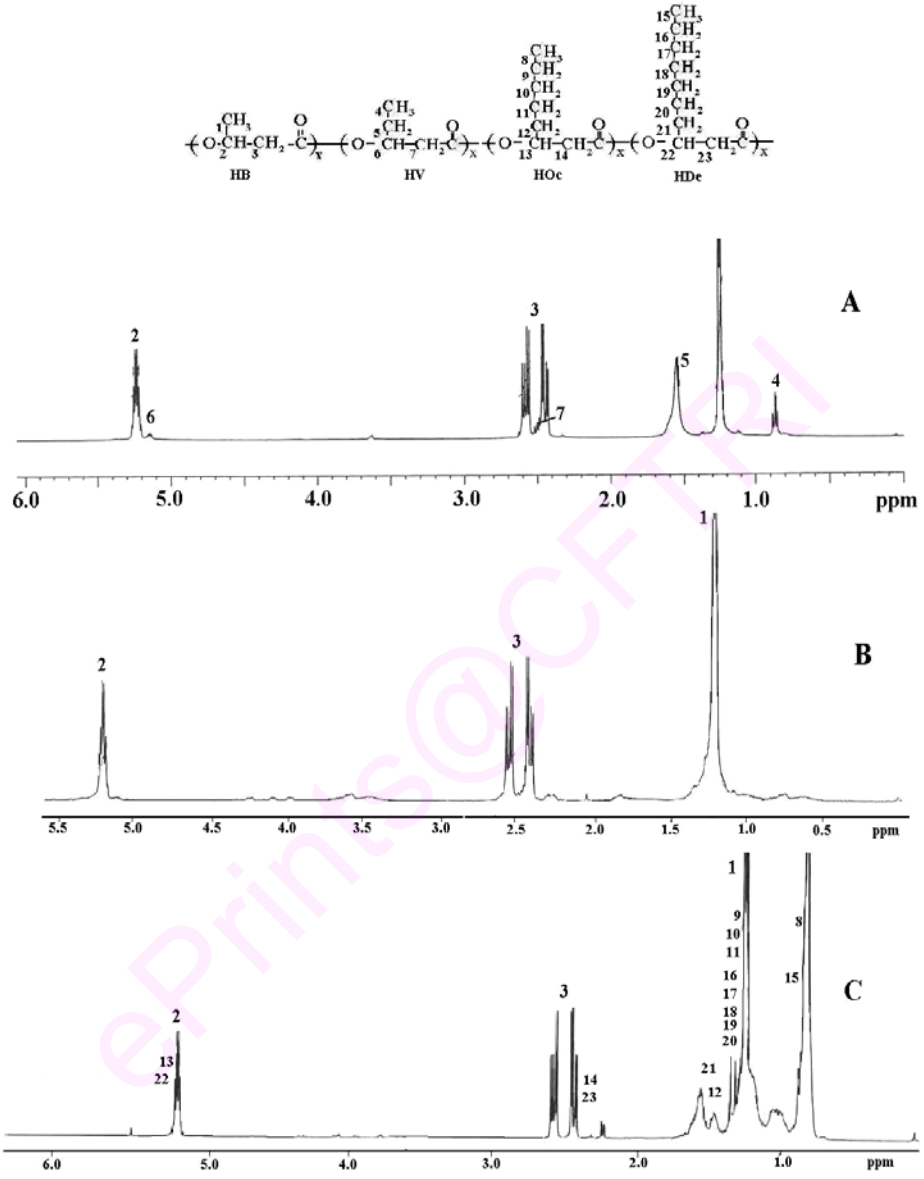


Figure 2.21: ^{1}H NMR spectrum of PHA: A) Standard P(HB-co-HV); B) PHA isolated from *B. flexus* grown on sucrose as main carbon source and C) PHA isolated from *B. flexus* grown on sucrose as main carbon source and rice bran oil as co carbon source.

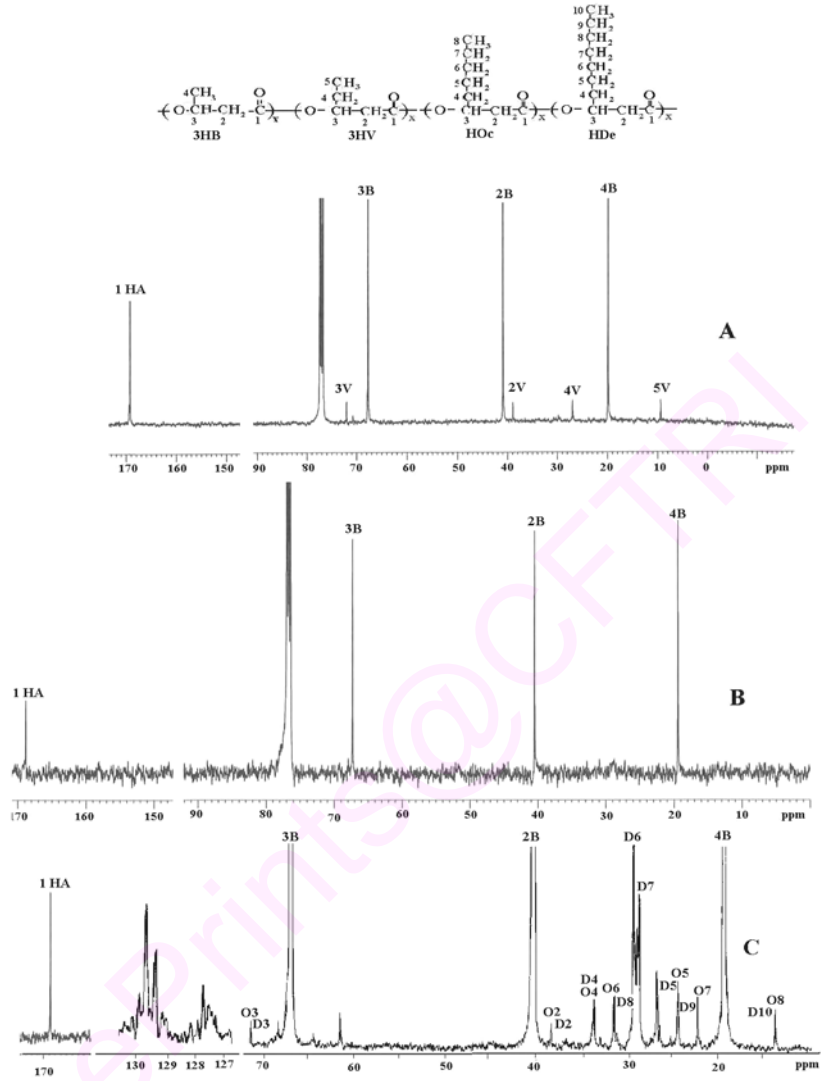


Figure 2.22: ^{13}C NMR spectrum of PHA: A) Standard P(HB-co-HV); B) PHA isolated from *B. flexus* grown on sucrose as main carbon source and C) PHA isolated from *B. flexus* grown on sucrose as main carbon source and rice bran oil as co carbon source (Assignment of peaks: 1HA common peak C=O of 3HB, 3HV, HOc and HDe; H=Butyrate; V=Valerate; O=Octanoate and D=Decanoate).

Table 2.8: ^{13} C chemical shifts of PHA monomers isolated from *B. flexus* cells cultivated on rice bran oil as carbon source.

Carbon	C4	C5	C8	C10
1	168.78	168.78	168.78	168.78
2	40.87	38.48	38.70	38.70
3	67.65	71.58	71.00	71.58
4	19.78	26.77	33.72	33.72
5		9.00	24.54	25.10
6			31.25	29.34
7			22.13	28.98
8			13.74	31.57
9				22.33
10				13.74

Differential scanning calorimetry (DSC) was used to find the melting temperature of the polymer. It is a thermo analytical technique, which is widely used to analyze the polymer, to check their purity, melting point and glass transition temperature. It involves energy changes that can be detected to great sensitivity. When there is a flow of heat the sample undergoes physical transformation such as phase transition. There will be a difference in the heat flow between the samples as the reaction may be exothermic and endothermic. DSC measures the absorption or release of heat during such transition. Standard P(HB) and PHA isolated from rice bran oil were used for DSC and data is shown in Fig. 2.23. The melt temperature of standard P(HB) was 176 ^{0}C. The melt temperature decreased to 169 ^{0}C in PHA isolated from *B. flexus*.

Molecular weight of the polymer as determined by viscosity measurements was 1.7 x 10 ^{5} daltons for PHA isolated from sucrose fed cells. Slight increase in molecular weight to 1.9 x 10 ^{5} daltons was observed in PHA isolated from sucrose and rice bran oil containing medium.

2.3.8.2. Fermentor cultivation *B. flexus*

The bacterium was cultivated in a fermentor using sucrose as main carbon source and also with rice bran oil as co carbon source. Results show that in cultivation medium containing rice bran oil as co carbon source there was an enhancement in biomass (4 g/l) and PHA (2.2 g/l) production compared to that of only sucrose medium (2.8 g/l and 1.2 g/l, correspondingly) (Fig. 2.24). Data indicated that rice bran oil supported growth as well as PHA production in *B. flexus*. This may be due to fatty acids as well as other carbon or nitrogenous components that were present in the unrefined oil. PHA film that was formed after solvent casting is shown in Fig. 2.25.

2.3.9. Pathway involved in PHA biosynthesis

Several pathways are involved in the production of PHA copolymers by various bacteria. To determine whether fatty acid path way was involved in the monomer unit synthesis of PHA in *B. flexus*, PHA production was observed in the presence of acrylic acid. Acrylic acid is a known inhibitor of certain enzymes in β-oxidation pathway (Qi *et al.,* 1998). In the present study acrylic acid was added at 0.1 g/l at 18 h of culturing period to palm oil effluent, rice bran oil and control media. Addition of acrylic acid had visible effect on the cell growth and the growth decreased by 36-56 % compared to controls. Similarly there was a decrease in the PHA concentration of biomass by 23-46% (Table 2.9).

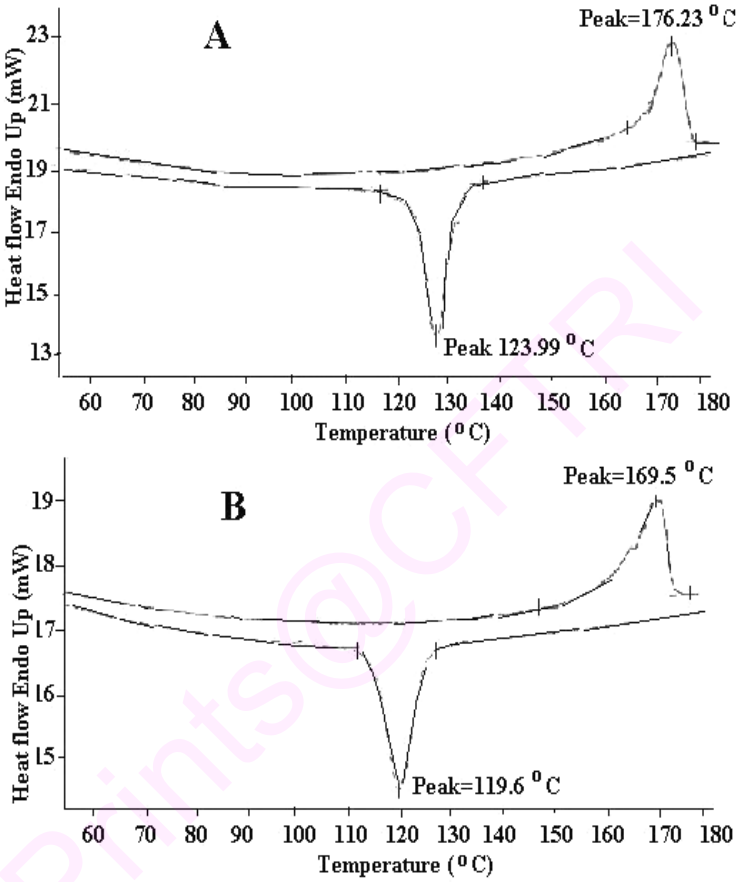


Figure 2.23: DSC of PHA: A) Standard PHB; B) PHA isolated from *B. flexus* cells cultivated on sucrose as main carbon source and rice bran oil as co carbon source.

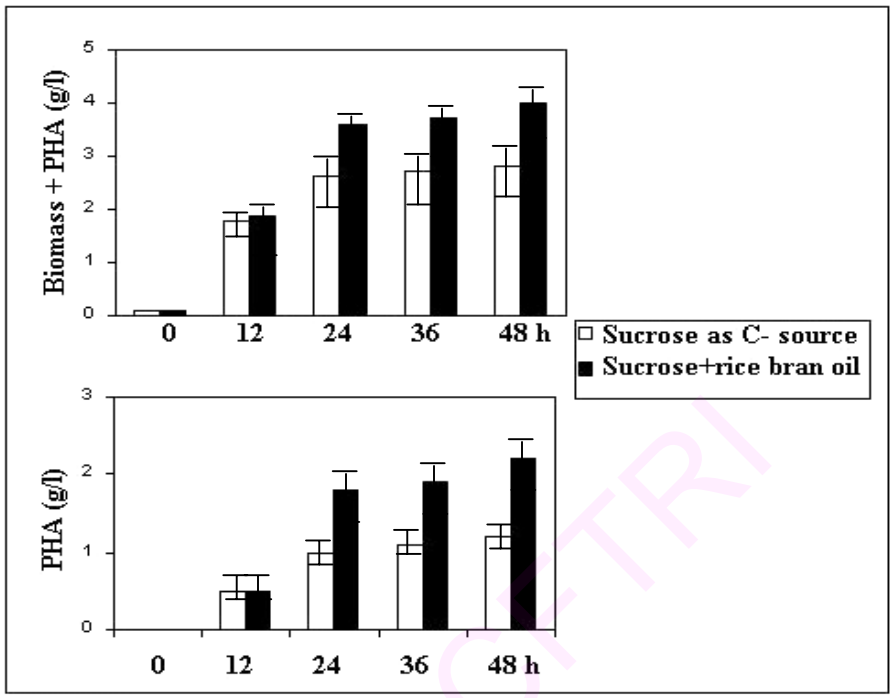


Figure 2.24: Fermentor cultivation of *B. flexus* using sucrose and sucrose + rice bran oil as carbon sources.

Figure 2.25: Polyhydroxyalkanoate produced by *B. flexus* formed into a film after chloroform casting method.

Table 2.9: Effect of acrylic acid on PHA accumulation by *B. flexus* in the presence of sucrose and rice bran oil containing fatty acids as co-carbon sources.

Substrate	Acrylic acid	Biomass (gl/l)	PHA (gl/l)	PHA (% biomass)	% Reduction* 1	2	3
Sucrose	-	1.4±0.21	0.56±0.10	40±0.62	-	-	-
Sucrose	+	0.9±0.14	0.28±0.20	31±2.12	36	50	23
R B oil	-	1.8±0.20	0.97±0.20	54±1.00	-	-	-
R B oil	+	0.8±0.20	0.25±0.20	31±2.00	56	75	43

R B oil=Rice bran oil; *Reduction in yields due to addition of acrylic acid to the culture:- 1=Yield of biomass; 2=Yield of PHA; 3=PHA content of biomass.

PHAs having 3, 4 and 5 hydroxyalkanoic acids have been produced by bacteria such as *Alcaligenes eutropha*, *Azotobacter vinelandii,* and PHAs with C4 to C12 carbon monomers are produced by *Pseudomonas* spp. (Brandl *et al.,* 1990). Copolymers of PHAs with P(HB) are better suited for various practical uses. *Bacillus* spp. are reported to produce C4 to C8 monomers (Brandl *et al.,* 1990, Caballero *et al.,* 1995, Tajima *et al.,* 2003), from a large variety of carbon sources and fatty acids (Valappil *et al.,* 2007; Shamala *et al.,* 2003; Anil Kumar *et al.,* 2007). PHA synthesis occurs due to structurally related as well as unrelated carbon sources (Valappil *et al.,* 2007). PHB-co-HHx has been synthesized in *Bacillus* spp. using ε-caprolactone, hexanoate, octanoate and decanoate (Labuzek and Radecka 2001; Tajima *et al.,* 2003). *Bacillus cereus* is found to produce 2-4 mol% of hydroxyheptonoate and hydroxyoctanoate from heptanoic and octanoic acids, respectively (Caballero *et al.,* 1995). *Bacillus thermolevorans* degraded n-alkanes and it is suggested that the strain was able to degrade these through terminal oxidation path way followed by a β-oxidation pathway. The resulting fatty acids are converted to acyl CoA derivatives that are metabolized through β-oxidation pathway (Kato *et al.,* 2001). In our study overall results showed that homopolymer of P(3HB) was synthesized in *B. flexus* cells fed with only sucrose as main carbon source (Figs. 2.19, 2.21 and 2.22). PHA copolymer of polyhdroxy(butyrate-co-valerate-co-octanoate) was produced in palm oil effluent containing medium (Fig. 2.16) and polyhdroxy(butyrate-co-octanoate-co-decanoate) with rice bran oil as co carbon substrate (Figs. 2.21 and 2.22). Amount of mcl

PHA synthesized although was less, this study has shown that based on the fatty acid supplemented in the medium, mcl-PHA such as octanoate and decanoate can be synthesized by *Bacillus* spp. also. PHA was characterized by FTIR, GC, GCMS and NMR methods. Presence of medium chain length PHA in copolymer was confirmed by GC-MS and Supplementation of acrylic acid, an inhibitor of β-oxidation pathway resulted in reduction in biomass as well as PHA yield.

Bacterial PHA are synthesized mainly through two pathways: a) It is known that P(HB) is synthesized from acetyl coenzyme A wherein β-ketothiolase catalyses condensation of two acetyl coenzyme A molecules to acetoacetyl–CoA that is subsequently reduced to hydroxybutryl CoA by acetoacetyl CoA reductase. P(HB) is then produced by the polymerization of hydroxybutryl CoA by the action of PHB synthase (Madison and Huisman, 1999; Tsuge, 2002). Further these authors have reported that the HV unit, which is found as one of the components of copolymer, is produced either from propionic acid or valeric acid through β-oxidation and deacetylation. In the absence of these typical HV precursors, HV most likely would be produced by some of the bacterial strains by the methylmalonyl-COA pathway where other carbon substrates other than propionic acid and valeric acid, can be utilized. b) PHA 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. c) 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 (Huijberts *et al.,* 1994). 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). *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 (Steinbuchel and Eversloh, 2003).

Synthesis of PHA in bacterial cell depends on the synthase activity and also precursors present in the cell. The precursor supply can be enhanced by supplementation of fatty acids, fatty acid synthesis and control of fatty acid degradation pathway (Reeta

2008; Reeta *et al.,* 2008). In *P. putida* it has been shown that both β-oxidation and fatty acid biosynthesis can function independently and simultaneously in generating precursors for PHA synthesis (Huijberts *et al.,* 1994). Acrylic acid inhibits enzymes involved in β-oxidation such as acyl CoA synthase and 3-ketoacyl-CoA thiolase (Huijberts *et al.,* 1994; Lu *et al.,* 2003). In recombinant *E. coli* having *phaC* and *phaJ*, PHA accumulation increased due to blocking of β-oxidation path way by acrylic acid which resulted in more enoyl CoA leading to channelisation of this towards PHA synthesis. While recombinant harbouring *phaC, phaJ* and *yafH* (acyl CoA dehydrogenase gene), PHB-co-HHx content decreased when acrylic acid was added to the culture suggesting that acrylic acid inhibited over expression of *yafH* (Lu *et al.,* 2003). In the present study decrease in PHA synthesis was also observed due to acrylic acid addition when the culture was growing on sucrose alone as carbon source (Table 2.9). Acrylic acid suppressed PHA production in medium containing oil or fatty acids as co carbon substrate along with sucrose as main carbon source. Free fatty acids such as dodecanoic acid, tridecanoic acid, hexadecanoic acids and octadecanoic acids were present both in acrylic acid treated and untreated cells cultivated in sucrose by supplementation of fatty acid as co carbon source. Presence of these was observed by GC-MS. This confirmed that fatty acid degradation rather than synthesis was inhibited by acrylic acid and P(HB) synthesis partly occurred through β-oxidation path way irrespective of carbon source supplied for growth. Cell dry weights were considerably higher without acrylic acid, which indicated the involvement of fatty acid pathway in bacterial growth. From this it can be concluded that precursors for P(HB) synthesis are derived: a) 50% through actyl CoA-acetoacetyl CoA-PHA synthesis steps b) 50% through β-oxidation pathway. It has been reported that hydroxyacyl coenzyme A thioesters required for quantitative and qualitative productivity of PHA are supplied by fatty acid biosynthesis and degradation pathway (Eggnik *et al.,* 1992). When fatty acids in the form of rice bran oil was fed to cells that were growing on sucrose, extra metabolic flux of fatty acid degradation appears to have channelised towards enhanced growth, P(HB) and some quantity of mcl-PHA production, compared to sucrose fed cells (Fig. 2.21). Rice bran oil that was provided as co carbon substrate for example contained: Oleic (43%) C18:1; linoleic (35%) C18:2 and palmetic acid (15%) C16. The substrate contained up to C18:2, however carbons in the monomers found even though in small

quantities were C8 and C10. Using *Pseudomonas* species it has been reported that the monomer present in the polymer is always lower than that present in the substrate (Bassas *et al.,* 2008). Presence of unsaturated monomer peak that could not be identified (Fig. 2.22) at 129 ppm may not be due to longer side chain monomer but may be derived from lipid groups. Such unidentified peaks have been reported to occur in bacterial PHA (Fukui *et al.,* 1998). Supplementation of butyrate could result in formation of P(HB) only whereas in the presence of valeric, octanoic and decanoic acids the bacterium synthesized valerate. McCool and Cannon 2001 have shown that phaB is essential for PHA biosynthesis in *B. megaterium* and β–oxidation pathway is involved in PHA synthesis. In the present study, BLAST search using the sequence of *B. flexus* revealed that it clustered with other strains such as *Bacillus megaterium* and *B.simplex*. The results confirm that the same pathway for monomer supply as that of *B. megaterium* exists in *B. flexus*. Based on the above data, proposed metabolic pathway for PHA biosynthesis in *B. flexus* is shown in Fig. 2.26. The figure indicates the involvement of fatty acid metabolism in P(HB), P(HV) and mcl-PHA synthesis in *B. flexus*. Synthesis of P(HB) from sugar is also indicated.

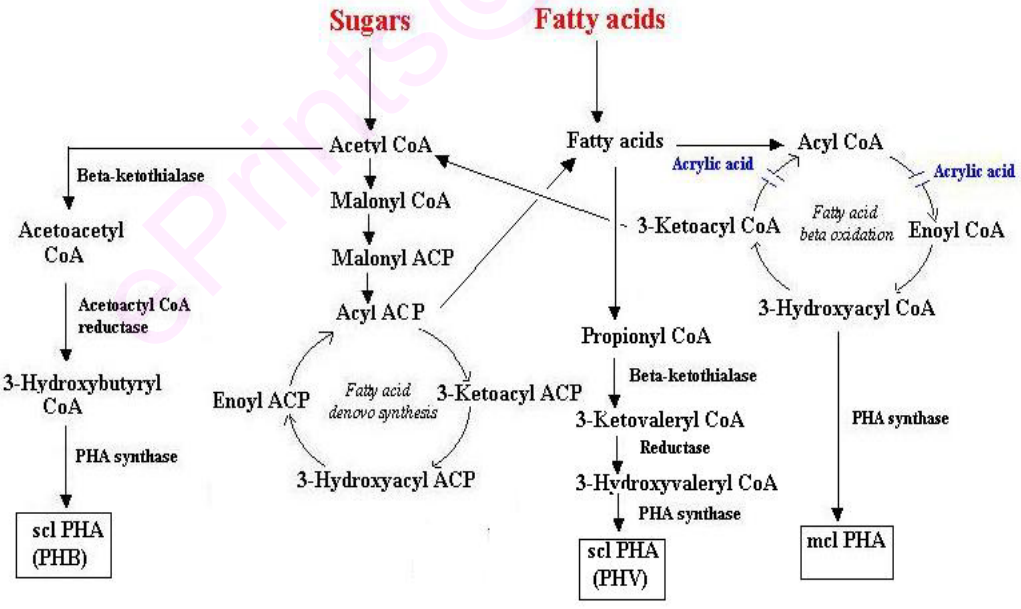


Figure 2. 26: Proposed metabolic pathway for PHA synthesis in *B. flexus*.
(Pathway proposed for other PHAs by Tsuge, 2002 is considered)

Conclusion

Results have indicated that *B. flexus* can utilize various nitrogen sources, carbon sources, economic substrates, fatty acids and plant oils for P(HB) or PHA copolymer synthesis. By using acrylic acid, it was confirmed that the chemical inhibited fatty acid degradation pathway and P(HB) synthesis partly occurred through β-oxidation path way irrespective of carbon source supplied for growth. PHA copolymer of polyhdroxy(butyrate-co-octanoate-co-decanoate) was synthesized from rice bran oil as a co substrate and the polymer was characterized by GC, FTIR, GC-MS and NMR techniques. The data indicates the possibility of utilization of *B. flexus* for PHA copolymer production.

Chapter 3

Isolation of PHA from bacterial cells

Introduction

The synthetic non-biodegradable polymers that are produced from petrochemical sources are accumulating in the environment at an alarming rate and are causing pollution problems. Even though PHAs are ecofriendly they are relatively costlier compared to synthetic plastics. They are accumulated intracellularly and hence their extraction from the biomass is a critical step for economic production. The cell hydrolysis by chemicals such as sodium hypochlorite leads to reduction in molecular weight of the polymer there by affecting the polymer quality and simultaneously generates chlorine-containing effluent (Berger *et al*., 1989; Hahn *et al*., 1993; Jacquel *et al*., 2008). In addition to this, the proportion of non- polymeric cell material to be separated is more, which renders filtration or centrifugation steps of such material difficult. As an alternate to this, enzymatic digestion method has been evolved for the extraction (Holmes, 1990; Kshama and Shamala, 2006; Kshama, 2005) The biomass is solubilized in stages using various enzymes such as pepsin, trypsin, bromelin, pappain etc, individually or in combinations.

Forth-coming sections deal with cell wall nature of *B. flexus* used in the experiment, effect of nutrient on cell wall composition and different methods of isolation of PHA from *B. flexus* cells. These methods include solvent extraction, alkali digestion enzymatic extraction and aqueous two phase system. Effect of gamma irradiation on cell lysis and modification of polymer are also worked out.

Part A: Chemical method of PHA isolation

A. 3.1. Introduction

PHA being intracellular, isolation and purification are major steps in the downstream process of the polymer. Numerous separation processes are employed for the recovery of PHA. These involve extraction by organic solvents such as chloroform, methelene chloride, dichloroethane, dichloromethane (Ramsay *et al.,* 1994). The use of solvents to recover PHA is generally hazardous and explosive. Baptist (1962) used a mixture of solvents such as chloroform/methanol to precipitate PHA. The solvent extraction is more useful in lab-scale experiment rather than pilot plant.

A halogenated chemical sodium hypochlorite is used to digest the non-PHA cellular material, which is known to degrade the polymer. Optimizing the conditions of sodium hypochlorite can reduce the degradation. Dispersions of chloroform and hypochlorite were used to overcome the problem (Hahn *et al.,* 1994). By this method three separate phases were obtained which included an upper phase of hypochlorite solution, middle phase in which non-PHA cell material and disrupted cells accumulated and the chloroform phase containing PHA. Then chloroform layer is separated and polymer is precipitated. By this method degradation of the polymer can be reduced.

Use of surfactant for cell hydrolysis is reported by several investigators (Chen *et al.,* 1999). The surfactant alone cannot give high recovery yield and also causes problems in wastewater treatment. Combination of surfactant and hypochlorite has been suggested for extraction (Dong and Sun, 2000).

Mechanical disruptions such as sonication, homogenizer was used to isolate the PHA (Yoshida *et al.,* 1995). High-pressure homogenizer has been used to disrupt the microbial cells but several drawbacks are associated as the micro ionization cell debris hinders the separation of solid-liquid phase (Chen *et al.,* 1999).

The purpose of present work is to improve the cell disruption to get maximum recovery of the product with higher purity. Nature of cell wall of the bacterium and effect of nutrients on cell wall composition were studied. Effect of chloroform, sodium hypochlorite and alkali were compared for cell wall digestion and extraction of the polymer.

A. 3.2. Materials and methods

A. 3.2.1. Microorganism and inoculum

B. flexus inoculum was prepared in 10 ml portions of sterile nutrient broth (Himedia, Mumbai, India) that was contained in test tubes. The test tubes were inoculated with 2-3 loop full of fresh culture from nutrient agar slants. The inoculated tubes were incubated at 30 ^{0}C and 250 rpm for 24 h. The inoculum contained 2 x 10^4 cfu/ml. *B. flexus* (MTCC 2909) was used for comparison.

A. 3.2.2. PHA production in shake flasks

PHA production was carried out in inorganic nutrient medium (IM) in 500 ml capacity Erlenmeyer flasks in triplicate. IM medium contained (g/l): Na_2HPO_4 $2H_2O$, 2.2; KH_2PO_4 , 1.5; $(NH_4)_2SO_4$, 1.5; $MgSO_4$ $7H_2O$, 0.2; sucrose, 20; pH 7.0. Culture conditions are explained under Materials and Methods (common for chapters), section M.5.

To find out the effect of organic and inorganic nutrients on growth, PHA production and cell wall composition, various combinations of the media were used: a) Control medium mentioned above, b) Complex organic medium (g/l): peptone, 5; yeast extract, 2.5; beef extract, 2.5; $(NH_4)_2SO_4$, 1.5; sucrose, 20; pH 7.0, c) In the IM medium $(NH_4)_2SO_4$ was replaced by yeast extract (2.5 g/l) or peptone (5 g/l) or beef extract (2.5 g/l). Inoculated flasks were incubated at 250 rpm and 30 ^{0} C for 72 h. Experiments were carried out in triplicate.

A. 3.2.3. Isolation of cell wall

Cell wall was isolated according to the method reported by Schnaitman (1981). Biomass was subjected to ultrasonic disruption in a commercial instrument, under cool conditions. The disrupted cells were centrifuged at 6000 rpm for 5 min to pellet the intact cells. The supernatant, which contained peptidoglycan along with other cell components was further centrifuged at 8000 rpm for 10 min. Peptidoglycan was purified by successive treatments with proteolytic enzymes, lipases, DNase and RNase. The charged particle that may adhere to the peptidoglycan was removed by washing with 1M NaCl

and water and the purified pelleted cell wall obtained after centrifugation at 8000 rpm for 20 min was lyophilized.

A. 3.2.4. Estimation of glucosamine

Reagents:

Solution A: Acetyl acetone (1.5 ml) + 50 ml of 0.7M Sodium Carbonate solution

Solution B: p – Di methyl amino benzaldehyde (1.6 g) + 30 ml of conc. HCl + 30 ml of ethanol.

Purified cell wall (10 mg) was hydrolyzed in a sealed tube with 6N-distilled HCl (10 ml), at 110 ^{0}C for 24 h (Gouda *et al.,* 2006). Glucosamine was estimated in the hydrolysate (Good and Bessman, 1964). For this, known amount of sample was taken and made up to 0.5 ml by distilled water, 0.5 ml of solution A was added and boiled in water bath at 80^0 C for 1 h. 5 ml of ethanol and 0.5 ml of solution B were added and allowed to stand for 1 h and O.D. was measured at 535nm. Calibration curve was prepared by using 10-100 µ g of N-acetyl glucosamine.

A.3.2.5. Estimation of amino acids

Amino acids were analyzed in the cell wall hydrolysate in duplicates after precolumn derivatization by using phenylisothiocyanate (Bidlingmeyer *et al.,* 1987). The phenyl thiocarbamoyl amino acids were analyzed by Pico-Tag amino acid analysis system (Bennett and Solomon, 1986).

A. 3.2.6. Estimation of total sugars

Total sugar of the cell wall was analyzed by phenol sulphuric acid method [Materials and methods (common for chapters), section M.6.11].

A. 3.2.7. Plate assay for antibiotic sensitivity

Plates containing nutrient agar (Himedia, Mumbai, India), IM agar (IM with 1.8% agar) and complex organic medium with 1.8% agar (composition described above) were lawned with 0.2 ml of culture broth that contained 2 x 10^4 cfu/ml of *B. flexus.* Antibiotic octodiscs (Himedia, Mumbai, India) containing tetracyclin (10 mcg), sulphamethaxazole

(25 mcg), trimethoprim (125 mcg), erythromycin (5 mcg), fusidic acid (10 mcg), gentamycin (10 mcg) and clindamycin (2 mcg) was placed on the agar surface and the plates were incubated at 37 ^{o}C up to 48 h. Clearance zone surrounding the individual antibiotic was measured in mm. Experiment was carried out in triplicate.

A. 3.2.8. Analysis

Estimation of biomass, GC analysis of PHA, NMR, assessment of polymer purity by crotonate assay and GC analysis were carried according to the methods that are described under Materials and Methods (common for chapters), section M.6.

A. 3.2.9. Cell hydrolysis and isolation of PHA

Cells obtained from IM medium were used for experimentation. Various methods used for comparison of hydrolysis of cell and estimation of PHA are as follows:

a) Hypochlorite hydrolysis: PHA content of bacterial cells was determined gravimetrically after hydrolyzing the dried biomass with sodium hypochlorite solution and solubilising the released polymer by chloroform (Williamson and Wilkinson, 1958; Law and Slepecky, 1961).

b) Chloroform extraction: Dried biomass was refluxed with chloroform at 40 ^{0}C, for 2h. The chloroform layer was separated by filtration and extracted polymer was recovered by using hexane in 1:2 proportions and the precipitated polymer was dried at 45 ^{0}C to a constant weight.

c) Surfactant and chelating agents: Cells obtained by centrifugation were suspended in 1 ml of water, 0.6% of triton X 100 and 0.06% of EDTA were added to the suspension and pH was adjusted to 13 with 1 N NaOH solution and was incubated at 50 ^{o}C for 10 min. The hydrolysate was centrifuged and washed with acetone, diethyl ether and absolute alcohol, dissolved in chloroform and dried to a constant weight (Chen *et al.,* 1999).

d) Alkali hydrolysis: Dried biomass (100 mg) was suspended in water (5 ml) and pH was set to 8-11 by ammonia solution (25%, v/v)/NaOH/KOH. The suspensions were incubated at 50 ^{0}C for 10 min. The hydrolysates were centrifuged (6000 rpm 10 min), washed with acetone, dissolved in chloroform and insoluble material was removed by filtration. Chloroform layer containing PHA was evaporated to dryness and weighed.

A. 3.3. Results and discussion

A 3.3.1 Effect of nutrients on growth

Complex organic medium containing peptone, yeast extract and beef extract stimulated growth (3 g/l), and to find out their effect on growth and PHA, they were substituted individually to inorganic nutrient medium (Table 3.1). Enhanced growth was obtained in the presence of peptone (4.1g biomass/l) and yeast extract (3.9 g biomass/l) and poor growth was observed in beef extract medium (1.6 g biomass/l) compared to control inorganic medium (2 g/l). However maximum concentration of PHA in the cells was synthesized in the inorganic nutrient medium (50%). Cell shape and size were also affected by the nutrients present in the growth medium (Fig. 3.1). The results indicated that the cells harvested from inorganic medium and from beef extract medium which had poor growth lysed easily and the recovery of PHA was more in chloroform extraction or mild alkali digestion (86-100%) compared to those grown in organic nutrient medium containing peptone and yeast extract (32-56%). Shape of cells differed under different nutrients provided in the media. It is reported that under limitation in the supply of phosphate, mutant cells of *B. licheniformis* formed irregular spheres, which changed back to rods when phosphate was supplied (Forsberg *et al.*, 1973). This is assigned to the enzyme deficiency, which is linked to cell wall synthesis.

A.3.3.2. Effect of nutrients on cell wall composition of *B. flexus*

Studies were carried out to find out the effect of organic and inorganic nutrients on cell wall compositions of *B. flexus*. Total sugar concentration (mg /100 mg cell wall) of cell wall isolated from cells grown in inorganic nutrient medium and complex organic medium was 8 mg and 6 mg, respectively. Concentration of glucosamine was not affected by organic or inorganic nutrients (8.8 mg/100 mg of cell wall). The cell walls contained glutamic acid, alanine, arginine, valine, leucine, tyrosine, aspartic acid and isoleucine (Figs. 3.2 and 3.3. Table 3.2). Under peptone and yeast extract supplementation higher concentration of amino acids were observed in the cell walls compared to those cultivated in inorganic salts medium.

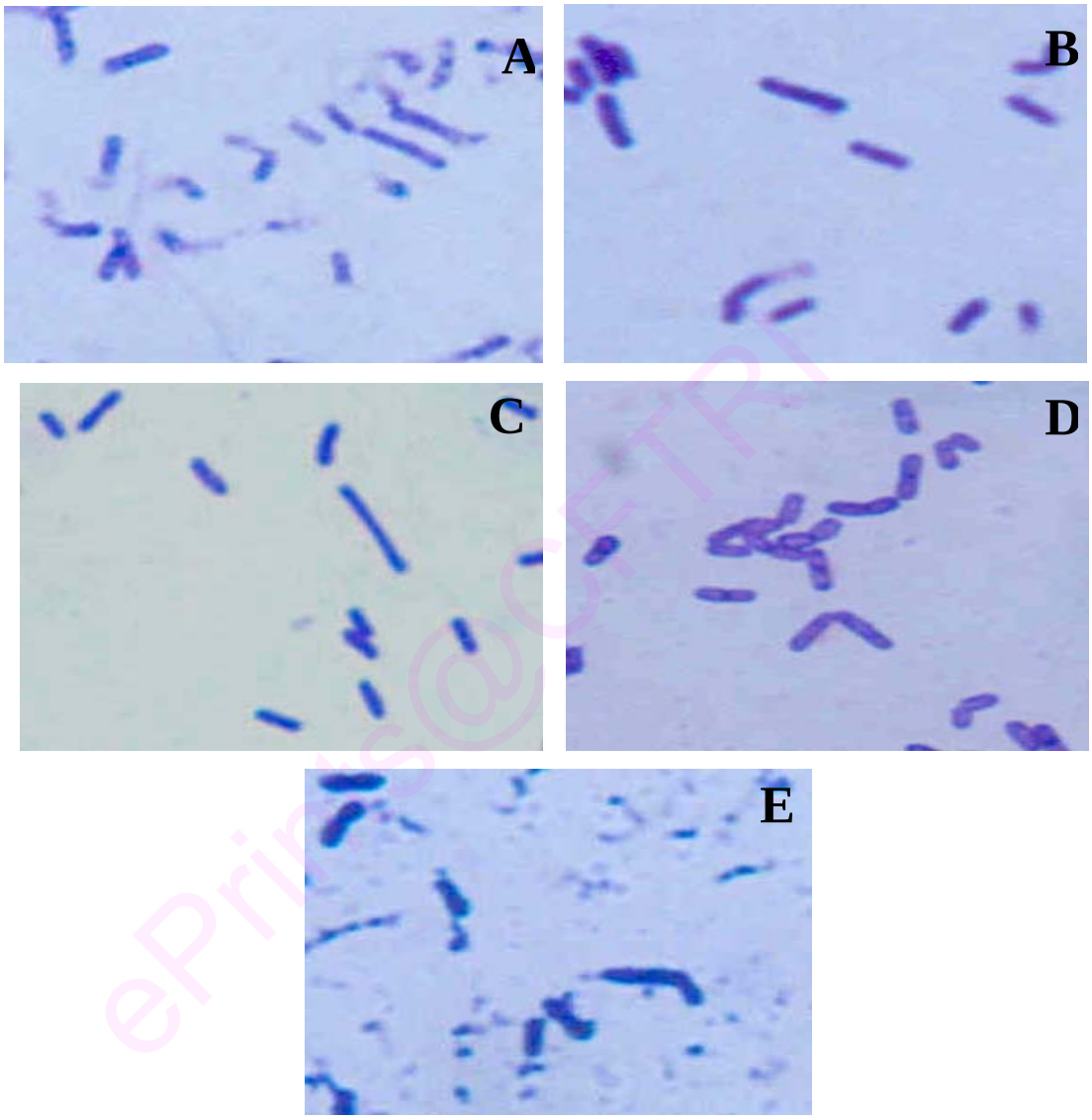


Figure 3.1: Stained cells of *B. flexus* cultivated in different nutrients and observed under microscope (100x). A–IM with ammonium sulphate, B – Beef extract, C – Complex medium, D – Peptone, E – Yeast extract (Details of media as under A.3.2.2).

Table 3.1: Production of PHA by isolated culture of *B. flexus* in various media and its extraction.

Medium	Cell size (µ m) and morphology	Biomass (g/l, w/v)	PHA (g/l)	PHA extracted (% of biomass)		
				*1	*2	*3
IM**	1 x 6±0.70 Pleomorphic	2.0±0.84	1.0±0.42	50	43	50
MTCC 2909 in IM	1 x 6±0.70 Pleomorphic	3.2±0.91	0.1±0.02	3.7	-	-
IM[a]	1 x 2±1.41 Small rods	3.9±0.91	1.6±0.35	40	13	13
IM[b]	1 x 3±0.70 Small rods	1.6±0.35	0.7±0.21	44	39	44
IM[c]	1 x 4± 0.70 Pleomorphic	4.1±0.28	1.6±0.28	30	17	12
Complex organic medium	1x 5± 0.70 Pleomorphic	3.0±0.84	1.4±0.42	48	38	23

**Inorganic nutrient medium with $(NH_4)_2SO_4$; IM[a] IM[b] and IM[c] media are similar to inorganic nutrient medium, without $(NH_4)_2SO_4$ but with supplementation of yeast extract (2.5 g/l); Beef Extract (2.5 g/l) and Peptone (5.0 g/l), respectively. *1=Hypochlorite hydrolysis; *2=Chloroform extraction; *3=Alkali (NH_4OH) hydrolysis.

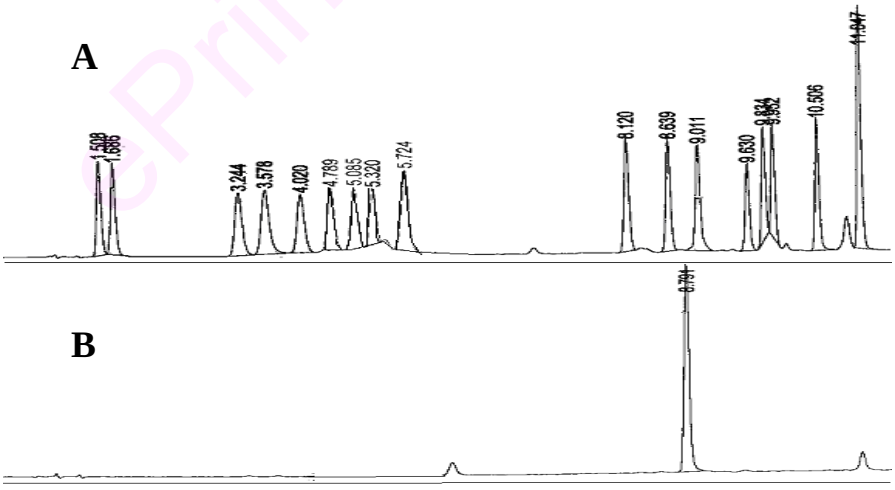


Figure 3.2: Profile of amino acids (A- Standard) and Diaminopimilic acid (B-Standard).

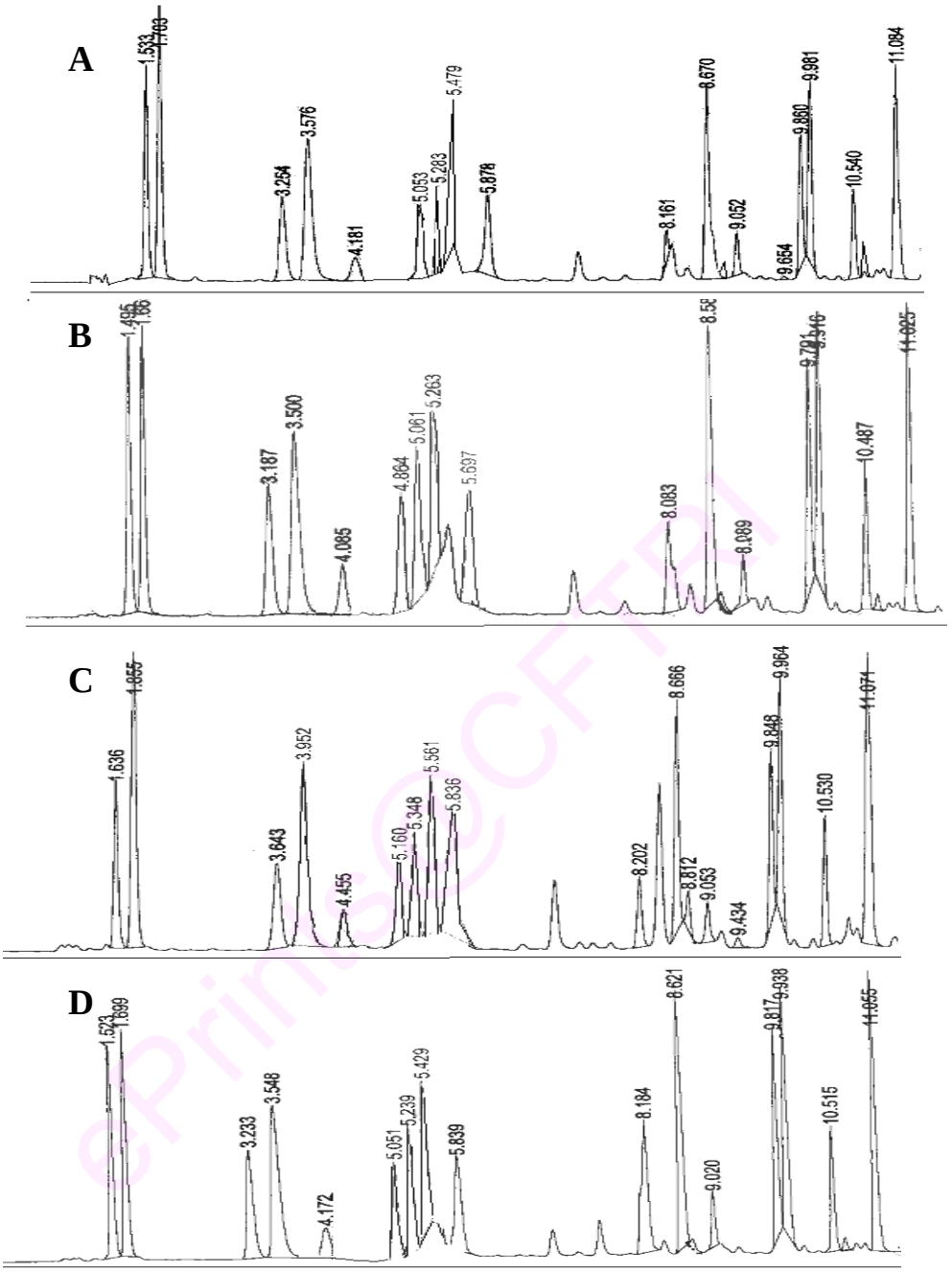


Figure 3.3: Amino acid profile of the cell wall of *B. flexus* grown in different media.
A – Nutrient broth, B – Yeast extract, C – Peptone, D – Inorganic medium (IM).
Details of media composition are given under A.3.2.2.

Table 3.2: Amino acid analysis of the cell walls isolated from *B. flexus* cultivated in inorganic and organic nutrient media.

Amino acids	Amino acid in isolated cell wall (mg/100 mg)				
	1	**2**	**3**	**4**	**5**
Aspartic acid	0.26	0.95	1.08	0.10	0.52
Glutamic acid	0.62	1.12	1.12	0.18	0.81
Arginine	0.27	0.89	1.05	0.09	0.42
Alanine	0.30	0.75	0.73	0.09	0.42
Tyrosine	0.13	1.00	0.6	0.03	0.07
Valine	0.26	0.95	0.98	0.10	0.48
Isoleusine	0.23	0.81	0.81	0.08	0.30
Leusine	0.33	1.06	1.1	0.13	0.44
Diaminopimilic acid	-	Present	Present	-	-

1=Inorganic nutreint medium (IM); 2= IM with Yeast Extract, 3=IM with peptone, 4= IM with Beef extract, 5=Complex organic medium; Composition of media is given under A.3.2.2.

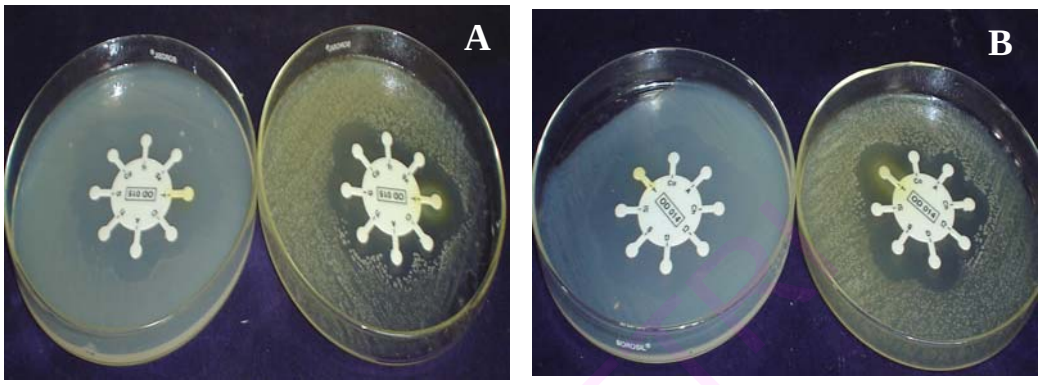


Figure 3.4: Difference in antibiotic sensitivity/resistance of *B. flexus* in different media. A & B – Cells grown in different media showing variation in the sensitivity/resistance for different antibiotics.

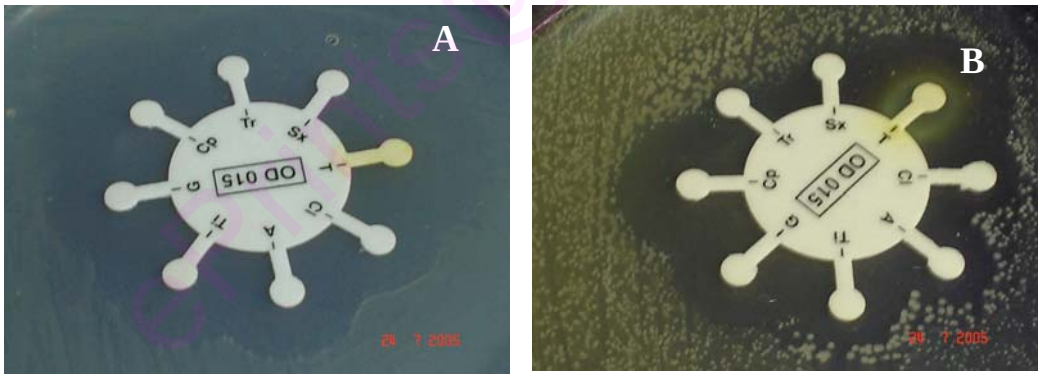


Figure 3.5: Magnified photograph of the culture plates showing variation in their sensitivity/resistance to the antibiotic on different media. (A=IM medium; B=Organic medium; same antibiotics were used in both the media).
T=Tetracycline; Cl= Colistin; A = Ampicillin; Ti = Ticarcillin; G = Gentamycin; Cp = Cephalexin; Tr = Trimethoprim; Sx = Sulphamethaxazole.

Table 3.3: Antibiotic sensitivity (clearance zone) of *B. flexus* cultivated in various media* for 48 h at 30 ^{0}C.

Antibiotics used (As octodisc)	Clearance zone (in mm) on agar plates		
	Nutrient agar	Inorganic medium	Organic medium
Tetracyclin (10 mcg)	40±1.41	40±1.41	30±0.70
Sulphamethaxazole (25 mcg)	22±2.82	40±1.41	20±0.70
Trimethoprim (125 mcg)	22±2.12	22±1.41	28±0.70
Erythromycin (5 mcg)	16±0.70	16±0.70	16±0.70
Fusidic acid (10 mcg)	12±1.41	24±0.70	12±1.41
Gentamycin (10 mcg)	12±1.41	22±0.70	12±0.70
Clindamycin (2 mcg)	32±0.00	16±0.70	32±0.70
Penicillin G (1 unit)	24±1.41	24±0.70	24±1.41

*Details given under materials and methods in the text

Cells grown in the presence of peptone and yeast extract contained detectable amounts of diaminopimilic acid in their cell walls, which was totally absent in those grown in the inorganic salts medium. The general features and composition of cell wall of *Bacillus* spp. have been worked out (Hughes *et al.,* 1970). The mucopeptide contains diaminopimilic acid, glutamic acid, alanine, N-acetylglucosamine, N acetylmuramic acid and amide groups. The cell wall also contains N acetylgalactosamine, phosphorous, glycerol and glucose. The cell wall peptide has four amino acids and it is usually made up of alanine and glutamic acid. Different peptidoglycan structures are formed due to substitutions of diamino acids at the third position of the peptide. The most common

diamino acids are lysine and diaminopimilic acid (DAP). DAP containing peptide participates in cross bridge formation between DAP and alanine or between two DAP residues. Such cross bridge formations provide structural stability to the cell. It is difficult to disrupt the cell walls of gram-positive bacteria such as *Bacillus* spp. due to the presence of thicker peptidoglycan layer, which is cross-linked. The formation of peptidoglycan is dependent on the uptake of nitrogen and its metabolism to provide the required peptides for peptidoglycan synthesis. Variations are known to occur in the chemical composition of the cell wall of *B. subtilis* during growth in different media (Young, 1965). Two or three fold increase in cell wall thickness has been found after incubation of *B. subtilis* cells in a glucose-amino acids-salts medium (Hughes *et al.,* 1970). Earlier it has been reported that the growth phase and the composition of the growth medium lead to the variation in the chemical composition of the cell wall. The composition and fine structure of the vegetative cell wall peptidoglycan from *Bacillus subtilis* were determined by analysis of its constituent muropeptides. The structures of 39 muropeptides, representing 97% of the total peptidoglycan, were elucidated. About 99% analyzed muropeptides in *B. subtilis* vegetative cell peptidoglycan have the free carboxylic group of diaminopimelic acid amidated. This amount is, however, dependent on the composition of the growth media. *B. subtilis* peptidoglycan is incompletely digested by lysozyme due to de-acetylation of glucosamine. The cross-linking index of the polymer changes with the growth phase. It is highest in late stationary phase (Atrih *et al.,* 1999).

In our study, the presence of DAP in the cell walls of cells grown in peptone and yeast extract containing medium confirmed that the cell wall is more stable compared to those grown in inorganic medium. Complex organic medium which contained ammonium sulphate as one of the nitrogen sources also lacked DAP, had lower concentrations of amino acids in the cell wall, allowed easier extractability of the polymer, compared to peptone/yeast extract grown cells, which may be due to changes in the metabolic activities in the presence of inorganic nitrogen source.

Cells grown in inorganic salts medium plate, when compared with those grown on complex organic medium-showed susceptibility or resistant to certain antibiotics (Fig. 3.4. and 3.5, Table 3.3). Antibiotic sensitivity or resistance involves various mechanisms

(Mims *et al.,* 1998). Changes in the sensitivity observed in the present study may be due to the drug inactivation through enzymes, altered uptake due to changes in the outer membrane and the channelization through which the molecules move into the cell and altered target site or antibiotic binding proteins, which are based on the metabolic activity and nutritional status of the cell.

The results demonstrate that the composition of cell wall of *B. flexus* is significantly influenced by the composition of the growth medium. Cells grown in inorganic medium lysed easily and this can be further exploited for easier recovery of the intracellular PHA.

A.3.3.3. Isolation of PHA

Various methods were used for the isolation of PHA. The cells were harvested from inorganic nutrient medium for the purpose.

A.3.3.3.1 Digestion by sodium hypochlorite

Sodium hypochlorite is commonly used for hydrolysis of cells containing PHA. However it causes severe degradation of PHA resulting in the reduction of molecular weight (Berger *et al.,* 1989). PHA isolated from the cells was 54% of biomass weight and showed 88% purity, and had a molecular weight of 7.4×10^5 (Table 3.4). It is known that the decrease in molecular weight is due to saponification of PHA granules, which decomposes into soluble products such as monomers (Hahn *et al.,* 1994).

A.3.3.3.2. Solvent extraction

Use of solvent for the extraction of PHA is one of the traditional methods. The solvent acts on the permeability of cell membrane and then it solubilises PHA. Lemoigne first described the use of solvent for PHA isolation by using *Bacillus megaterium* cells. Later Vanlautem and Gilain reported that the best results with PHA extraction could be obtained with the solvents carrying at least one chlorine atom and one hydrogen atom at functional carbon atom (Vanlautem and Gilain, 1982). In the present study 43% PHA (of biomass weight) was extracted by using chloroform. Purity of the sample was 98% after hexane precipitation (Table 3.4). The molecular weight of the PHA obtained from solvent

extraction was high compared to other methods of extraction. This is because solvent extraction is a nondestructive method for polymer extraction and solubilization.

A. 3.3.3.3. Isolation by dispersions of sodium hypochlorite and chloroform

Cell hydrolysis by using a mixture of sodium hypochlorite and chloroform resulted in separate phases of hypochlorite (top) and chloroform (bottom) wherein the PHA granules released from the cells dissolved in the chloroform phase and non – PHA cellular material settled in the interphase. Chloroform phase was removed and PHA was precipitated. By using optimum condition of 37 ^{0}C, 1:1 volume ratio of sodium hypochlorite and chloroform and 1 h incubation treatment, 57% of the PHA (of biomass weight) with 97% purity was recovered (Table 3.4). Quantitative and qualitative yields were higher compared to only hypochlorite digestion (A.3.3.3.1).

A.3.3.3.4. Use of surfactant and chelating agent for the extraction of PHA.

The monomers of surfactants are known to get inserted in a lipid bilayer leading to the rupture of cell membrane (King *et al.,* 1991). The addition of a chelating agent to the surfactant enhances the rate of release of PHA. The addition of chelating agent destabilizes the cell membrane by forming complexes with divalent cations. In this study the cells were treated with 1mM of EDTA and 0.5M of triton X 100 at pH12 to lyse the cells. PHA extracted was 37% (of biomass weight), with 97% purity (Table 3.4).

A.3.3.3.5. Alkali extraction of PHA

PHA recovery by different alkalis i.e., NaOH, KOH and NH_4OH at various pH were carried out to find the optimal pH and alkali for the recovery of PHA. Amongst different alkalis tested for cell hydrolysis, NH_4OH efficiently digested non-PHA cellular material at pH 11, to give 50% of PHA (of biomass weight) with 98% purity. Recovery was less with NaOH and KOH (30% and 20%, respectively), compared to NH_4OH (50%) (Fig. 3.5).

Table 3.4: Comparison of PHA yield and purity obtained by different extraction methods from *B. flexus* cells.

	Hypochlorite	Chloroform	Chloroform+ Hypochlorite	Surfactant + chelating agent	Alkali (NH_4OH)
PHA%	54	43	57	37	50
Purity %	88	98	97	97	98
Mol wt	7.39×10^5	1.7×10^6	ND	ND	1.3×10^6

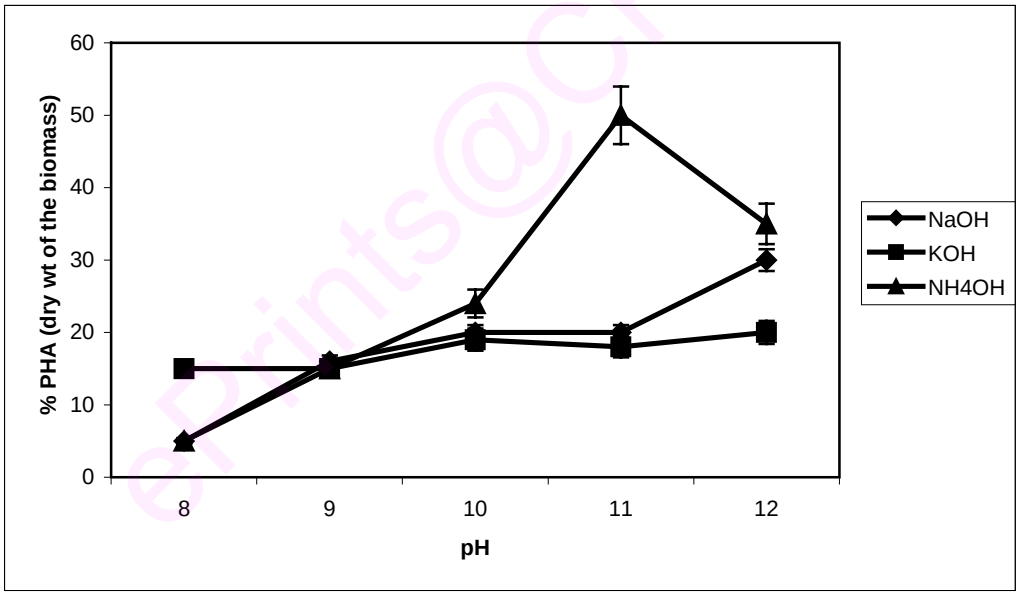


Figure 3.6: Extraction of PHA from *B. flexus* using various alkalies at different pH.

A.3.3.3.6. Characterization of polymer

GC and NMR analysis of the polymer obtained by different extraction methods revealed that there was no variation in the chemical composition of the polymer.

Conclusions

Bacillus spp. cell wall is resistant and not easily hydrolysable. However cell wall thickness is dependant on nutrition status of the cell. Organic nutrient medium results in cells having thick walls and cells cultivated in inorganic nutrients have relatively thin cell walls that can be lysed easily. Several methods have been reported for the recovery of PHA. Efficient and economical recovery might be possible by digestion with inexpensive chemicals and enzyme. Therefore in this study digestion of cells using various methods were investigated. Even though extraction was efficient with hypochlorite-chloroform mixtures, for large-scale operations more solvents are required which are expensive and hazardous to handle. Extraction by surfactants is also expensive compared to alkali digestion. From this point of view ammonium hydroxide method appeared to be economical and advantageous compared to other extraction methods. This is due to low cost of chemical involved; low amount used and short recovery process.

Part B: Enzymatic hydrolysis of *B. flexus* cells

B.3.1. Introduction

PHA granules are intracellular and several methods have been developed to recover them. Enzymatic digestion method was developed (Holmes and Lim, 1990) as an alternative to solvent extraction. Proteolytic enzyme can actively hydrolyse cell wall proteins (Greenberg, 1995). A combination of methods involving treatment with heat, alcalase enzyme and SDS assisted by EDTA has been used for the recovery of PHA from *Pseudomonas* sp. (DeKonig and Witholt 1997). Harrison *et al.*, (1991) reported complete lysis of *Ralstonia eutropha* cells upon treatment with lytic enzymes obtained from *Cytophaga* sp. at 37.5 ^{o}C, pH 7.3 for 60 min with out any mechanical processing. Purified PHA has been obtained by using 2% bromelain at 50 ^{o}C and pH 9.0 (Kapritchkoff *et al.,* 2006). The cell lytic culture of *Microbispora* sp. could be directly grown for a shorter period without any additional nutrient supply in the fermented broth containing *Sinorhizobium meliloti* cells having PHA and the lytic biomass obtained after growth was easily separated by filtration and optimum yield of PHA was recovered from the hydrolyzed broth (Kshama and Shamala, 2006). The enzyme obtained by the cultivation of *Microbispora* sp. could also be used for hydrolysis of *S. meliloti* cells to recover PHA (Kshama and Shamala, 2006). Present study deals with application of cell lytic enzyme for the hydrolysis of gram +ve cells of *B. flexus*, which are more resistant to hydrolysis, and characterization of the lytic enzyme.

B.3.2. Materials and methods

B.3.2.1. Growth of *B. flexus*

B. flexus cells were grown in PHA production medium for 72 h at 250 rpm and 30 ^{0}C [Materials and methods (common for chapters), section M.5, production medium 1 of Table 3]. Harvested cells were used for isolation of PHA by enzymatic method.

B.3.2.2. Growth of the *Microbispora* sp.

Microbispora sp. was isolated from local soil sample and maintained on nutrient agar slants at 4 ^{0}C. Inoculum of ***Microbispora*** sp. was prepared by transferring cells from 24 h old slant to the nutrient broth containing 200mg% of *B. flexus* cells. *B. flexus* cells were used as substrate for lytic enzyme production. The flask was incubated at 30^{0} C and

250 rpm for 48 h. After the growth, ***Microbispora*** sp. cells could be easily filtered. Filtrate rich in lytic enzyme was used for cell hydrolysis.

B.3.2.3. Extraction of PHA from *B. flexus* by using commercial enzyme and culture filtrate of *Microbispora* sp.

Enzymes used for cell hydrolysis were: Lysozyme (Sigma, USA), lytic activity 55000 u/mg protein; alcalase 2L (Novozyme, Denmark), protease activity 22000 U/ml and *Microbispora* sp. enzyme, protease activity of 30 U/ml and lytic activity of 84 U/ml. *B. flexus* biomass (100 mg) was hydrolysed with 30 units of protease/ 80 units of lytic acitivity/ml of alcalase, lysozyme and *Microbispora* culture filtrate at 37 ^{0}C for 1 h and the hydrolyzed sample were suspended in chloroform and mixed well. The chloroform phase was separated from the cell debris. PHA was recovered by precipitation by adding 2 volumes of hexane. The precipitated PHA was collected and dried.

B.3.2.4. Inhibition of lytic activity by bovine serum albumin (BSA)

From above experimentation it was observed that the lytic activity of *Microbispora* sp. culture filtrate on *B. flexus* cells could be due to proteases. In order to confirm this, the reaction mixture containing 100mg of *B. flexus* cells in 5ml buffer (phosphate buffer, pH 7) was supplemented with BSA (5, 25 50 mg%, w/v) and hydrolysis was carried out at 37 ^{0}C for 1h. Sample without BSA served as control. O.D. was measured at 620 nm.

B.3.2.5. Growth of *Microbispora* sp. in various media

Different combinations of media and medium components were tested for growth and enzyme production by *Microbispora* sp. Nutrient broth (Himedia, Mumbai, India) was supplemented with *B. flexus* cells (200 mg%)/2% (v/v) soya milk/ 2% (v/v) of soya whey/2% (v/v) of milk whey. Yeast extract (2 g/l) was supplemented with sucrose (10 g/l)/*B. flexus* cells (200 mg%). Beef extract/Peptone (2 g/l) were used along with sucrose (10 g/l) as carbon source. Complex medium containing organic substrates (A.3.2.2.) was also used for experimentation. Media (50 ml) were sterilized in 250 ml capacity Erlenmeyer flasks and inoculated with *Microbiapora* sp. culture and incubated at 250

rpm and 37 ^{o}C for 48 h. Biomass was removed by filtration and protease activity was measured as described below (B.3.2.7.). Biomass was dried to a constant weight at 60 ^{0}C.

B.3.2.6.Optimization of lytic conditions

Microbispora sp. was grown in the nutrient broth with 200 mg% of *B. flexus* cells as mentioned above. Optimization of the *B. flexus* cell lysis was carried out in cell suspension (100 mg biomass/5 ml) at different pH (6, 7, 8), temperature (30, 40, 50 ^{o}C) and volume of the filtrate (10 – 50 µl). Incubations were carried out at 150 rpm for 8 h. Chloroform was mixed with lysate, solvent layer was removed and dried to a constant weight. Lytic activity was defined in units as: 1000 x (initial O.D. at 620 nm-final O.D. at 620 nm).

B.3.2.7. Protease assay

Protease assay was carried out to find out the protease activity in the culture filtrate of *Microbispora* sp. The protease enzyme cleaves casein to form tyrosine, which reacts with Folin's reagent in alkaline condition to give green colour.

1% of casein solution was prepared in phosphate buffer (pH 8); one ml of this was mixed with 1 ml of test sample. The mixture was incubated at 37 ^{0}C for 20 min. 3 ml of 5% of trichloro acetic acid was added and mixed. Precipitate that was formed was removed by filtration. 1 ml of filtrate was mixed with 2 ml of 0.2N NaOH and 0.6 ml of Folin's reagent and incubated for 20 min in dark before measurement of colour at 620 nm.

B.3.2.8. Protein estimation

Protein estimation was carried out according to Lowry's method using alkaline copper sulphate and Folin ciocalteu's phenol reagent through absorbance measurements at 660nm. Bovine serum albumin was used as standard [Materials and methods (common for chapters), section M.6.12].

B.3.2.9. Purification of enzyme

Ammonium sulphate (SRL, AR) was added at 50, 60, 70 and 80% saturation level to 250 ml each of the *Microbispora* sp. culture filtrate to precipitate the protein present in the culture filtrate. The mixture was kept at 4 ^{0}C, over night. The precipitate was collected by centrifugation and dissolved in 10 ml of Tris-HCl buffer (pH 7.2). The precipitate was checked for its protease activity and protein content. The precipitate was then dialyzed in double distilled water.

B.3.2.10. Separation of proteins by column chromatography

Separation is based on the fact that proteins are macromolecules whereas the salts are low molecular weight substances. During gel filtration solutes are separated primarily on the basis of their molecular size. Due to molecular sieving effect, the large molecules are eluted from the column first followed by compounds of smaller molecular mass.

B.3.2.10.1. Protocol

Sephadex G-100 (2g) was suspended in 100 ml of Tris-HCl buffer (50 mM, pH7.2) and kept for 3-4 h at room temperature for swelling. Excess buffer was decanted along with any suspended fine particles. The slurry was filled into a glass column (1.8 x 100 cm) and excess of buffer was drained out. The dialyzed sample mixture was gently applied uniformly over the bed and it was allowed to pass through the gel. Known volume of mobile phase (Tris-HCl buffer 7.2) was made to pass constantly from the reservoir. Sample fraction (2 ml) was collected manually. O.D of the fractions were measured at 280 nm. Respective fractions were pooled based on the peak obtained against OD. Protein content and enzyme activity for each pooled sample was analyzed.

B.35.2.11. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

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 before use.

Preparation of separating gel (30 ml)

15 ml of separating gel buffer, 12 ml of acrylamide stock, ammonium persulfate 50 µl, TEMED 50 µl were mixed and 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.

Preparation of stacking gel (10 ml)

Stacking gel buffer 1.25ml, stock acrylamide for stacking gel 0.75ml, ammonium persulfate 50 µl, TEMED 50 µl, distilled water 6 ml were mixed and poured over the separating gel and allowed to polymerize for 30 min.

Sample preparation

Purified sample of protease (from *Microbispora* sp. culture filtrate) (200 µl) was mixed with sample buffer (to get 1X sample buffer in a mixture), vortexed thoroughly and boiled for 3 min, cooled and 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 stacking gel. Current was increased to 50mA for separation gel.

Staining and destaining of the gel

Fixing solution

Methanol – 50%

Acetic acid – 12%

Formaldehyde – 0.59%

Ethanol – 50%

Sodium thiosulphate 0.2 g/l

Silver nitrate and formaldehyde – 2 g/l and 750 µl/l

Sodium carbonate and formaldehyde – 60 g/l and formaldehyde 500 µl/l

Protocol

Gel was fixed in fixing solution over night, washed in 50% ethanol for 20 min and in 30% ethanol for 30 min, then treated with sodium thiosulphate for 1min. It was rinsed with distilled water for 2-3 times. Colour was developed in sodium bicarbonate solution, when protein bands became visible then the reaction was stopped by adding 3 ml of 2% acetic acid directly at the corner of the staining container.

Destaining

The gel was repeatedly washed in 10% acetic acid and stored in 5% acetic acid.

Documentation of gel

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

B.3.2.12. Characterization of enzyme

The amino acid sequence of the protease produced by *Actinomycetes* was collected from the NCBI and three-dimensional structure of the protease was elucidated using SWISS-PORT protein modeling tool (swissmodel.expasy.org).

B.3.3. Results and discussion

B.3.3.1. Extraction of PHA by enzymatic digestion

Commercial enzymes such as alcalase (protease), lysozyme and cell lytic culture broth of *Microbispora* sp. was used to hydrolyse the cell walls of *B. flexus* to liberate PHA from the cells. Data in Table 3.5 indicate that the *Microbispora* culture filtrate showed optimum cell lytic activity compared to lysozyme.

B.3.3.2. Inhibition of lytic activity of culture broth by bovine serum albumin (BSA)

As commercial protease and culture filtrate of *Microbispora* gave almost similar results of cell hydrolysis followed by isolation of PHA, experiment was carried out to confirm the presence of protease in the culture filtrate and its effect on cell hydrolysis. By incorporation of BSA at 5, 25 & 50 mg% level in hydrolysis broth it was observed that the cell lytic activity reduced with concomitant increase in the BSA level (Fig. 3.7). The enzyme utilized the readily available substrate BSA than bacterial cells, thus the cell lytic activity was reduced indicating that protease was involved in cell lysis. Further experiments were carried out to enhance the protease activity in the culture broth and to purify the enzyme.

B.3.3.3. Optimization of medium for optimal production of protease by *Microbispora* sp.

The lytic enzyme producing actinomycetes species (*Microbispora* sp.) was grown in media containing various substrates for the production of the lytic enzyme. Various organic medium constituents and substrate such as beef extract, yeast extract, peptone, soya whey, soya milk, dairy whey and bacterial cells were used (Table 3.6). Culture grew well in nutrient broth agar plate containing *B. flexus* cells (Fig. 3.8 A). Good growth and maximum protease activity (10.5 IU) was obtained in the medium that contained *B. flexus* cells as substrate (Table 3.6). Bacterial cells were completely utilized by 72 h fermentation period as observed visually and under microscope (Fig. 3.8 B and 3.9). Soya whey also supported the production of the lytic enzyme (8.25 IU). The results reveal that optimal cell hydrolytic activity is achieved by using the bacterial cells as substrate.

Table 3.5: Comparison of the commercial enzyme and culture filtrate of *Microbispora* sp. for *B. flexus* cell lysis.

Enzyme	Alcalase (protease)	Lysozyme	*Microbispora* sp. broth
PHA (% of biomass)	48	11.7	54

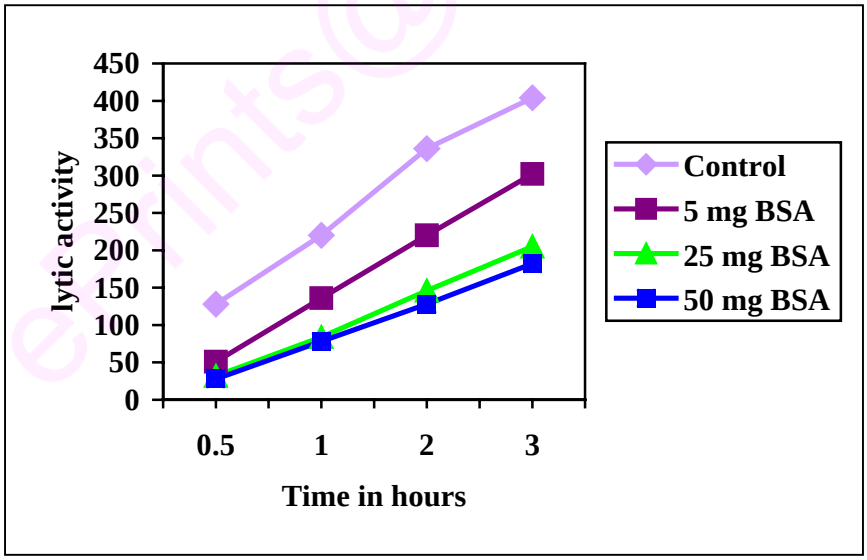


Figure 3.7: Inhibition of lytic activity of *Microbispora* sp. culture filtrate against *B. flexus* cells by BSA.

Table 3.6: Growth and enzyme production by *Microbispora* sp. in various media.

Media	Biomass of *Microbispora* sp. (dry wt g/l)	Protease activity in culture filtrate µg/ml/min
Nutrient broth	1.49	3.5
Beef extract+sucrose	0.8	2.5
Yeast extract+sucrose	1.95	4.5
Complex medium	2.58	2.8
Peptone+sucrose	1.9	-
Nutrient broth+*Bacillus* cells	1.03	10.5
Yeast extract+*Bacillus* cells	1.09	3.5
Nutrient broth+Soya milk	4.89	5.5
Nutrient broth+Soya whey	2.11	8.25
Nutrient broth+ Dairy whey	2.34	-

Media compositions are given under B.3.2.5.

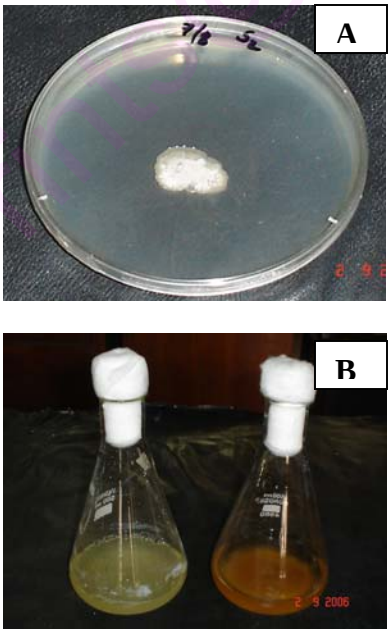


Figure 3.8: Growth of *Microbispora* sp. A – Nutrient agar plate containing *B. flexus* cells as substrate; B – Growth of culture at '0' h and 72 h in nutrient broth with *B. flexus* cells as substrate.

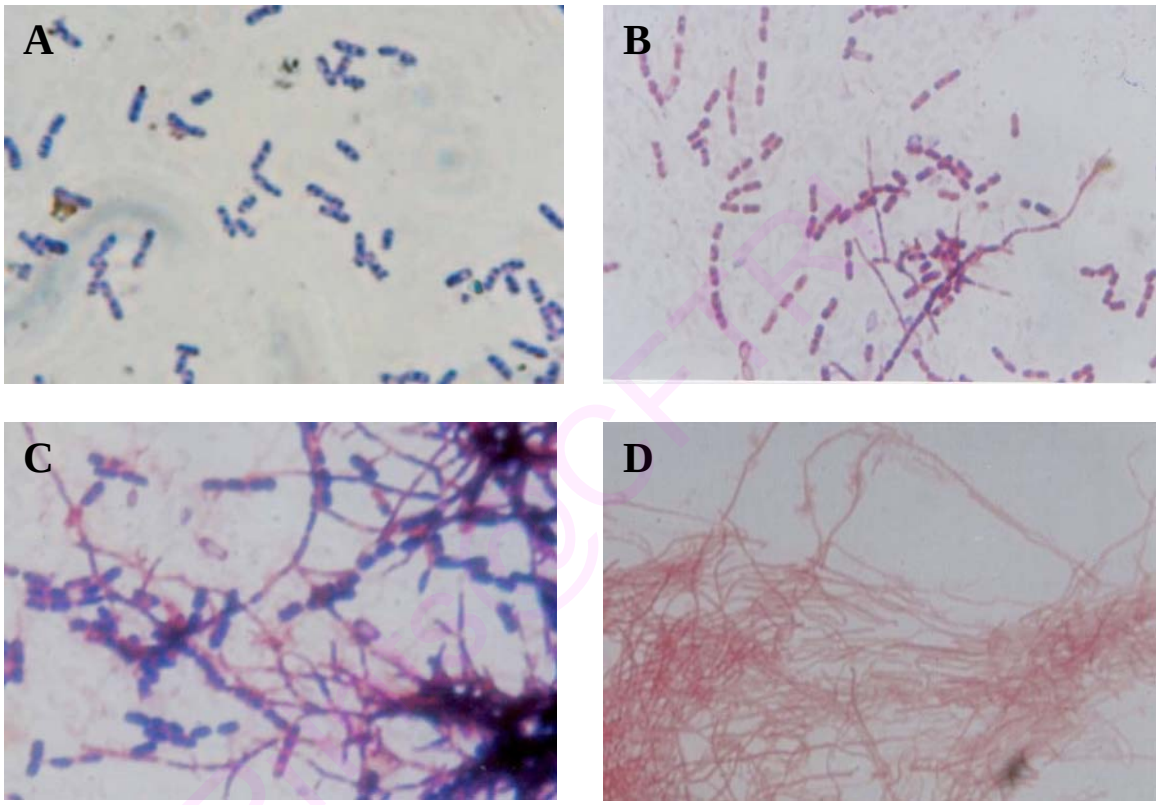


Figure 3.9: Growth of lytic culture of *Microbispora* sp. in nutrient broth containing *B. flexus* cells, as observed under microscope (100 x): A- '0' h, B - 24 h, C – 48 h and D – 72 h of fermentation.

B.3.3.4.Optimization of lytic conditions

Conditions for lytic activity by *Microbispora* sp. culture filtrate on *B. flexus* cells was optimized. Parameters optimized were pH, temperature and volume of culture filtrate required for optimal cell hydrolysis. Maximum cell lytic activity was observed at pH 6-7 and 50 ^{0}C using 10 µl of culture filtrate and 100 mg of *B. flexus* biomass in 5 ml test sample (Fig 3.10.).

B.3.3.5. Purification of enzyme

Lytic enzyme produced by *Microbispora* sp. in the culture filtrate was precipitated by 80% saturation of ammonium sulphate. Precipitated enzyme was dialyzed to remove the salts and enzyme was fractionated using sephadex G 100. Fractions collected were checked for the cell lytic activity (Fig. 3.11) and positive fractions were pooled and lyophilized. The molecular weight of the enzyme was confirmed by SDS-PAGE analysis. This revealed that the purified enzyme had a mol wt of ~ 40 KD (Fig. 3.12). The purified enzyme showed 10 folds more activity compared to crude component (Table 3.7.).

B.3.3.6. Characterization of enzyme

The amino acid sequence of the protease produced by *Actinomycetes* showed putative hits with 66 pdb (Protein Data Bank) protein templates. Sequence showed the presence of two peptides in their locus (Fig 3.13.) one is from 34-142 residues and another one is from 160-265. The highest homology was found with 2d4na and1nsoA.pdb with 53 and 45% sequence identity. The proposed 3D structure of the protein suggested that the polypeptide might fold in to one helix and seven plated sheet elements connected by various loops (Fig 3.14).

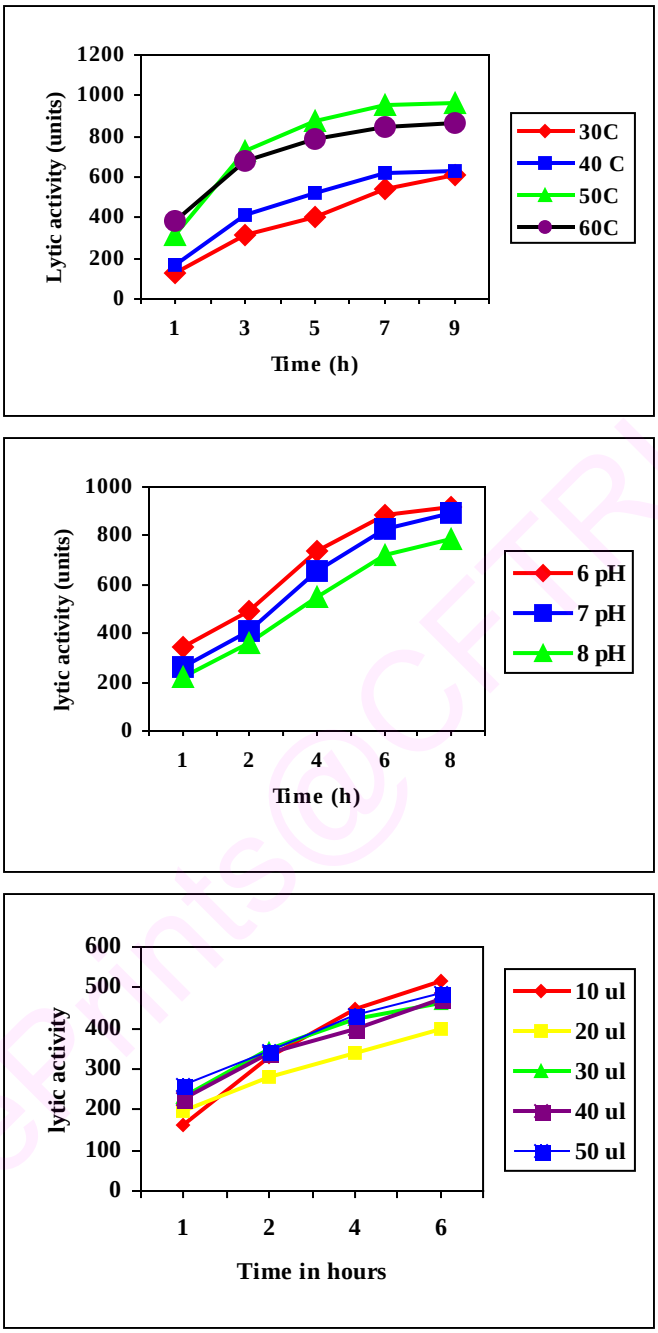


Figure 3.10: Optimization of the conditions for lytic activity of *B. flexus* cells by *Microbispora* sp. culture filtrate.

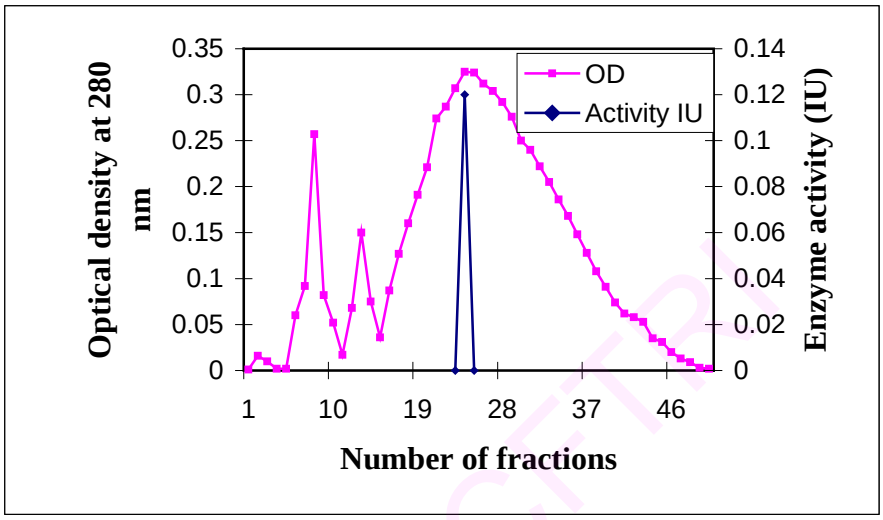


Figure 3.11: Fractions of enzyme of *Microbispora* sp. obtained by sephadex G-100 column chromatography and peaks showing *B. flexus* cell lytic activity regions.

Table 3.7: Cell lytic activity of *Microbispora* sp. enzyme against *B. flexus* cell hydrolysis at different levels of purification.

Enzyme	Protein (mg/ml)	Total activity	Specific activity
Crude	8.8	102	0.01
Precipitated (80% saturation of $(NH_4)_2SO_4$	6	580	0.02
Purified	4.5	1450	0.12

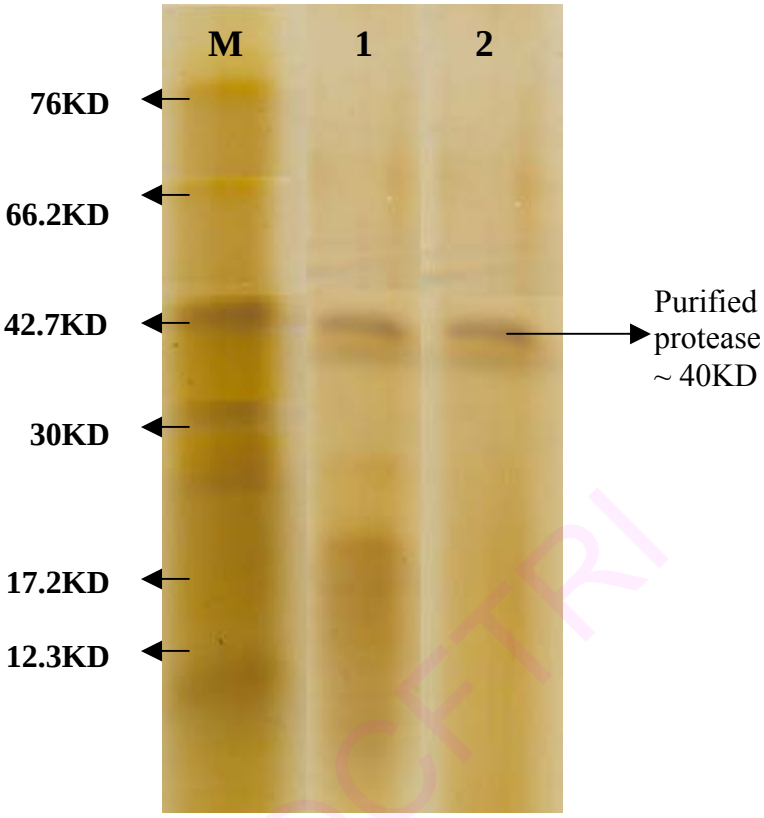


Figure 3.12: SDS-PAGE analysis of the enzyme obtained by *Microbispora* sp. culture filtrate. M=Marker; 1=Culture filtrate; 2= Purified protein.

Figure 3.13: Peptide regions in the amino acid sequence locus of protease.

Figure 3.14: 3D model of the peptides present in the amino acid sequence of protease.

Actinomycetes play an important role in ecosystem by enhancing soil fertility as well as in destruction of toxic substrates, which are the products of other organisms. Their ability to decompose organic matters is due to the synthesis of various enzymes, which can hydrolyze wood, lignin, cellulose, chitin, wax and microbial cells. *Actinomycetes* spp. can lyse many living and dead bacteria. Welsch 1942 showed that killed gram-negative cells were readily dissolved by sterile filtrates of *Actinomycetes.* The lysis of dead gram-negative cells was brought about by proteolytic enzyme system, which consisted of a protease and peptidase. The initial lysis of the cells is reported to be due to action of protease (Muggleton and Webb, 1952). The main function of these hydrolytic enzymes is to release nutrients from the substrates that are required for growth of the cells. *Bacillus* sp. is reported to produce a complex of hydrolyases composed of chitinase, lipase protease etc when grown in chitin containing medium, which could hydrolyse certain fungal species (Helisto *et al.,* 2001). Preferential induction of such lytic activity makes it evident that the hydrolases are involved in the dynamics of antagonism.

Bacteriolytic enzymes have been isolated from various microorganisms such as *Streptomyces, Bacillus subtilis, Flavobacterium, Chalaropsis, Myxobacter* sp. etc. Most of these lyse gram +ve bacterial cells (Ensign and Wolfe, 1965). It has been reported that in the genus Arthrobacter some of the species are susceptible to lytic enzyme of *Myxobacter* sp., and others are resistant to the enzyme attack. The difference in susceptibility is apparently due to macromolecular outer layer. Various mechanisms of cell lysis are reported based on peptidoglycan degrading specificity and are broadly classified as 1) glycosidases 2) N acetylmuramyoyl-l-alanine amidases and 3) endopeptidases. The preoteases (trypsin, chymotrypsin, papin and bromelain), β-glycosidases (cellulases) and lysozyme were the enzymes studied to cleave the peptidoglycan- β-1,4-glycosidic links located between N-acetyl glucosamine (NAG) and N acetylamine muramic acid (NAM) residues and peptide links of tetrapeptides that connect the polymeric chain of NAG and NAM which forms very rigid structure of bacterial cell wall (Bugg, 1997).

It has been observed that the lysozyme can be effective in the presence of EDTA (a chelating agent which destabilizes the outer membrane) and proteinase K (cell wall lytic enzyme) and surfactant SDS (solubilises outer membrane), which enable lysozyme to

reach the peptidoglycan layer. Lysozyme is a costly enzyme and addition of adjuvants will further enhance the cost of enzymatic hydrolysis. Achromopeptidase exhibits higher bacteriolytic activity and is more potent than the lysozyme. However a *Lysobacter* sp. has been reported to hyper produce endopeptidases (Ahmed *et al.,* 2003).

Bromelain and pancreatin have been used to hydrolyse *R. eutropha* cells to release PHB (Kapritchkoff *et al.,* 2006). Proteases are more efficient than β-glycosidases as they act up on peptidoglycan and other cell proteins. Trypsin is specific for peptide linkages with lysine whereas bromelain has broad specificity. It has been observed that utilization of pancreatin is more economical for cell hydrolysis compared to chymotrypsin, trypsin or bromelain (Kapritchkoff *et al.,* 2006).

In the present work Actinomycetous culture was used for cell hydrolysis. The isolate was identified as *Microbispora sp.* which produced white aerial mycelium, brownish-orange diffusible pigment. New species of Microbispora have been reported from the local soil sample (Rao *et al.,* 1987). Earlier studies using the same isolate has shown that the lytic enzyme produced was active in lysing gram –ve cells of *Rhizobium meliloti* (Kshama and Shamala, 2006). The enzyme produced by the organism was also used optimally to hydrolyse the gram+ve cells of *B. flexus* in the current study. Data reported in the literature indicate that the lytic enzyme produced by a specific strain is either active against gram+ve or gram -ve bacterium (Ensign and Wolfe, 1965). However we have observed that both gram +ve and gram –ve cells can be hydrolysed by the enzymes secreted by a single strain of microorganism which indicated wide spectrum of activity and various substrate specifities. This may be because *R. meliloti* or *B. flexus* cells were used as substrates for the production of cell lytic enzyme. However the studies have shown that both the lytic enzymes were proteases.

Lysozyme has been used for peptidoglycan hydrolysis, however this enzyme was not effective against *B. flexus* cell wall hydrolysis.

Fractionation of ammonium sulphate precipitate revealed the presence of three proteins, among which one showed the bacteriolytic activity, which was accompanied by proteolytic action, which are inherent to proteases, hydrolyzing the peptide bonds between the amino acids. The mechanism used to cleave a peptide bond CO-NH, -C(=O)NH by amino hydrolysis (addition of water molecule) and formation of amino acid

residues so that it can attack peptide carbonyl group. The enzyme was separated and their properties were studied. Hydrolysis of *Bacillus* peptidoglycan by this lytic enzyme was accompanied with increase of reducing sugars and amino groups.

The proteolytic activity of the enzyme was confirmed by the use of BSA along with the *Bacillus* cells. The enzyme utilized the readily available substrate BSA, there by inhibiting the cell lysis. In the absence of BSA the enzyme degraded the bacterial cells, which was a sole substrate in the medium completely. The purified enzyme was optimized for temperature, pH and substrate concentration. The enzyme was found thermotolerant and stable in wide temperature (28-55 ^{0}C) and pH range (6-9). The maximum activity was recorded at 50 ^{0}C at 7.2 pH. The polyacrylamide gel electrophoresis with SDS and β mercaptoethanol showed homogeneity of the purified enzyme and its molecular weights was ~ 40000 daltons.

Conclusions

Enzyme produced by *Microbispora* sp. hydrolysed the gram +ve cells of *B. flexus*, which are more resistant to hydrolysis. By incorporation of BSA in hydrolysis broth it was observed that the cell lytic activity was due to proteases. Maximum protease activity was secreted by *Microbispora* sp. in medium containing *B. flexus* cells as substrate. Optimal cell lytic activity was observed at 50 ^{0}C and pH 6-7. The enzyme was purified to homogeneity by column chromatography and the purified enzyme had a mol wt of ~ 40 kD and it showed 10 folds more activity compared to crude component. 3D structure of the protein suggested that the polypeptide might fold in to one helix and seven plated sheet elements connected by various loops. Overall results have indicated the utility of *Microbispora* sp. enzyme for hydrolysis of *B. flexus* cells.

Part C: Separation of PHA by aqueous two phase system

C.3.1. Introduction

PHA is intracellular and hence acceptable method of isolation from the cell is essential for economic recovery of the product. A number of methods for the recovery of PHA have been developed (Lee, 1996). Sodium hypochlorite is used for cell hydrolysis, which leads to the reduction in molecular weight of the polymer. Enzymatic digestion of cells has been developed as an alternative to chemicals but this again consists of several steps before the purified product is obtained (Holmes and Lim, 1990; Kshama and Shamala, 2006). Most of the methods require large amount of chemicals, which includes organic solvents for solubilization and isolation of the product. As an alternative to these, possibility of application of aqueous two-phase system for the isolation of PHA was explored.

Albertson first introduced aqueous two-phase system (ATPS) in 1950s for the partition of macromolecules and cell particles. Now a vast variety of methods currently exist for separation and characterization of biomolecules. The special feature of the aqueous two-phase system is that the solvent used in both the phases is water. This feature provides the suitable environment for biomolecule in both the phases. There is a diverse application of ATPS for biotechnological downstream process. But the use of these system has so far been limited probably due to the poor predictability and analysis of the parameters affecting the partitioning (Bensch *et al.,* 2007). The present section deals with isolation of PHA from bacterial cell using ATPS.

C.3.2. Materials and methods

C.3.2.1. Polyhydroxyalkanoate production

Inoculum, preparation, composition of production medium and fermentation conditions is dealt in [Materials and methods (common for chapters), section M.5.4, M.5.5. production medium 1, Table 3). This inoculum contained 1.5 x 10^3 CFU/ ml. Cultivation was carried out at 30 ^{0}C and 250-rpm for 72 h. Experiment was carried out in

fifty flasks of 500 ml capacity, and biomass was obtained from pooled up fermented broth.

C.3.2.2. Estimation of biomass and quantification of PHA

Estimation of biomass and quantification of PHA content of the bacterial cells by GC method, has been described earlier [Materials and methods (common for chapters), sections M.6.1 and M.6.6).

C.3.2.3. Lysis of PHA containing *B. flexus* cells and separation of PHA by ATPS

Suitability of various ATPS systems for PHA isolation was tested using sodium hypochlorite digested cells (mentioned below). The systems were selected from the reported polymer-polymer and polymer-salt types (Hofsten and Baird, 1962; Raghavarao *et al.,* 1995), which consisted of: a) PEG 4000/Dextran 50; b) PEG 4000/maltodextrin; c) PEG 4000/phosphate; d) PEG 6000/phosphate and d) PEG 8000/phosphate.

B. flexus cells were lysed by three different methods, in triplicate, and PHA was isolated from the lysate by ATPS as described below:

a) Sodium hypochlorite digestion and optimization of ATPS conditions:

Fresh *B. flexus* cells (1 g dry weight basis) was suspended in 50 ml of 5% sodium hypochlorite solution and incubated at 37 ^{0}C, 150 rpm for 1h. The hydrolysate was centrifuged at 2900 g for 15 min; sediment containing cell materials and PHA granules was washed with water and mixed with 100 ml of ATPS mixture. The system was selected from the phase diagram (Fig. 3.15), which is given by Zaslavasky (1995). The selected phase formed two phases in equal volume. One hundred ml of aqueous phase mixture used in the experiment contained phosphate buffer (9.7g % of K_2HPO_4 and KH_2PO_4 in the ratio of 1.82:1.0) and 12% (w/v) PEG 8000. The mixture was homogenized thoroughly and allowed to stand for 30 min. Optimization of ATPS separation was carried out a) By varying the pH of the system (pH 5-10) at room temperature of 28 ^{0}C, b) By holding the system (pH 8.0) at different temperatures during phase separation (4, 15, 25 and 35 ^{0}C). Under optimized condition (pH 8.0 and room

temperature of 28 ^{0}C), PHA that partitioned to the top phase was centrifuged at 2900 g for 15 min, washed with acetone and dried at 60 ^{0}C to a constant weight. Control values for PHA were obtained by extracting of sedimented and acetone washed material with chloroform at 45 ^{0}C, 2 h, 150 rpm, precipitation of the polymer from clear chloroform layer using 4 volumes of methanol and recording the dry weight.

b) Sonication:

Fresh *B. flexus* cells (1 g dry weight basis) was suspended in 50 ml of water and sonicated for 10 min (5 cycles). The cell lysate was suspended in 100 ml of ATPS mixture (pH 8.0 at room temperature of 28 ^{0}C) and handled further as mentioned above. Another set of sonicated sample was centrifuged, washed with acetone, extracted with chloroform as mentioned above.

c) Hydrolysis by *Microbispora* sp. culture filtrate:

Microbispora sp. which was used for enzymatic hydrolysis of PHA containing *B. flexus* cells was used in the experiment (Section B.3. described above). Inoculum was prepared by transferring cells from fresh culture to 100 ml sterile nutrient broth containing *B. flexus* cells (0.2% on dry weight basis). The flask was incubated at 30 ^{0}C and 250 rpm for 48 h. After cultivation, *Microbispora* sp. cells was easily removed by filtration. Filtrate was used for lysis of *B. flexus* cells. Lytic activity, which was due mainly to protease present in the culture filtrate, was assayed as mentioned in section above (Section B.3.2.7.). Calculation of lytic activity against *B. flexus* cells is explained under Section B.3.2.6.

For enzymatic hydrolysis of biomass and separation of PHA from other cell material, PHA containing cells of *B. flexus* (1 g on dry weight basis) and culture filtrate of *Microbispora* sp. (50 ml) were added to the ATPS mixture detailed above and the final volume was kept constant at 100 ml. The mixture was incubated at 200-rpm at 37 ^{0}C for 2 h. At the end of incubation period the mixture was allowed to stand at room temperature of 28 ^{0}C for 30 min. Protease and PHA were found in the top PEG phase and residual cell material was found at the bottom phase. The phase volumes were measured. The top PEG phase that contained PHA was centrifuged at 2900 g for 15 min

to sediment PHA granules. The sediment was washed with water and acetone and dried to a constant weight at 60 ^{0}C. In a control experiment, *B. flexus* cells were also hydrolysed with culture filtrate as mentioned above without ATPS system and PHA was extracted using chloroform as mentioned earlier.

C.3.2.4. Separation of protease

In the above experiment protease also partitioned into upper PEG phase along with PHA. After the removal of PHA by centrifugation, the supernatant was assayed for protein concentration and protease activity (Section B.3.2.7. and B.3.2.8)

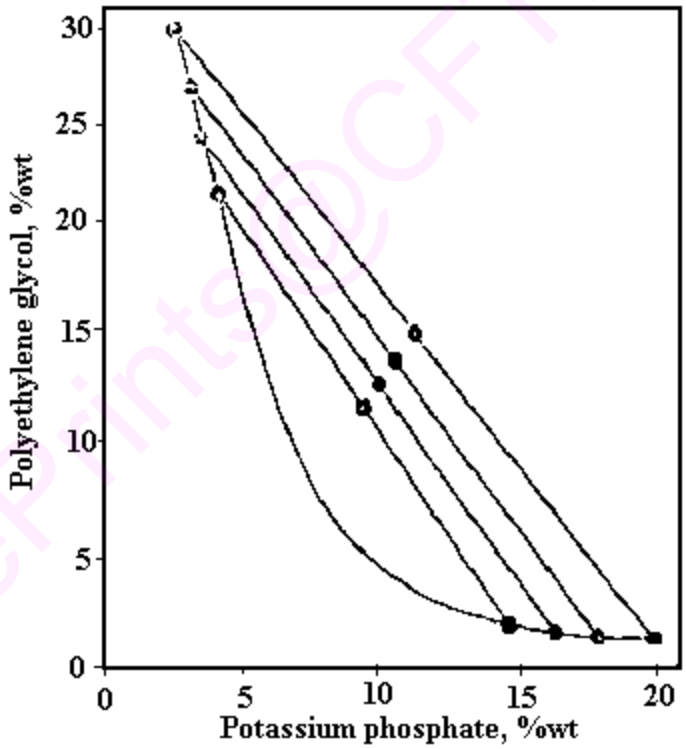


Figure 3.15: Phase diagram of polyethylene glycol 8000, (Sigma Aldrich) and potassium phosphate. The mixture contains K_2HPO_4 and KH_2PO_4 in the 1.82:1.0 ratio (Zaslavasky, 1995).

C.3.2.5. Quantification and characterization of PHA

Purity of PHA was measured by crotonic acid assay [Materials and methods (common for chapters), section M.6.10.) Purity of the sample was calculated using standard PHA graph .

Purity of the polymer was also checked by GC analysis. GC analysis of PHA, FTIR, NMR and molecular weight determinations were carried out as detailed under [Materials and methods (common for chapters), sections: M.6.4; M.6.6; M.6.9 and M.6.13). P(HB-co-HV) containing 5 mol% of hydroxyvalerate (Sigma Aldrich, USA) was used as standard for calibrations.

C.3.2.6. Determination of partition coefficient, purification factor and yield

The partition coefficient (Porto *et al.,* 2007) was determined as the ratio of the yield (for PHA) or activity (Protease) in the top phase to the bottom phase by applying:

$$K = Y_t/Y_b$$

Where Y_t is yield of PHA in top phase and Y_b is the PHA in bottom phase. Purification factor was calculated as the ratio of the specific activity in the top phase to the specific activity of fermented broth prior to separation, using the formula:

$$PF = \frac{A_t / C_t}{A_i / C_i}$$

Where A_t and A_i are the activity of the enzyme in top phase and in the initial stage in the broth, respectively. C_t and C_i are total protein concentrations, expressed as mg/ml, in the top phase and initial broth, respectively.

The activity yield was determined as the ratio of total activity in the top phase to that of the fermented broth, using the formula:

$$\text{Activity yield} = \frac{A_t / V_t}{A_i / V_i} \text{ X } 100$$

Where V_t and V_i are the volumes of the top phase and the initial extract, respectively.

C.3.3. Results and discussion

C.3.3.1. ATPS partition of cell lysate obtained from sonication, hypochlorite and enzyme hydrolysis

B. flexus produced 2.5 g/l of biomass with 1.3 g/l of PHA under shake flask conditions. GC analysis showed that the polymer produced on sucrose was mainly composed of polyhydroxybutyrate with traces of hydroxyvalerate copolymer. Bacteria accumulate PHA intracellularly and hence isolation of polymer includes hydrolysis of cell as a preliminary step. A number of methods have been developed for the recovery of intracellular PHA. This includes mechanical, chemical and biological or enzymatic methods. In the present study ATPS was used for isolation of the polymer.

ATPS systems have been successfully used for the recovery of many biomolecules (Bensch *et al.,* 2007; Shibusawa *et al.,* 2007). Several factors such as polymer molecular weight, concentration of polymer and salt, pH, influences biomolecule partition in ATPS. These factors are inherent to the particular choice of the system components. Molecular mass, charge, hydrophobicity of the separating molecule also affects the partitioning. Interaction between the system and target molecule is also an important criteria. In PEG-phosphate system partitioning of the biomolecule depend on the 'volume exclusion effect' and 'salting out effect' of the system (Rabelo *et al.,* 2004). PEG-dextran two-phase system with relatively small molecular weights was used to separate the cell and cell content (Hofsten, and Baird, 1962). In the current study ATPS involving PEG and phosphate has been used to separate PHA from cell lysate.

In a preliminary experiment it was found that amongst various aqueous-aqueous systems such as PEG 4000/dextran, PEG 4000/maltodextrin, PEG 4000/phosphate, PEG 6000/phosphate and PEG 8000/phosphate tested for partitioning of PHA from residual biomass material, polyethylene glycol 8000 and potassium phosphate system was best suited for the purpose. In PEG/dextran system cell debris and PHA separated together in the interphase. Maltodextrin was used as a cheaper ingredient instead of costlier polymeric material such as dextran. In this system maltodextrin formed a turbid phase and interfered with PHA granule separation. In lower molecular weight PEG (4000-6000)/salt systems cell debris collected at the bottom phase and PHA separated into

interphase. Due to this it was difficult to separate PHA from other cell debris. Higher molecular weight PEG (8000) gave optimum results, which is discussed below.

Results indicated that recovery of PHA from sonicated cells was low compared to other methods of cell hydrolysis (Table 3.8). This may be due to incomplete cell lysis. Molecular weight of PHA obtained after hypochlorite digestion was low compared to enzymatic cell hydrolysis. It is known that cell hydrolysis by chemicals such as sodium hypochlorite leads to generation of chlorine containing effluent and also reduces the molecular weight of the polymer (Kshama and Shamala, 2006). Alternatively enzymatic digestion method has been used for the isolation of PHA from bacterial cells (Holmes and Lim, 1990). Culture filtrate of actinomycetes which is rich in lytic enzyme has been employed for the hydrolysis of PHA containing cells of *Sinorhizobium meliloti* to release PHA so that the quality of PHA is not affected (Kshama and Shamala, 2006). The final step of PHA isolation from hydrolysed samples invariably involves the usage of solvent such as chloroform for isolation of the product. In the present study PHA was isolated from cell lysate using ATPS, which is a non-organic solvent method of PHA isolation.

The typical composition of PEG and phosphate was selected from the phase diagram (Fig. 3.15) available in the literature (Zaslavasky, 1995). Partition coefficient of the PHA was calculated as the ratio of yield obtained in the top phase to the bottom phase. Sodium hypochlorite digested *B. flexus* cells were used for optimization of pH and temperature for phase separation in PEG-phosphate system (Fig. 3.16). Partition coefficient of the PHA obtained was analyzed as the ratio of yield obtained in the top phase to the bottom phase. Temperature and pH of the system have a great impact on the partition of the PHA, where both cell debris and PHA are found in interphase at low pH and temperature. By keeping temperature for separation constant at 28 ^{0}C and varying the pH, differences were observed for separation of cell material and PHA. At pH 5.0-cell lysate settled down completely in the bottom phase, at pH 6.0 they accumulated as interphase and at pH 7.0 they were unevenly distributed in both the phases. Alkalinity enhanced the partitioning and recovery of PHA. At pH 8 and above, PHA was completely partitioned to the top phase (Fig. 3.17). Similarly by maintaining pH at 8.0 better separations were obtained at higher temperature (25 ^{0}C and above) compared to lower

temperatures. The actual mechanism of partition behavior of PHA has not been studied so far.

PHA containing cells were subjected to sonication, sodium hypochlorite and enzymatic hydrolysis by using *Microbispora* sp. culture filtrate for *B. flexus* cell lysis mixed withPEG/phosphate was allowed to stand for phase separation under optimized conditions (pH 8.0, room temperature of 28 ^{0}C). PHA partitioned in the upper PEG phase and other cell material settled in the bottom phase (Fig. 3.17). PHA yield (% of dry biomass weight) obtained after partitioning in the upper PEG phase with sonication, hypochlorite and *Microbispora* sp. culture filtrate treatment was 19%, 51% and 45%, respectively (Table 3.8). Corresponding values for controls (chloroform extraction, without ATPS separation) were 20, 58 and 49%. Sonication and enzymatic cell hydrolysis resulted in product of better purity compared to purity of PHA isolated from hypochlorite digestion. PHA isolated from control sample of hypochlorite digestion was less pure, which may be due to contamination of lower molecular weight degradation products that were formed by chemical hydrolysis and interference of salt. The molecular weight of the polymer isolated from hypochlorite digestion was also low compared to samples obtained after enzymatic hydrolysis (Table 3.8).

Economics of PHA production depends on various factors such as raw materials and bacterium used for fermentation, production process, method of cell disruption and isolation of PHA, PHA quality and quantity synthesized in the bacterial cell etc. Development of economic and easy method of extraction is one of the important factors for overall economics of polymer production. Several PHA recovery methods from various bacteria are reported in the literature and an over view is shown in Table 3.9. Extractability of PHA from the cells using solvents results in pure PHA without change in the molecular weight (Brandl *et al.*, 1990). This extraction requires large amount of solvent, which also causes environmental pollution. Sodium hypochlorite has been used as an alternative, however it leads to degradation of the polymer (Brandl *et al*., 1990). As an alternative to this, sodium hypochlorite-chloroform dispersions have been adopted successfully in large scale to obtain PHB of high yield and purity (Choi and Lee, 1997). However it has also been reported that from point of economics, surfactant-hypochlorite method of isolation resulted in lowest price of PHB of $ 4.75/kg (Choi and Lee, 1997). It

is known that cell hydrolysis by chemicals such as sodium hypochlorite leads to generation of chlorine containing effluent and also reduces the molecular weight of the polymer (Kshama and Shamala, 2006). Alternatively enzymatic digestion method has been used for the isolation of PHA from bacterial cells (Mayerhoff *et al.,* 2004). Culture filtrate of actinomycetes, which is rich in lytic enzyme, has been employed for the hydrolysis of PHA containing cells of *Sinorhizobium meliloti* to release PHA so that the quality of PHA is not affected (Kshama and Shamala, 2006). But the use of expensive chemicals and complex process does not seem to be economical (Choi and Lee, 1997). Pure PHA can be recovered if the cells are ruptured by simple method, which does not lead to polymer degradation. Use of simple chemicals and easier methodologies are required for economic production. Lowering of PHA production cost to 3.66$/kg has been achieved by using alkali digestion of recombinant *E. coli* (Choi and Lee, 1999). However this method is applicable only to bacterial cells such as *E. coli*, which have fragile cell walls. Many of the methods involve multi steps for cell rupture and isolation of PHA from non-PHA cell materials

Table 3.8: Comparison of PHA yields and purity obtained after different methods of *B. flexus* cell hydrolysis and subsequent separation by ATPS

Method of *B. flexus* cell hydrolysis		PHA isolated (% of dry biomass)	Purity of isolated PHA (%)	Molecular weight
1. Sonication	A	20.3±2.08	92±2.0	
	B	19.0±2.64	99±1.0	
2. Na-Hypochlorite	A*	58.0±2.50	80±1.0	
	B	51.0±1.00	90±2.0	5.5×10^5
3. Culture filtrate**	A	49.3±2.50	95±1.0	
	B	45.3±1.50	97±1.0	1.4×10^6

A = Controls, PHA extracted from respective cell lysates by chloroform extraction; B = PHA isolated from respective cell lysates by ATPS in PEG phase; *Standard method used for PHA quantification; ***Microbispora* sp. culture filtrate containing protease was used for *B. flexus* cell hydrolysis.

In the present study preliminary experiment has been carried out to find out the possibility of hydrolyzing and extracting PHA in a single system containing cell lytic enzyme and ATPS. The final step of PHA isolation from hydrolyzed samples invariably involves the usage of solvent such as chloroform for isolation of the product. PHA was isolated from cell lysate using ATPS, which is a non-organic solvent method of PHA isolation.

Table 3.9. Comparison of various methods of cell rupture and isolation for PHA from various bacteria.

Bacteria	Cell rupture	PHA isolation	PHA recovery (%)	Mol. wt. of PHA	Reference
Rhodospirillum rubrum	Hypochlorite	Centrifugation	89	Degraded	Brandl *et al.*, 1990
Rhodospirillum rubrum	Hot chloroform	Chloroform; centrifugation	92	No change	Brandl *et al.*, 1990
Alcaligenes eutrophus	Surfactant + hypochlorite	Centrifugation	95	Slight change	Choi and Lee, 1997
Alcaligenes eutrophus	Hypochlorite + chloroform dispersion	Chloroform; methanol, water	90	Slight change	Choi and Lee, 1997
Aeromonas hydrophila	Dry cell powder	Ethyl acetate; hexane/heptane	-NG-	-NG-	Chen *et al.*, 2001
Recombinant *Escherichia coli* (Fragile cells)	NaOH (0.1 N)	Centrifugation	90	Slight change	Choi and Lee, 1999
Sinorhizobium meliloti	Lytic (protease) enzyme	EDTA + surfactant; centrifugation	94	-NG-	Kshama and Shamala, 2006
Bacillus flexus	Lytic (protease) enzyme + ATPS	ATPS; centrifugation	92	No change; Protease recovered as by product	Present work

NG: Data not given

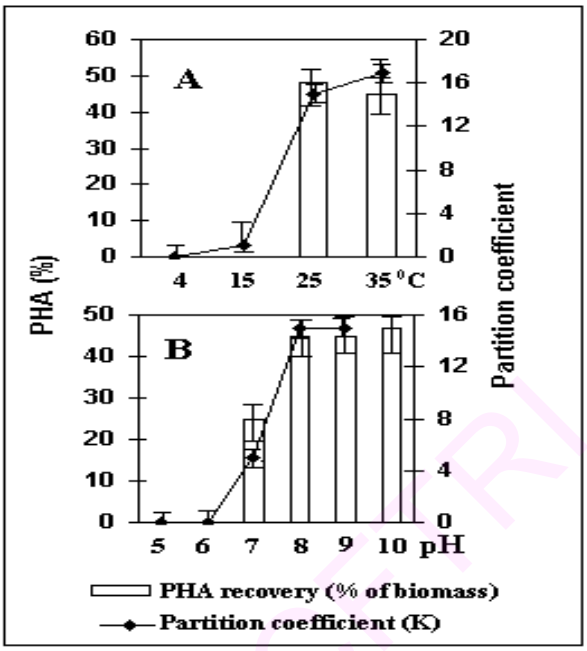


Figure 3.16: Partition coefficient (K) and % recovery of PHA from hypochlorite digested *B. flexus* cells separated into PEG phase of ATPS: A) At pH 8.0 and different temperatures of 4 to 35 ^{0}C; B) At room temperature of 28 ^{o}C and different pH ranging from 5 to 10.

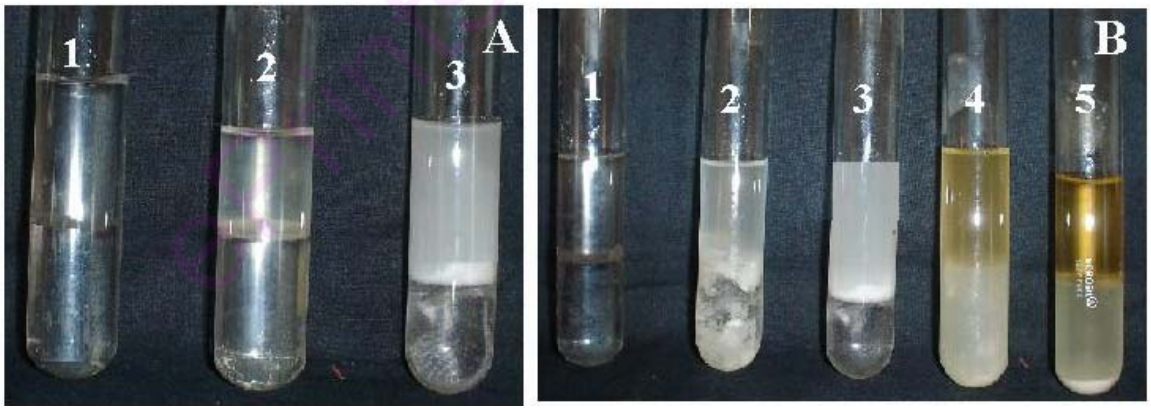


Figure 3.17: Aqueous two phase system of PHA extraction from *B. flexus* cells. A= Showing the separation of PHA at different pH (1: Blank, 2: pH 7 and 3: pH 10); B= PHA extracted by ATPS by different methods at optimized conditions [1: Blank system; 2: Sonicated cells; 3: Hypochlorite extracted cells; 4: Enzyme digested cells and 5: Undigested cells (Control)].

C.3.3.2. Partitioning of protease in ATPS

Fermentation of *Microbispora* sp. in the presence of *B. flexus* cells produced 3 U/ml protease. When PHA containing cells were digested with this culture filtrate in the presence of PEG and phosphate, protease partitioned along with PHA in the upper phase. Activity yield of the enzyme was Y = 672%, with a purification factor PF = 9.1.The activity enhanced by 7 folds (Table 3.10).

ATPS has been used for partitioning of several enzymes and partition behavior of the protease is also known (Porto *et al.,* 2007). In this study protease present in the *Microbispora* sp. culture filtrate that was used for cell hydrolysis was also separated as a by-product during PHA recovery in the top phase at alkaline pH. It is known that the pH of the system influences ionizable groups of protein and alters its surface charges, hence enzymes above isoelectric point partition to the top phase as pH increases (Ravindra Babu *et al.,* 2008). Activity yield of the enzyme was Y = 672%, with a purification factor PF = 9.1 with 7 fold enhancement in the activity. Such enhancement in activity yield value higher than 100% has been frequently reported for enzyme extraction using aqueous two-phase system (Porto *et al.,* 2007; Narayan *et al.,* 2008). Pancera *et al.,* (2002) reported that PEG could influence the activity of enzyme by altering the structure of enzyme active site. Elimination of enzyme inhibitors from PEG phase during extraction is also known to enhance the activity (Mayerhoff *et al.,* 2004). The phosphate present in the system acts as a buffering salt, which favors the enzyme activity and the cells act as a substrate. Protease has been recovered with PF = 2.5 - 5.0 and Y = 132-255% by PEG/Citrate system (Porto *et al.,* 2007), peroxidase with PF = 1.98, 3.7, 18.36, 2.33 and the activity yield Y=160, 976, 80, 616% in three phase system (Narayan *et al.,* 2008). ATPS system used in the current study ensured separation of PHA, higher yield and purification of protease, simultaneously.

Table 3.10: Recovery of protease present in the culture filtrate of *Microbispora* sp used for cell hydrolysis of *B. flexus* during ATPS separation of PHA.

Sample	Protease activity U/ ml	Protein mg/ ml	Specific activity/ mg protein	Purification of protease (fold)	Purification factor (PF)
Culture filtrate*	3±1.00	1.3±0.51	0.03±0.01	-	-
PEG phase of ATPS	21±1.00	1.0±0.50	0.21±±0.10	7	9.1

* *Microbispora* sp. culture filtrate containing protease was obtained by the cultivation of the organism in shake flask culture.

C.3.3.3. Characterization of PHA

PHA isolated by ATPS was analyzed by GC for its purity and characterized by FTIR and ^{1}H NMR. FTIR spectrum showing C=O stretching at 1724 Cm^{-1}, O-C-O, CH and CH_2 in the band area of 1320-1150 Cm^{-1}, CH_3 at 1381 Cm^{-1}, CH stretching and C-H-O hydrogen bands at 2850 – 3050 Cm^{-1} confirmed that the polymer isolated was PHA (Fig 3.18).

The ^{1}H NMR spectrum (Fig. 3.19) showed a doublet at 1.29 ppm attributed to methyl group, a doublet of quadruplet at 2.57 corresponding to methylene and methyne signal at 5.2, which confirmed that the polymer analysed, was polyhydroxybutyrate (PHB). The results obtained are in accordance to that reported in the literature (Kshama and Shamala, 2006). From these results it can be concluded that the material separated from PEG phase of ATPS is exclusively PHA, which is in the form of PHB.

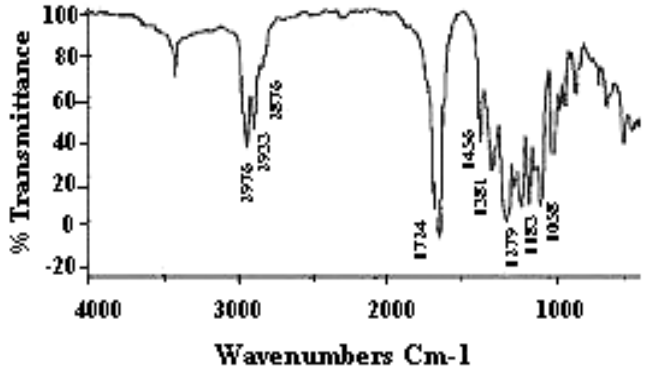


Figure 3.18: FTIR spectrum of PHA isolated from *B. flexus* cells in which cells were hydrolysed by *Microbispora* sp. culture filtrate and PHA was separated from PEG phase of ATPS.

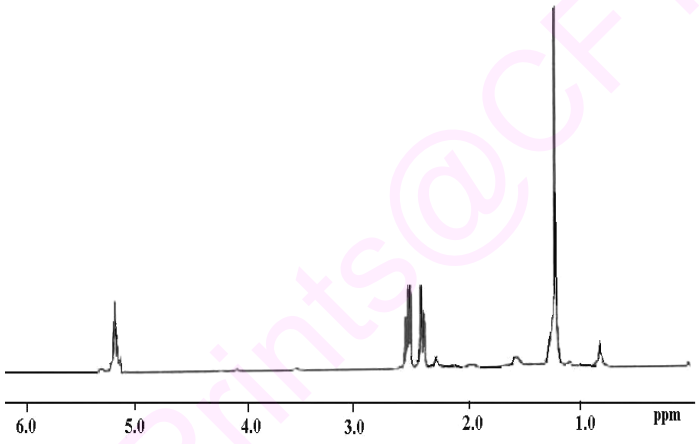


Figure 3.19: ^{1}H NMR spectrum of PHA isolated from *B. flexus* cells in which cells were hydrolysed by *Microbispora* sp. culture filtrate and PHA was separated from PEG phase of ATPS.

Conclusions

The effect of different extraction methods; pH and temperature on ATPS partitioning of bacterial cell lysate containing PHA has been studied. PEG 8000/potassium phosphate was found to be the most suitable system for the separation and concentration of PHA and protease. Data obtained indicated that by using PHA containing cells of *B. flexus, Microbispora* sp. culture filtrate containing protease in ATPS system, cell hydrolysis, partitioning of the polymer and recovery of protease to several fold purity can be achieved simultaneously in a single system. The results on the whole prove that ATPS is a potential and powerful separation step in the overall enzyme and PHA protocol. However the actual mechanism of partition behavior of PHA requires to be studied.

Part D: Effect of gamma irradiation on cell lysis and modification of PHA

D.3.1. Introduction

P(HB) is the most commonly synthesized PHA of the bacterial cell. This homopolymer is brittle and its mechanical properties can be improved by co polymerization with other alkanoates (Ashby *et al*., 1998; Doi *et al.*, 1995; Matsusaki, 2000; Kshama and Shamala, 2003). Synthesis of co-polymer is induced by supplementation of co-substrates such as fatty acids to the medium. Plant oils have been used as alternative substrates for fatty acids (Akiyama, *et al.,* 2003; Kahar *et al.,* 2004). PHA is intracellular and it is isolated after the digestion of the cell wall by physical, chemical or enzymatic methods (Kshama and Shamala, 2006).

Recently research has been focused on modifications of polymer for various applications. Polymer modification by ionizing radiation has advantages over chemical modifications. The radiation modification includes: radiation cross-linking, radiation induced polymerization and also degradation. Radiation affects the properties of polymeric materials, depending on the intensity and type of radiation, nature of the polymer and mechanism of reaction. Radiation has been used to modify a few polymeric materials (Chmeilewski *et al.,* 2005; Bassas *et al.,* 2008; Dufresne *et al.,* 2001). Attempt has been made to improve the mechanical properties of poly(3-hydroxyoctonoate-co-undeconoate), a medium chain length polymer produced from *Pseudomonas oleovorans,* through exposure to gamma radiation and it was observed that the double bonds took part in the formation of irradiation cross links in the film (Dufresne *et al.,* 2001).

The objective of the present work was to use *B. flexus* for PHA production, to improve polymer properties by using plant oil as a co carbon substrate for fermentation and by exposing PHA containing cells to gamma irradiation. Usefulness of gamma irradiation of whole wet cells for improvement of polymer properties and also cell lysis, which leads to easier isolation of the polymer, has been worked out.

D.3.2. Materials and methods

D.3.2.1. Cultivation of *B. flexus*

Inoculum preparation of *B. flexus* was carried out as explained earlier (Chapter 2, section 2.5.4). The inoculum contained 2 x 10^4 CFU /ml.

PHA production was carried out in a jar fermentor (Bioflo 110, New Brunswick Scientific Co. USA) of 3 l capacity, containing 1.8 l of PHA production medium [Materials and methods (common for chapters), Table 3, medium 1) and 200 ml inoculum. Sucrose was used at 20 g/l. 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 through cascading effect automatically. Fermentation experiment was carried out at 30 ^{0}C in duplicate a) by using sucrose as main carbon source b) by addition of castor oil 2 g/l (in 10 ml water) as co carbon source to sucrose containing medium, after 18 h of fermentation. Castor (*Ricinous communis*) oil contained: Ricinoleic acid (85%), stearic acid (1%), oleic acid (6%), linoleic acid (5%), palmitic acid (1%) and linolenic acid (1%). Fermentation was carried out up to 48 h.

D.3.2.3. Estimation of biomass and quantification of PHA

The cells were harvested by centrifugation at 7000 rpm for 15 min, washed with distilled water and with hexane/petroleum ether to remove oil and dried at 80 ^{0}C in airflow drier to a constant weight.

The PHA content of the cells was initially estimated by gravimetric method by subjecting dry cells to hydrolysis using 5% sodium hypochlorite [Materials and methods (common for chapters), section M.6.2).

D.3.2.4. Gamma irradiation of *B. flexus* cells

Method of cell disruption and subsequent extraction of PHA is crucial for retention of certain physical and chemical properties of the polymer. Gamma irradiation of wet biomass was carried out to assess the extractability of the intracellular PHA and to improve the polymer property, simultaneously. The wet biomass of known dry weight that was obtained after centrifugation of the culture broth (5 g on dry weight basis, in 100

ml capacity beaker covered with aluminum foil) was exposed in duplicate to 5, 10, 20 and 40 kGy dosage of gamma irradiation (Gamma chamber 5000, BARC, India, with Cobalt 60; model 444 TBQ, 12000 kCi, irradiation volume 5000 cc, diameter of sample chamber 20.5 cm). The irradiated biomass was suspended in chloroform and homogenized. Clear chloroform layer was obtained after centrifugation and the polymer was precipitated from this using 4 volumes of methanol. PHA was collected and dried to a constant weight. As the unirradiated samples did not yield measurable quantities of PHA by this method, PHA was extracted from the control samples held in chloroform at 37 ^{0}C, 150 rpm, for 2h.

D.3.2.5. Scanning electron microscopy

SEM of cells harvested in duplicate and exposed to different dosages of gamma irradiation (10, 20 and 40 kGy) was carried out using LEO-435 VP scanning electron microscope [Materials and methods (common for chapters), section M.6.3). The scanned images were captured at a magnification of 1000 X at 15 kV.

D.3.2.6. Characterization of PHA

FTIR spectroscopy, Gas Chromatography and ^{1}H NMR spectroscopy and molecular weight determination of PHA samples were carried out as described earlier [Materials and methods (common for chapters), sections: M.6.4; M.6.6; M.6.9 and M.6.13].

D.3.2.7. Preparation and analysis of PHA films

Film was prepared by solvent casting technique in duplicate by using the polymer obtained after extraction of cells that were cultivated in sucrose, sucrose with castor oil as co substrate and gamma irradiated cells. PHA (1.5 g) was dissolved in chloroform (60 ml) and the solution was poured on to a balanced glass plate (12 x 12 cm). The solvent was allowed to evaporate at room temperature of 28-30 ^{0}C, and the film was peeled off, cut into ten strips of 1 x 10 cms. Thickness was measured by using micrometer, (Model 549E, testing machines inc. New York, USA) and the thickness was expressed in terms of gauge (100 gauge=25 μm).

Tensile strength measurement was carried out using 10 strips of films of known thickness in Universal Testing Machine (LX5, Lloyd, UK), at 50mm/min. Tensile strength was calculated as maximum load in N/cross sectional area in mm^2 and expressed in terms of MPa.

D.3.3. Results and discussion

D.3.3.1. Synthesis and isolation of PHA

B. flexus produced 45 % PHA of biomass on sucrose as the sole source of carbon (Fig. 3.20). PHA production was enhanced to 55% by addition of co carbon substrate such as castor oil to cultivation medium. P(HB), which is commonly produced in the bacterial cells, is brittle and the copolymers of P(HB) are known to possess better mechanical properties. Various co carbon substrates are used for the production of copolymers. Synthesis of copolymer in bacteria occurs when certain co substrate or precursor is present in the fermenting medium (Haywood *et al.,* 1991). In the presence of sucrose as carbon source *B. flexus* produced mainly P(HB). Castor oil was used as a co substrate to enhance growth and induce copolymer production. GC-MS analysis confirmed the presence of PHA copolymer in castor oil fed cells and the polymer contained P(HB) and hexanoate in 96:3 mol% of PHA (Fig. 3.21).

Retention of the polymer property depends both on the technique applied for cell disruption and subsequent method of extraction of PHA. To isolate the intracellular polymer, sodium hypochlorite is generally used for the hydrolysis of cell wall and chloroform for the recovery of the polymer. It is known that hypochlorite-purified samples undergo reaction with chlorine causing chlorination at the double bond of PHA. Additionally there is depolymerization and number average molecular weight decreases. If only chloroform is used for PHA isolation then the number average molecular weight and double bond content is retained and polymer quality is better as compared to those prepared by hypochlorite method (Dufresne *et al.,* 2001). Mechanical destruction of whole cells using French press also reduces molecular weight. Once the polymer is optimally released from the cells, then several other methods of isolation can be followed such as differential centrifugation, liquid-liquid extraction, solvent extraction and non-

solvent precipitation etc. (Brandl *et al.,* 1990). In the present study chloroform extraction was used as an example. Data in Table 3.11 shows that PHA recovery of 18-20% from whole unirradiated cells was achieved by high temperature solvent extraction for longer periods, whereas mere homogenization of irradiated cells (10kGy) with chloroform resulted in maximum isolation of the polymer (54% of biomass weight). This was due to cell damage that occurred during irradiation (Fig. 3.22). Exposure of cells to higher dosages of irradiation did not result in additional yields. However it was observed that after 5-kGy of gamma-exposure, PHA extractability from the cells cultivated on sucrose and sucrose with castor oil was 25 and 27% of biomass weight, respectively.

D.3.3.2. Characterization and properties of PHA isolated from gamma irradiated cells

Gamma radiation processes have several advantages compared to chemical methods for improvement in properties of various materials. Absorption of radiation energy leads to free radical initiation, which can be controlled by dose rate of radiation (Bhattacharya, 2000). The physical state of the polymer appears to be important for cross-linking by irradiation. It has been shown that the formation of starch-based film is influenced by its physical state (Zhai *et al.,* 2003). Both cross linking and scission reactions are known to occur due to change in radiation dosage (Bhattacharya, 2000).

PHA isolated from gamma irradiated biomass was compared with control samples for characterization by FTIR and ^{1}H NMR (Figs. 3.23-3.25). FTIR spectrum of 10-40 kGy irradiated samples isolated from *B. flexus* cultivated on castor oil as co substrate showed a broad band at 3600-3200 Cm^{-1} the formation of which is reported to be due to oxidation products such as hydroperoxide and hydroxyl (OH^{-}) groups during irradiation. The weak bands at 1690-1650 Cm^{-1} corresponded to C=C stretching and this disappeared in irradiated sample which may be due to loss of double bonds in cross-linking process. This might have caused shift in the neighboring carbonyl band of ester C=O from 1723 Cm^{-1} to 1724 Cm^{-1} in irradiated PHA. Bands in the region 3015-2950 Cm^{-1}, those in 2945-2925 Cm^{-1} and 2880-2850 Cm^{-1} are assigned to CH_3 asymmetric stretching mode, CH_2 antisymmetry stretching mode and CH_3 symmetric stretching mode, respectively (Sato *et al.,* 2004a). The usual CH_3 asymmetric band appears in the region 2975-2950

Cm^{-1}. Recently it has been reported that appearance of CH_3 asymmetric band above 3000 Cm^{-1} indicates the presence of hydrogen bond (C-H···O) between CH_3 group and C=O group (Sato *et al*., 2004b). A band at 3004 Cm^{-1} in the irradiated PHA indicates the cross-linking between CH_3 group and C=O group.

^{1}HNMR spectrum of PHA isolated from sucrose medium, indicated signals characteristic of PHB, namely a doublet at 1.29 ppm, which is attributed to the methyl group, a doublet of quadruplet at 2.5 ppm due to methylene group and a multiplet at 5.28 ppm characteristic of methyne group (Fig. 3.24). Irradiation of PHA composed of only PHB did not show much improvement in increase in molecular weight or tensile strength of film. The polymer from unirradiated cells had a molecular weight of 1.5 x 10^5 and tensile strength of 18 MPa, which marginally increased on irradiation (10kGy) to 1.9 x 10^5 and 20 Mpa, respectively. ^{1}H NMR also did not indicate any change or shift in hydroxybutyrate resonance (Figs. 3.24. and 3.25). PHA copolymer obtained from castor oil medium showed resonance of hydroxybutyrate and hexanoate (Fig. 3.24). The resonance peaks due to side chain of hexanoate were more prominent in unirradiated sample (1.28-2.29 ppm) compared to irradiated sample, which indicate that the side chains are desirable if cross linking to be more effective. This is also substantiated by increase in molecular weight and a better tensile strength of film (2.3 x 10^5 and 35 MPa, respectively) compared to unirradiated sample (1.7 x 10^5 and 20 MPa, respectively). Molecular weight of PHA decreased at higher intensities of irradiation (40 kGy), which was due to the depolymerization of the PHA (Table 3.10). Polymer degradation may be due to the chain-scission reaction of irradiation. From the data it appears that there may be predominant cross linkage of the polymer at low doses and chain scission at higher doses. The polymerization and the degradation depend on the structure of the polymer (Chmeilewski *et al.,* 2005).

Table 3.11: Effect of gamma radiation on the extractability of polyhydroxyalkanoate from *B. flexus* cells and property of the film obtained from the polymer.

Sample	Radiation dosage (kGy)	PHA extracted (%)	Molecular weight (Daltons)	Tensile strength (MPa)
A	Control	18±0.5[a]	$1.5X10^5$±0.15	18.3±0.5
B		20±0.5 [a]	$1.7X10^5$±0.15	20.7±1.0
A	10	45±1.1[b]	$1.9X10^5$±0.25	20.2 ±0.7
B		54±1.5 [b]	$2.3X10^5$±1.70	35.2±0.8
A	20	45±1.1 [b]	$1.8X10^5$±0.25	17.7± 1.5
B		55±1.1 [b]	$1.9X10^5$±0.25	22.5±1.0
A	40	45±1.1 [b]	$1.4X10^5$±0.25	-
B		54±1.5 [b]	$1.4X10^5$±0.25	-

A=Cells cultivated with sucrose; B=Cells cultivated using sucrose and castor oil.
[a] % PHA of biomass (based on dry weight) extracted using chloroform at 37 ^{0}C for 2 h.
[b] % PHA of biomass (based on dry weight) extracted using homogenization of sample with chloroform at room temperature.

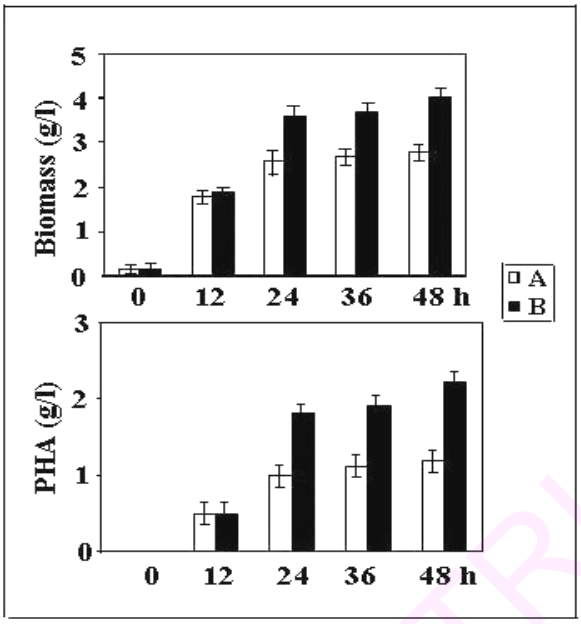


Figure 3.20: Fermentor cultivation of *B. flexus* using sucrose and castor oil as C-sources:A) Control: Bacterium cultivated in sucrose as main carbon source B) Bacterium cultivated in sucrose as main carbon source and castor oil (2 g/l) as co carbon substrate.

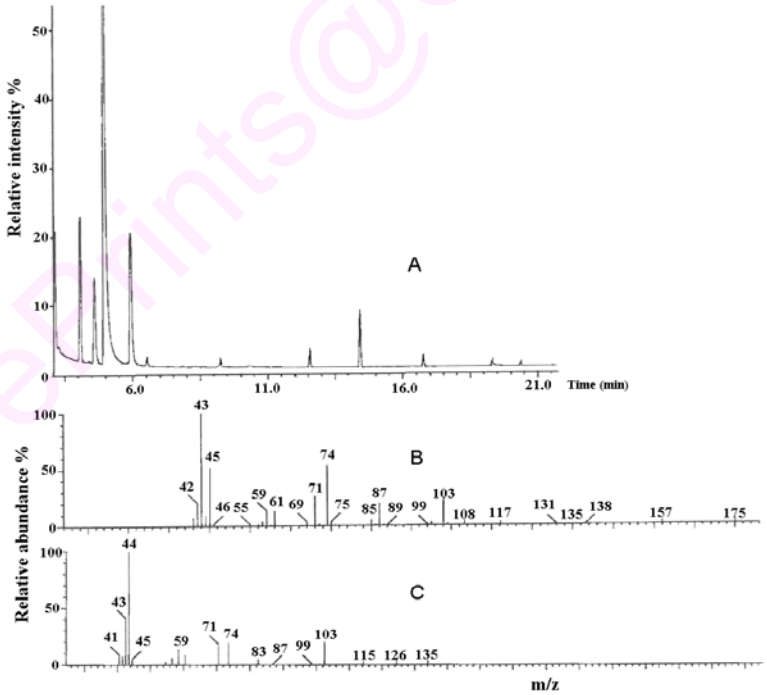


Figure 3.21: The total ion current chromatogram of the methanolysis product (A) of PHA obtained from cultivation of *B flexus* on castor oil as co substrate; Mass spectrum of methyl esters: B=3-hydroxybutyric acid; C= hexanoic acid.

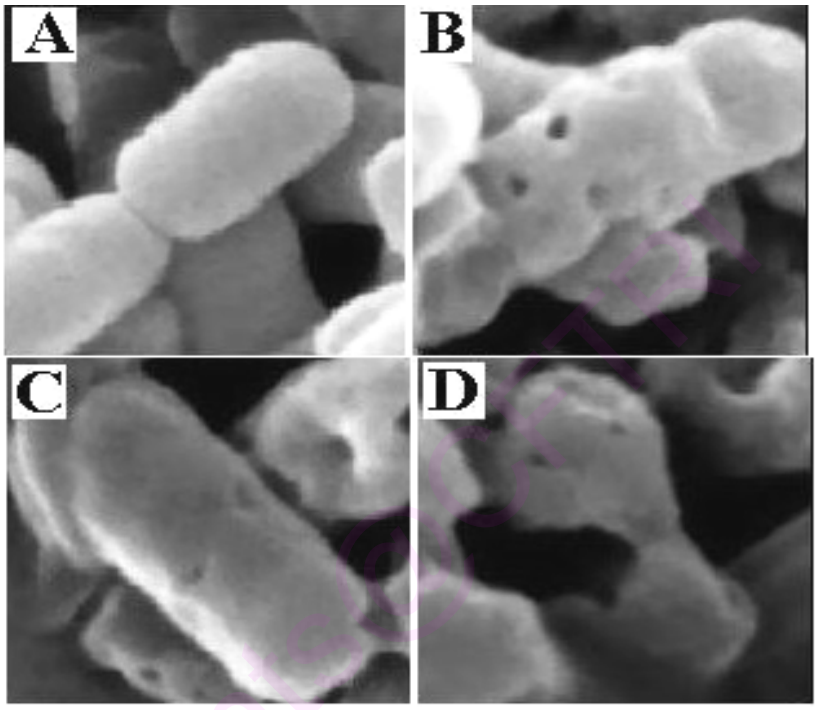


Figure 3.22: Scanning electron micrograph of *B. flexus* cells cultivated in sucrose as main carbon source and castor oil as co carbon substrate and exposed to different doses of gamma irradiation. Magnification 10.00Kx; A=Control; B=10kGy; C=20kGy; D=40 kGy.

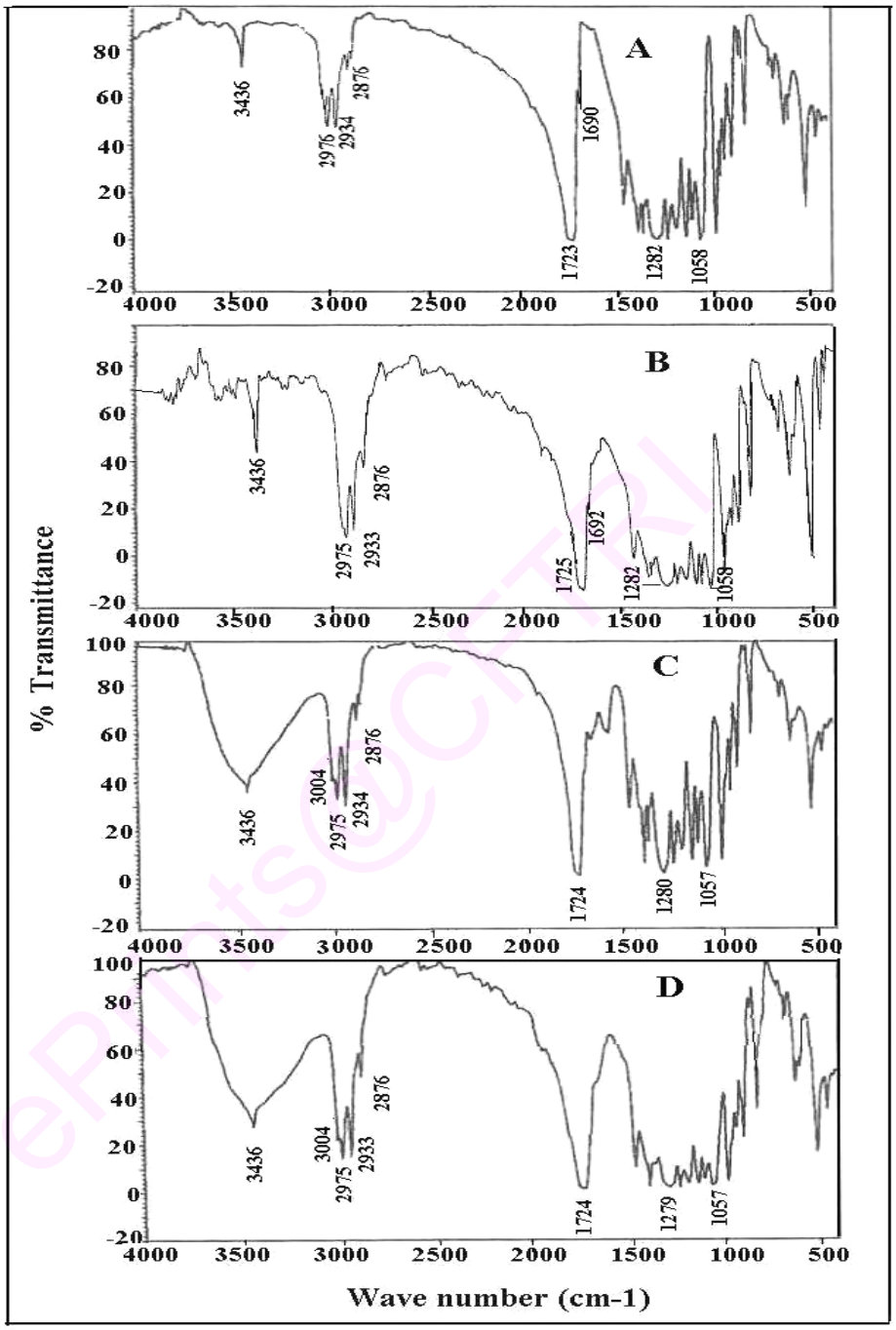


Figure 3.23: FTIR spectrum of PHA isolated from *B. flexus* cells:
A=Standard PHB; B=Standard P(HB-co-HV); C=PHA extracted from cells grown on castor oil as co carbon source and irradiated at 10 kGy; D=PHA extracted from cells grown on castor oil as co carbon source and irradiated at 40 kGy.

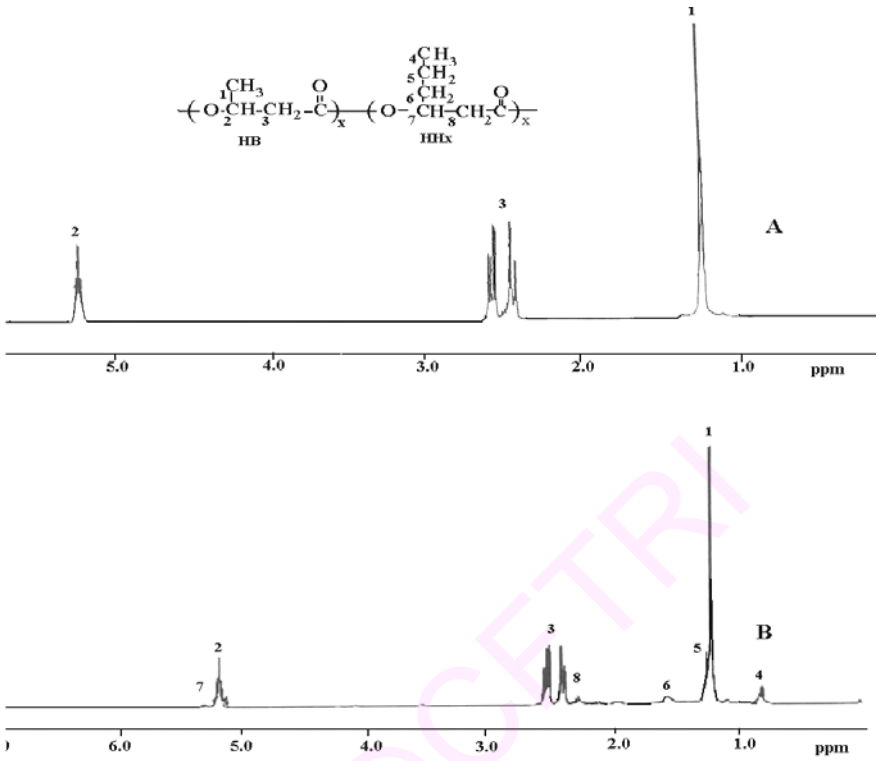


Figure 3.24: ^{1}H NMR of PHA extracted from *B. flexus* cells.
A) Standard-PHB (Control); B=PHA from cells grown on castor oil as co substrate.

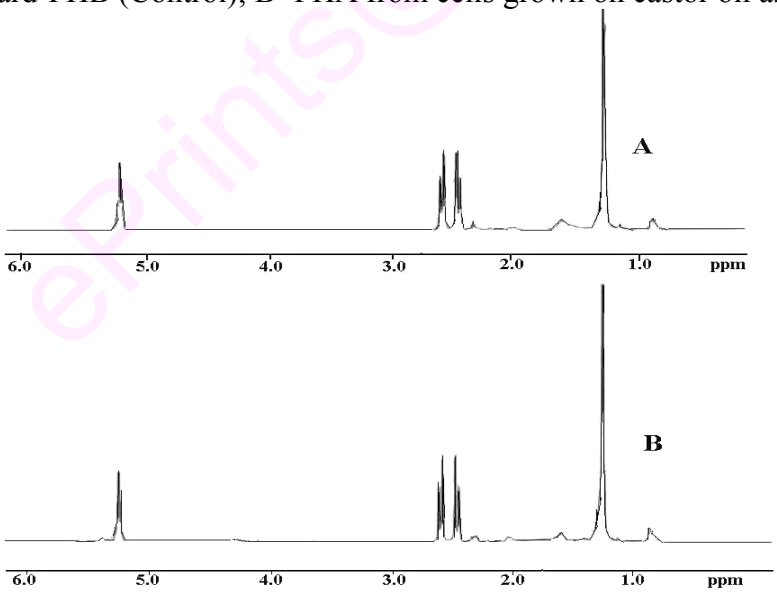


Figure 3.25: ^{1}H NMR of PHA extracted from *B. flexus* cells cultivated in castor oil as co substrate and exposed to different dosages of gamma irradiation: A=10 kGy; B=40 kGy.

Conclusions

Several methods have been applied to isolate PHA quantitatively from bacterial cells. It is observed that techniques used for cell disruption and subsequent polymer extraction is crucial for retention of physical and chemical properties. The advantage of gamma irradiation of PHA containing cells appears to be a) optimal disruption of cells at low dosage of irradiation b) easier extractability of the polymer c) improvement of property of the polymer in its nascent granular state d) retention of solvent solubility due to low degree of cross linking. Polymer quality of PHA composed of P(HB) was not much affected by irradiation. Although low degree of cross-linking occurred in PHA copolymer, there was an improvement in molecular weight and tensile strength of the polymer. Several types of PHAs are produced by a vast number of bacterial species and gamma irradiation at low dosage may be effectively utilized for cell disruption and improvement of property of the polymer, simultaneously.

Chapter 4

Cloning and expression of lytic gene in Bacillus flexus

4.1. Introduction

Since PHAs are stored within the cell, the development of an efficient recovery process including cell disruption is indispensable to reduce the cost of PHAs. A number of methods for the recovery of PHA have been developed (Lee, 1996). In most of these a solvent or hypochlorite is used for the extraction of PHA by digesting of the non-PHA cellular materials. An alternative process, which consists of thermal treatment of biomass, enzymatic digestion and washing with an anionic surfactant, has been developed (Holmes and Lim, 1990). However on an industrial scale these methods require large amounts of chemical reagents or enzymes.

Utilization of phage lysis genes to disrupt recombinant *Escherichia coli* cells that produce PHA has been reported (Fidler and Dennis, 1992). In this method, the ability of the bacteria to self-disrupt to release PHA without the addition of any solvents, hypochlorite and enzymes is conferred on *E coli* cells. Resch *et al.,* (1998) used the lytic gene of bacteriophage ϕX174 under the control of a thermo sensitive expression system. Hori *et al.,* (2002) have developed a self-disruptive strain of *Bacillus megaterium* by using lysis system of *Bacillus amyloliquefaciens* phage holin and endolysin that responds to substrate exhaustion.

Advances in genetic engineering has made possible to develop many techniques for genetic manipulation of *Bacillus,* which are more advanced compared to other bacteria including *E. coli*. *Bacillus* vectors have been designed to allow the recombinant genes to maintain in the host. This has not been possible in other *Bacillus* except *B. subtilis* because of transformability. A number of DNA repair systems have been detected and studied in *B. subtilis*. The ability to generate insertion mutants is one of the greatest technical strengths of *Bacillus*. *Bacillus* spp. are also important source for industrial enzymes, antibiotics, insecticide etc. They secrete several essential proteins into the medium for their easy growth, also their well proven safety have also made them prime candidates for the production of heterologous proteins.

B. subtilis as PHA production host has been reported earlier in which *B. subtilis* 1A304 was transformed with *B. megaterium* PHA biosynthesis genes (Law *et al.*, 2003). The recombinant *B. subtilis* grown on malt wastes produced 5% PHA in the cells.

In the present study, in order to establish a novel recovery system for PHA, a self-disruptive *B. flexus* strain was developed by introducing a gene cassette carrying the lytic system of lambda phage. The ability of the recombinant strain to accumulate PHA and to release the PHA granule to extra cellular environment after lysing the cell wall is investigated.

4.2. Materials and methods

4.2.1 Microorganisms and plasmids

Different *Bacillus* spp. such as *B. flexus, B. cereus, B. subtilis, B. endophyticus, Bacillus* sp hp 67, 43, hp 45, 88, 5150 (all isolated from soil) and *E. coli* (DH5α) were grown and maintained on nutrient agar slants. Plasmid vectors used in this study such as pTZ57 R/T and pSG1154 were obtained from lab stocks.

4.2.2. DNA manipulation

All the DNA manipulation experiments were conducted by following standard protocols (Sambrook *et al.*, 1989).

4.2.3. Screening of lytic gene

Different oligonucleotide primers for lytic system were designed (Table 4.1.) based on the NCBI sequence data for different *Bacillus* strains and lambda phage. The genomic DNA isolated from different *Bacillus* strains and λ-DNA were subjected to PCR using specific primers. The lytic gene was amplified from λ-Phage DNA under the PCR conditions 94 ^{0}C/1', 50 ^{0}C/1', 72 ^{0}C/1 for 35 cycles and final extension at 72 ^{0}C /10'. The PCR amplicon was confirmed by agarose gel electrophoresis using standard markers. The authenticity of the PCR product was also confirmed by carrying out nested PCR with internal primers and partial sequencing of the PCR product.

4.2.4. Cloning of λ-phage lytic gene

The amplicon obtained by the PCR reaction was purified using PCR clean-up kit (Sigma Aldrich, USA). The purified DNA was ligated with pTZ57 R/T sequencing vector (MBI fermentas). The components of the ligation mixture are as follows:

Purified DNA insert:	10µl
PTZ57R/T:	1 µl
10X ligation buffer:	2 µl
PEG 8000:	2 µl
T4 DNA ligase:	0.5 µl
Sterile water:	4.5 µl
Total:	20 µl

The ligation mixture was incubated overnight at 22 ^{0}C and was used to transform competent cells of *E. coli* DH5α. The transformants were screened on the basis of ampicillin resistance. Plasmid isolation from the transformed cells was carried out by alkali lysis method (Birnboim and Doly, 1979) and recombinant plasmids were screened on the basis of their movements in an agarose gel. The recombinant plasmid was named pTZλL.

4.2.5. Cloning of lytic gene into pSG1154 vector

For cloning and expression of lytic genes in *B. flexus*, the lytic genes from pTZλL plasmid were sub cloned into pSG1154 vector. A double digestion reaction with two restriction enzymes was performed, by mixing the following components:

pTZλL	10µl
TY/tango 10x buffer	3µl
*Eco*RI	1µl
Kpn I	1µl
Sterile water	15µl
Total	30µl

The mixture was incubated at 37 ^{0}C overnight. Heating at 60 ^{0}C for 30 min inactivated the enzymes. The digested fragments of pTZλL clone were separated on an agarose gel and the released insert was excised from the agarose gel and the DNA fragment was purified by gel extraction kit (Quiagen). The purified lytic gene was ligated with double digested pSG1154 vector (*Eco*RI-*Kpn* I) over night at 22 ^{0}C. The ligation mixture was later used to transform the competent cells of *E. coli* DH5α. The recombinant plasmids were screened based on their movements on agarose gel along with control plasmid. The recombinant plasmid was named as pSGλL

4.2.6. Transformation of *B. flexus*

B. flexus was transformed by standard protocol developed for *B. cereus* as follows:

4.2.6.1. *B. flexus* electro competent cells preparation (Mod. Silo-suh *et al.*, 1998)

- A colony of *B. flexus* was inoculated in to 10 ml of sterile Luria Bertani (LB, Himedia, Mumbai, India) medium contained in a 125 ml conical flask; the flask was incubated overnight at 30 ^{0}C at 250 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 30 ^{0}C at 250 rpm.
- The growth of the culture was monitored until it reached 0.3 OD_{600} (cell density of 1x 10^7 CFU / ml).
- The culture was chilled in ice for 10 min and 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 rpm).
- The supernatant was discarded and the pellet was suspended in 10 ml sterile ice-cold electroporation (EP) buffer and cells were pelletted by centrifugation.
- The supernatant was discarded and the pellet was suspended in 5 ml sterile ice-cold EP buffer, centrifuged and pellet was resuspended in 0.5 ml of EP buffer and used immediately.

4.2.6.2. Electroporation

4.2.6.2.1. 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 up to 200 ml (with milli Q water), filter sterilized and stored at 4 ^{0}C.

4.2.6.2.2. Protocol

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

a) Plasmid DNA was pipetted (0.05-1µg in p to 10µl in water or TE buffer) in to a sterile 1.5 ml micro centrifuge tube. The tubes were placed on ice and 100µl of electro-competent cells were added to 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) 2 ml LB broth was taken in a 17x100 mm 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 17x100 mm tube.

f) The pulse parameters were checked and recorded. The time constant was 8.6$^{m\ sec.}$ and the voltage was 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 spectinomycin (50µg/ml) and incubated overnight at 30 ^{0}C.

4.2.6.3. Isolation of plasmids

Alkali lysis method was carried out to isolate plasmids from overnight culture of transformed *B. subtilis* cells (Birnboim and Doly, 1979) with minor modifications (To the solution-I, 30 μg/ml lysozyme powder was added and the mixture was incubated at 37 ^{0}C for 30 min).

4.2.6.3.1. Integration of lytic gene into *B. flexus* genome

After a few generations of growth, the integration of the lytic gene into the amylase locus (*amyE*) of *B. flexus* genome was confirmed by checking the inability of the strain to degrade starch optimally. The strain was cultivated [Materials and methods (common for chapters), sections M.5.4. – M.5.6.) in 50 ml sterile PHA production medium [Materials and methods (common for chapters), Table 3, production medium 1), with starch as carbon source (20 g/l), for 72 h at 30 ^{0}C and 250 rpm. Wild strain was used as control. Culture broth was centrifuged (3000 g for 10 min), biomass was washed and dried to a constant weight at 70 ^{0}C.

4.2.6.3.2. Characterization of the lytic gene and gene product

The lytic gene sequence of lambda phage was analyzed using Clone Manager software and the restriction map of the gene was constructed. The deduced amino acid sequence of the gene was analyzed by Expasy proteomic tool to elucidate the three-dimensional structure of the lytic protein by SWISS-PROT protein modeling tool (swissmodel.expasy.org).

4.2.6.4. Expression of lytic gene and PHA production

Wild type cells and the transformants were grown in PHA production medium [Materials and methods (common for chapters), Table 3] at 30 ^{0}C and 250 rpm. Xylose was added as inducer at a concentration of 10 g/l after 48 h of growth. After 72 h of growth, culture broth was centrifuged, pellet was washed with acetone and suspended in chloroform, chloroform layer was separated and dried at 60 ^{0}C. The sediment was resuspended in chloroform and extraction was carried out at 45 ^{0}C for 2 h. Clear chloroform layer was collected and dried to recover PHA that was not released earlier in

to the culture medium. Cells were checked for cell-lysis by microscopy and by estimation of PHA released into the medium.

4.2.6.5. Optimization of PHA production by the recombinant *B. flexus* transformants and cell disruption

Recombinant *B. flexus* were optimized for PHA production by varying sucrose concentration (0.5, 1.0 %) in the media. Different levels of xylose (0.5 and 1.0 %) were added to the culture medium initially to optimize the minimum amount of xylose required for inducing the cloned genes. Other experimental details are as mentioned under 4.2.4.5 section above.

Table 4.1: Various oligonucleotide primers for lytic gene.

Primer name	Primer sequence
Hol 1 F	5’ **GGTACCTAACAAGGG**GTAATGAMMACRTTYGACAGCGCAT 3’
Hol 1 R	5’ TCATTTCGTTAACCCTTTTTGCTTAAGATTAAT 3’
DSB 1F	5’ **GGTACCTAAAGGAGG**ATGTTTGAATTCTTTCAT 3’
DSB 1R	5’ TCAGTCATCTTTTTTATCT**GAATTC** 3’
DSB 2F	5’ **GGTACCTAAAGGAGG**ATGGATCGTATTGATGTRTT 3’
DSB 2R	5’ TTAYWSAACATCTCCYT**GAATTC** 3’
DS λ F	5’ **GGTACCTAAAGGAGG**ATGAAGATGCCAGAAAAACA
DS λ R	5’TCATACATCAATCTCTCTCTCG 3’
	5’ CTATCTGCACTGCTCAT**GAA TTC** 3’

Significance of the colours used:

Restriction site KpnI

Stop codon

Ribosome Binding Site

Restriction site EcoRI

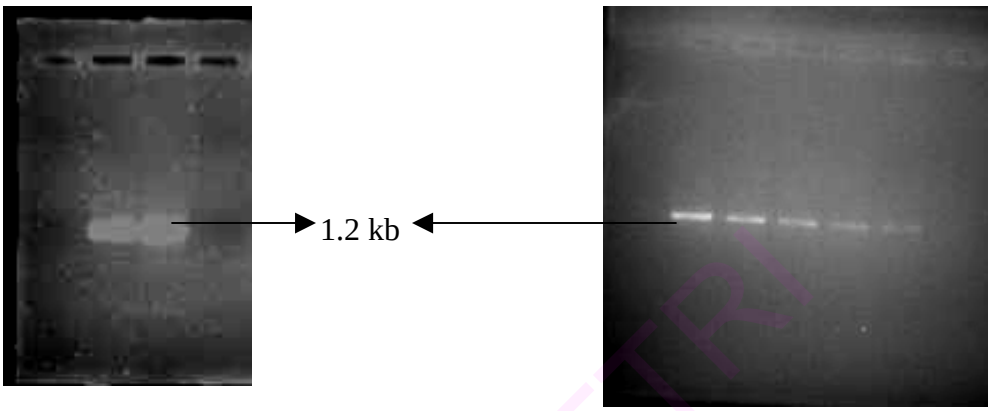
4.3. Results and discussion

4.3.1. Screening of lytic gene

Chromosomal DNA of different *Bacillus* spp. was extracted and was screened for lytic gene using different primers. Amplified fragment of 1.2 kb DNA was obtained from λ-phage DNA (Fig. 4.1.A). The PCR conditions were optimized in gradient PCR at different temperatures. The gradient amplification of lytic gene showed maximum yield of PCR product at 50 ^{0}C. The yield of PCR product reduced gradually when the annealing temperature was increased to 54 ^{0}C (Fig. 4.1.B). The PCR amplicon was confirmed by nested PCR using internal primers. A fragment of 1.2 kb DNA was observed on agarose gel, which was of expected size with reference to the standard DNA marker (Fig. 4.1.C). The authenticity of the PCR fragment was confirmed by nested PCR (Fig. 4.1.C) and by sequencing the PCR product. The sequence (Fig. 4.2) was compared with gene bank sequences by using various online tools like BLAST, Dialign and Clone manager (Fig. 4.5, Table 4.2).

4.3.2. Cloning of lytic gene into *Bacillus* expression vector pSG 1154

The purified lytic gene was cloned into pSG1154 (7.6 kb, Fig. 4.3), which is designed to clone the desired gene fragment under the control of a xylose-inducible promoter and also to integrate the cloned gene into the *Bacillus amyE* locus. The lytic gene of λ-phage was released from pTZλL clone and was sub cloned into pSG1154 vector. The recombinant plasmid was of expected size of 8.8 kb and which migrated slowly on an agarose gel when compared with the control plasmid pSG1154 containing no insert (Fig. 4.4). The recombinant plasmid was named as pSG1154λL and was checked further for the presence of lytic gene by restriction digestion and PCR. The restriction digestion of the recombinant plasmid resulted in the release of ~ 1.3 kb DNA insert. PCR using the pSGλL clone as a template DNA showed the amplification of lytic genes with expected size of 1.2 kb.



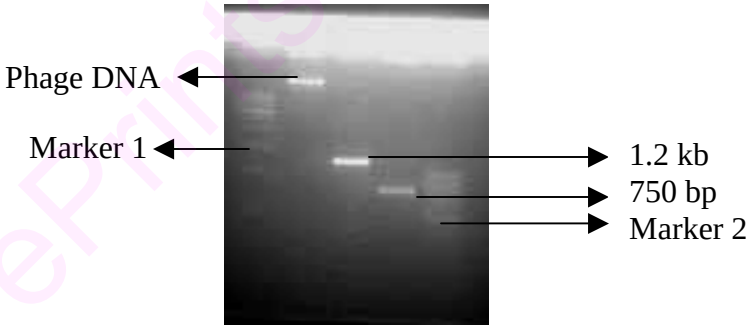
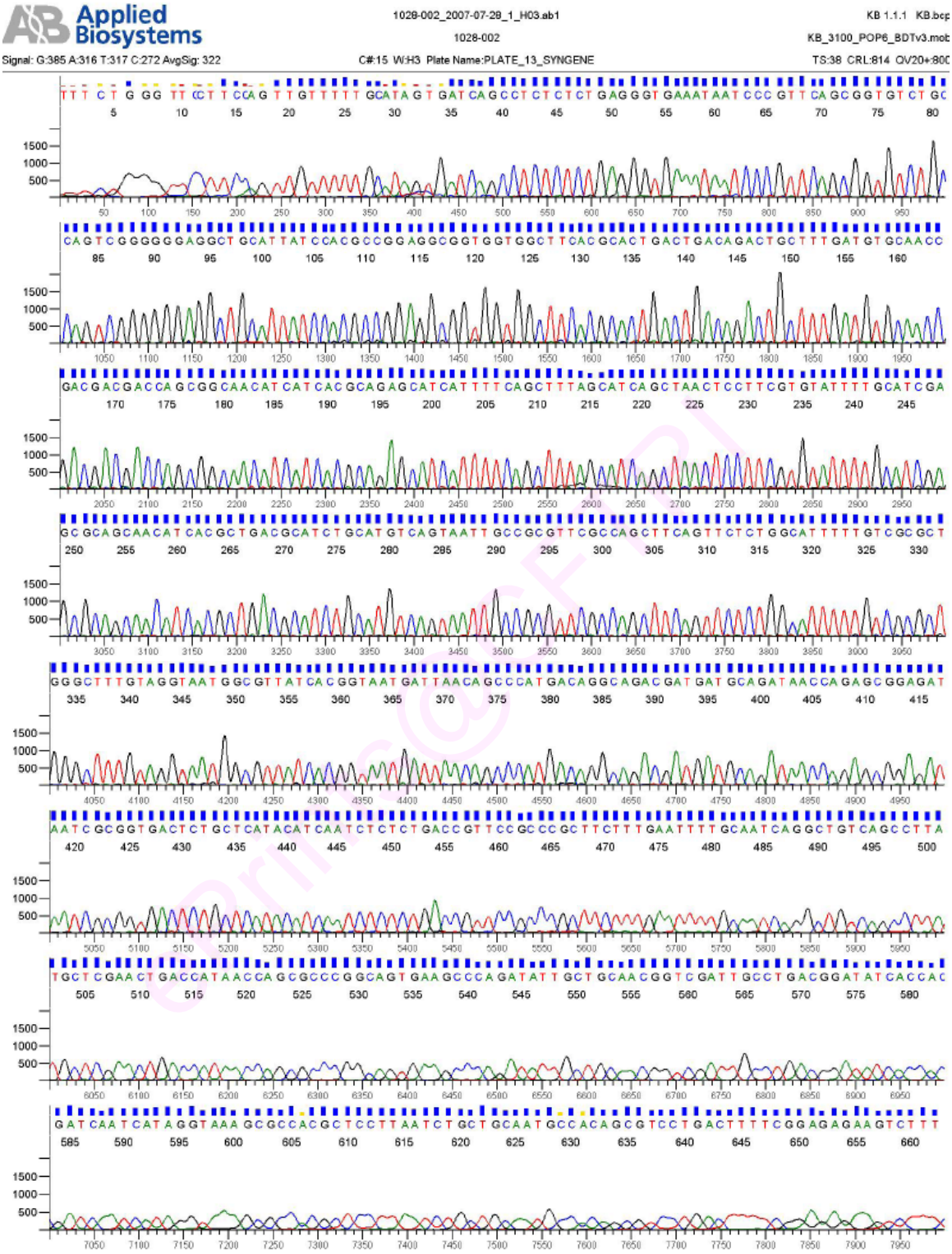


Figure 4.1: Screening and optimization PCR conditions for lytic gene. A–Amplified lytic gene from λ-phage DNA; B – Optimization of PCR conditions in gradient PCR; C - Gel photograph showing marker 1(8416, 6557, 4361, 2322, 2027, 1500,1000, 564, 300 bp), Phage DNA, complete lytic gene amplified (1.2 kb), lytic gene fragment obtained by nested primer and Marker 2 (1000 - 100 bp).



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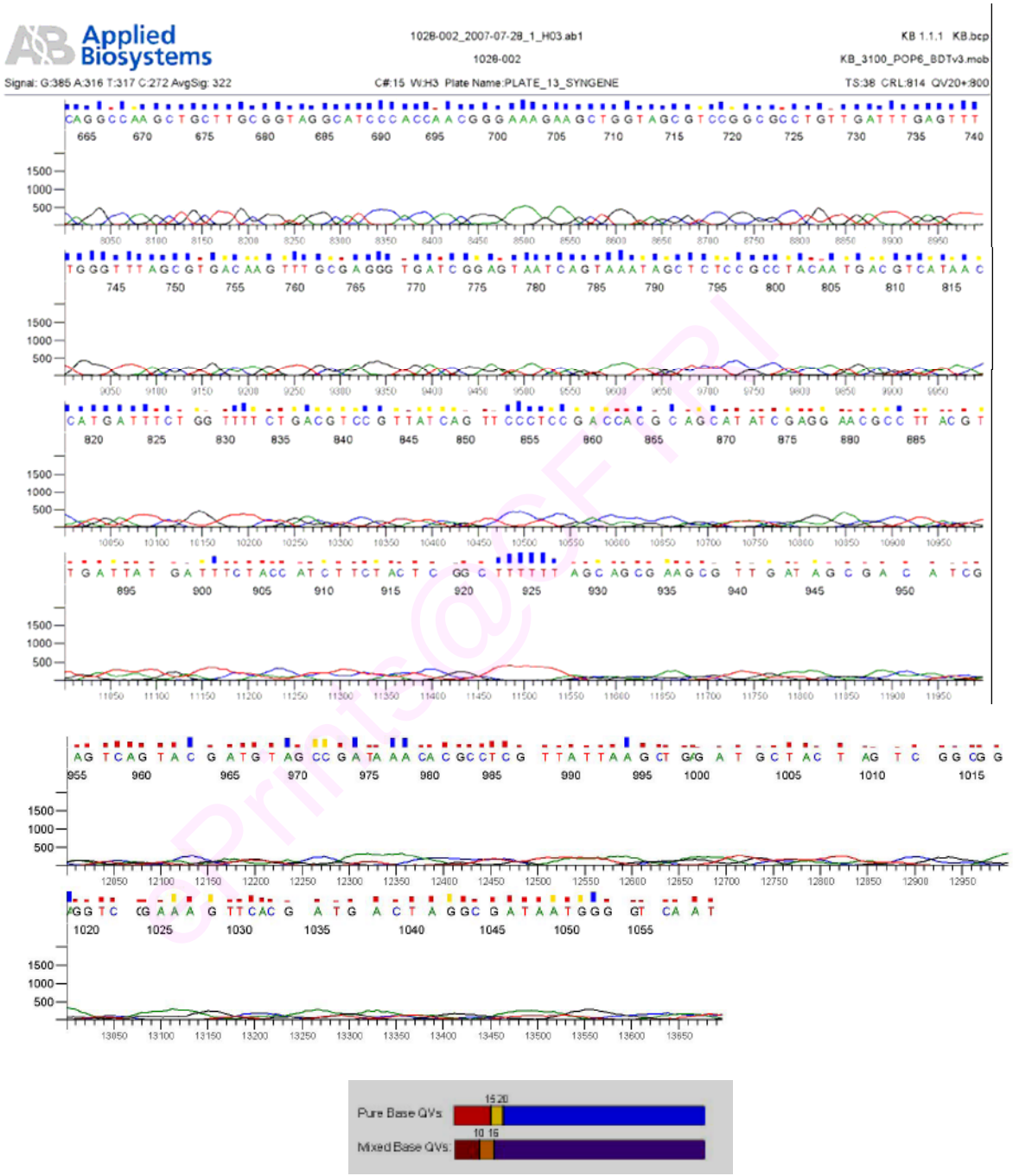

Figure 4.2: Electropherogram of the lytic gene sequence.

Table 4. 2: BLAST results of lytic gene isolated from lamda phage DNA.

NCBI	BLAST results		
Accession	**Description**	**Max score**	**Max ident**
U39286.1	Cloning vector TLF97-3, phage lambda lacZ translational fusion vector, complete sequence	1037	87%
U39285.1	Cloning vector TLF97-2, phage lambda lacZ translational fusion vector, complete sequence	1037	87%
U39284.1	Cloning vector TLF97-1, lambda phage lacZ translational fusion vector, complete sequence	1037	87%
U37692.1	Cloning vector lambda TXF97, lacZ transcriptional fusion vector, complete sequence	1037	87%
J02459.1	Enterobacteria phage lambda, complete genome	1031	87%
U02427.1	Cloning vector lambda EMBL3 SP6/T7, right arm	1031	87%
U02453.1	Cloning vector lambda EMBL3, right arm	1031	87%
AK157373.1	Mus musculus activated spleen cDNA, RIKEN full-length enriched library, clone:F830212M06 product:Antitermination protein Q homolog [Bacteriophage lambda], full insert sequence	1013	86%
AE017220.1	Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67, complete genome	931	87%
CP000711.1	Enterobacteria phage CUS-3, complete genome	917	88%
CP000468.1	Escherichia coli APEC O1, complete genome	917	88%
CP000243.1	Escherichia coli UTI89, complete genome	917	88%
AF069529.1	Bacteriophage HK97, complete genome	909	87%
AF069308.1	Bacteriophage HK022, complete genome	905	88%

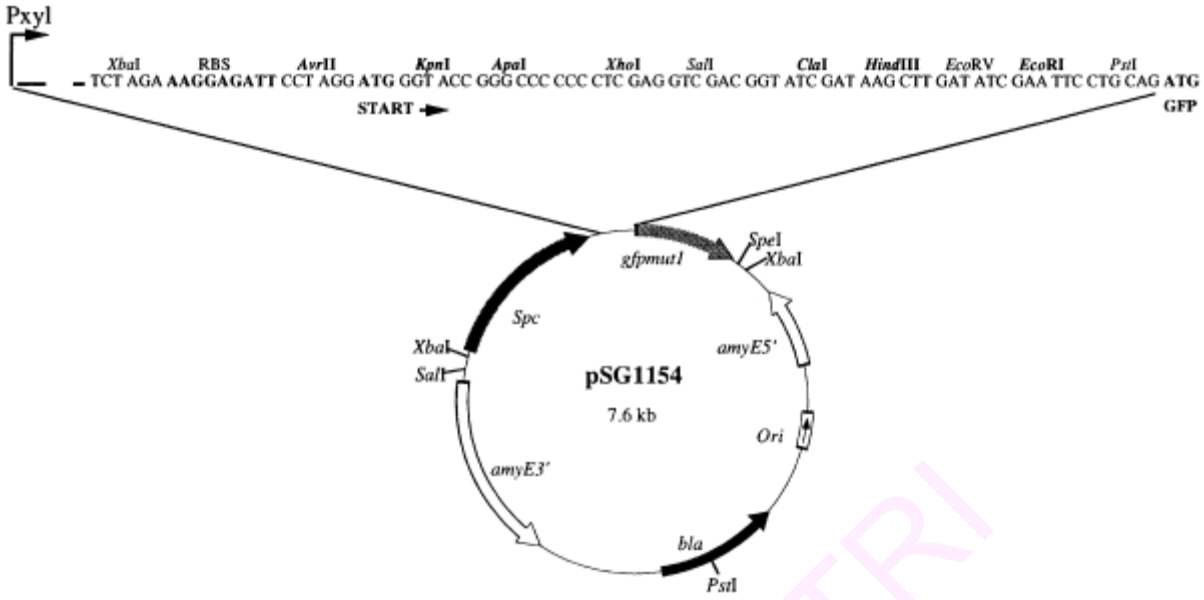

Figure 4.3: Map of pSG1154 vector

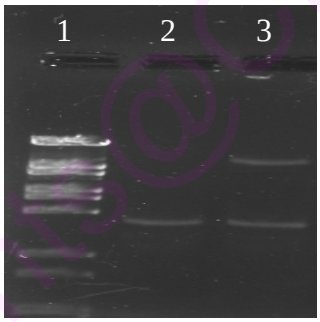

Figure 4.4.A: Gel photograph of the restriction digestion of pTZλl. 1 – Marker, 2 – Lytic gene (1.2 kb), 3 – Restriction digestion of pTZλl.

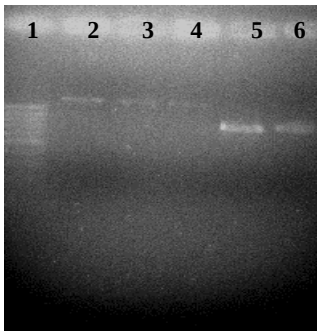

Figure 4.4.B: Gel photograph of plasmid containing lytic gene. 1 – Marker (8416, 6557, 4361, 2322, 2027, 1500, 1000, 564, 300 bp), 2, 3, 4-Recombinant plasmid pSG1154λl (8.8kb), 5-Self ligated plasmid, 6-Control plasmid without insert (7.6kb).

4.3.3. Transformation of *B. flexus* and integration of the lytic gene

The transformants were selected on the basis of spectinomycin resistance. The recombinant strain could not degrade starch optimally which suggested that the lytic gene had successfully integrated into the *B. flexus* genome. The degradation of the starch by the wild cells was very prominent, where as the recombinant was not efficient in the degradation (Fig. 4.5). The analysis of the *Bacillus* genome revealed that there are sequences homologous to amylase gene in the *Bacillus* genome. The reduced amylase activity of the recombinant strain may be due to disruption of target gene (*amyE*) and induction of less active homologous gene sequences present in the *Bacillus* genome.

In the present study in order to clone and express lambda lytic genes in *B. flexes* we have used a *Bacillus* integration vector pSG1154. The vector cannot replicate in a *Bacillus* and it is designed to specifically to integrate the cloned DNA into the amylase locus (*amyE*) present in the chromosomal DNA. The cloned DNA can undergo single or double crossover and integrate in to the *Bacillus* genome. Single cross over can result in the integration of whole plasmid into the *Bacillus* genome (Fig. 4.6). Inclusion of controllable promoter into the upstream of the target gene on the vector allows controllable gene expression of the cloned gene and also other genes present in the downstream in the same operon. In double cross over event the vector integrate in the genome via homologous recombination with the *amyE* fragments flanking either sides of the cloned gene. The recombination can result in strand exchange and the cloned gene can mobilize into the genomic DNA along with a spectinomycin resistant gene marker (Fig. 4.7). The ability of the recombinant to grow in the medium containing ampicillin proved that the integration of lytic gene has taken place through single crossover, in which entire plasmid will integrate in the target gene, which includes ampicillin resistant gene.

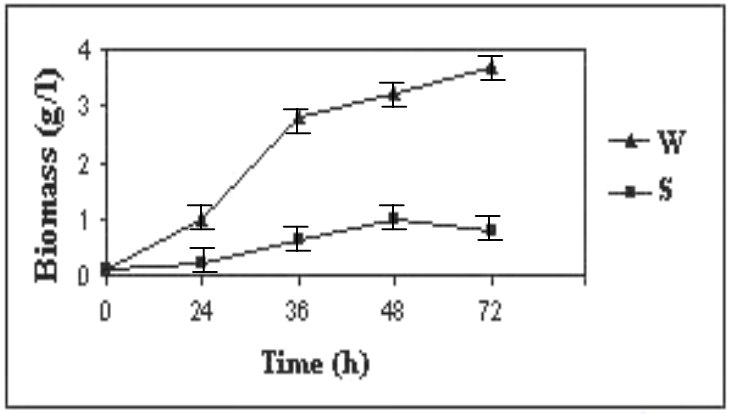

Figure 4.5: Growth of *B. flexus* in starch as carbon source:
W- Wild strain; S – Recombinant strain.

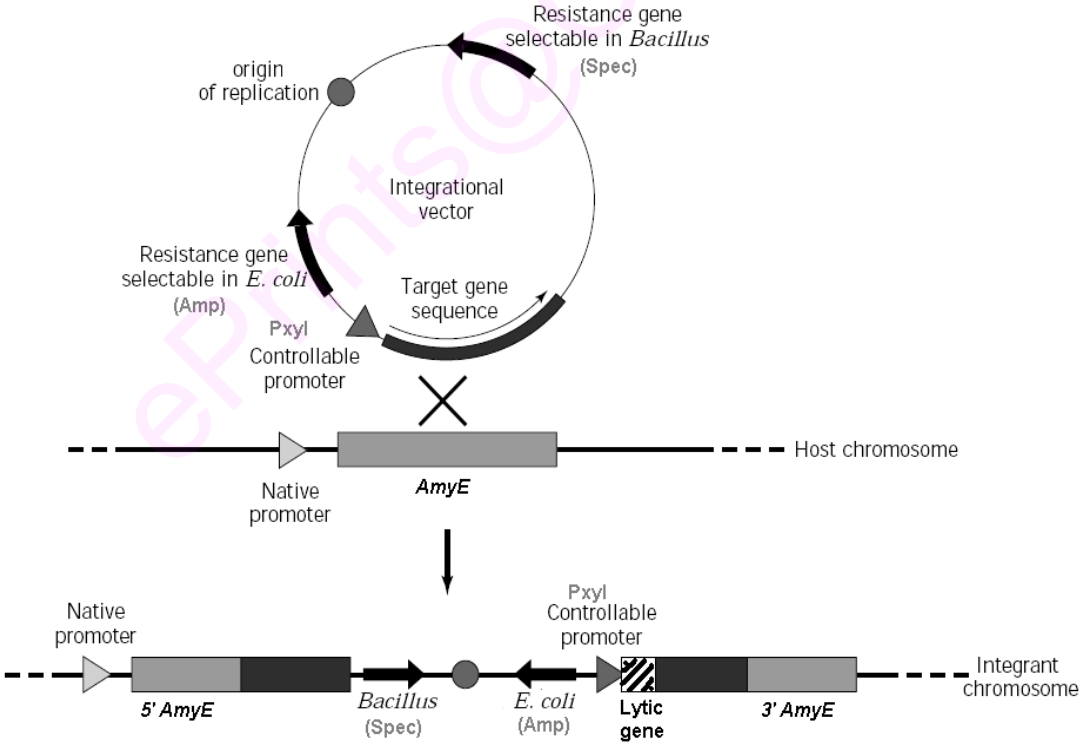

Figure 4.6: Single crossover recombination showing integration of whole vector into the host chromosome at the site of *amyE*.

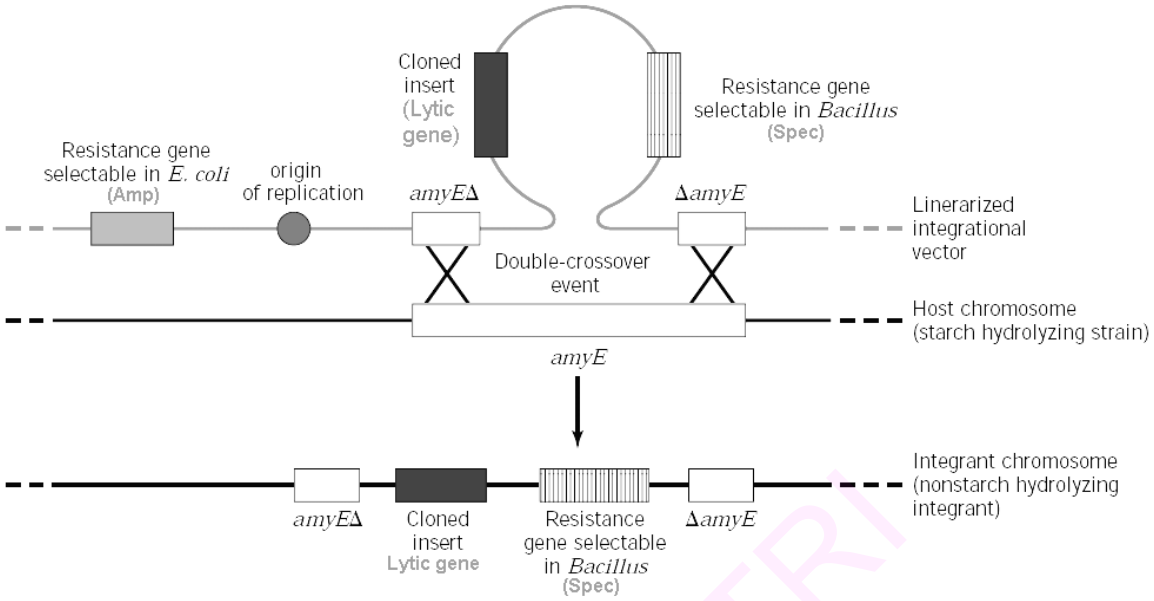

Figure 4.7: Double crossover recombination and integration of the gene of interest into 5' *amyE* and 3' *amyE* encoding α-amylase.

4.3.4. Characterization of the lytic gene and gene product

The lytic gene sequence of lambda phage was analyzed using Clone Manager software and the restriction map of the gene was constructed. The lytic gene sequence of lambda phage showed the presence three open reading frames (ORFs) and 17 restriction sites of various enzymes. The ORF3 contained two restriction sites and a maximum of 9 restrictions sites were found in ORF2 region (Fig. 4. 8).

The deduced amino acid sequence of the gene was analyzed by Expasy proteomic tool and which was used to elucidate the three-dimensional structure of the lytic protein by SWISS-PROT protein modeling tool (swissmodel.expasy.org). The amino acid sequence showed 100% homology with the bacteriophage lambda lysozyme complexed with a chitohexasacharide, X-Ray Resolution 2.60. The model residue range was from 106-259 (Fig. 4.9). The highest homology was found with 1d9ub.pdb (Protein data bank). The ribbon model showed that the polypeptide could fold into five alpha helices and two plated sheets. In the ball and stick model the amino acids in the polypeptide chain was arranged in such way that the hydrophobic amino acids were arranged into the core structure of the protein while the hydrophilic ones were exposed to the outer surface.

4.3.5. Expression of lytic gene in *B. flexus* and lysis pattern of the strain

The cloned lytic gene was under the control of a xylose inducible promoter. The expression of the lytic gene was induced by adding xylose at exponential and stationary phases of growth. It was observed that the growth of the B. *flexus* was affected after the addition of certain level of xylose concentration in the medium. A decrease in the OD_{600} was observed after the addition of xylose, where wild cells were normal even after the addition of xylose. This confirmed that the lytic system of the λ-phage was functional in *B. flexus*. It was clearly shown that the lysis could be induced after PHA accumulation in stationary phase (Fig. 4.7). This was confirmed by PHA quantification (Table 4.3).

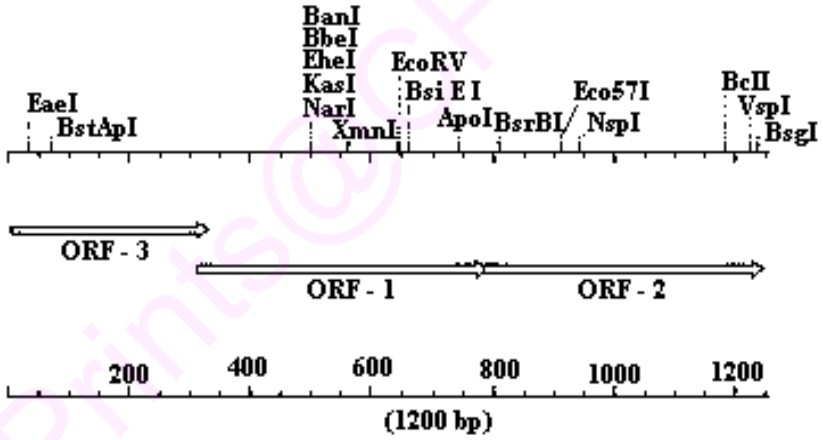

Figure 4.8: Restriction map of λ-phage lytic gene.

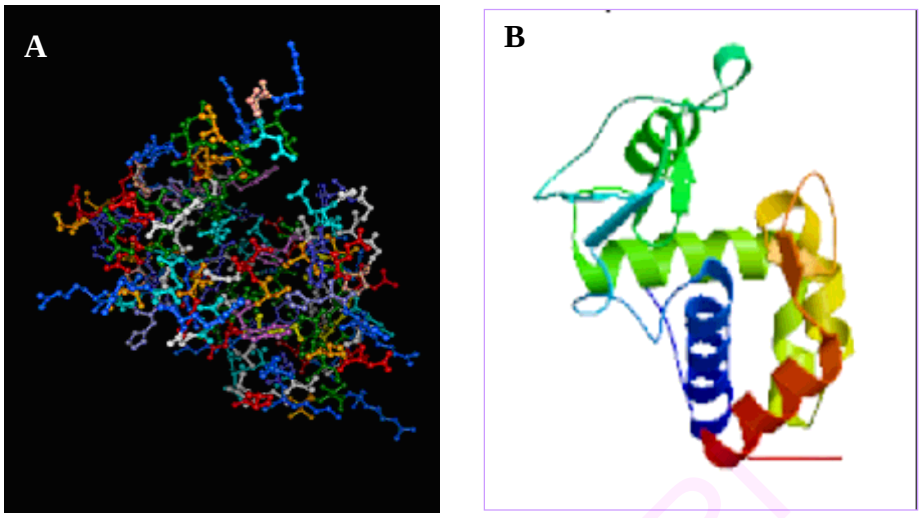

Figure 4.9: 3D model of the lytic enzyme of lambda phage.
A=Ball and stick model; B=Ribbon model.

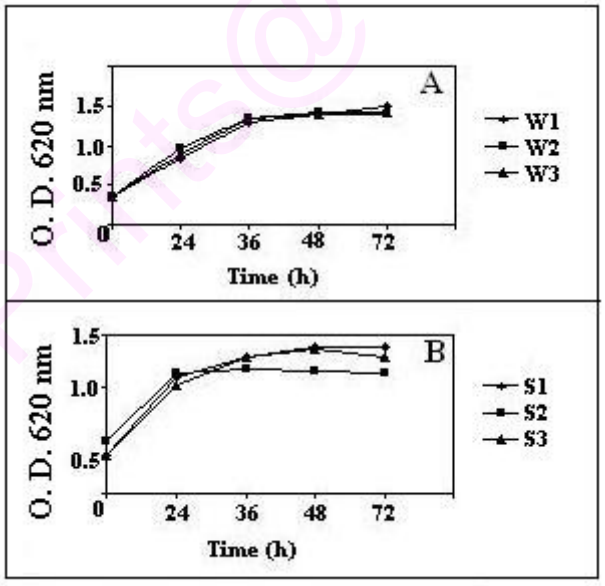

Figure 4.10: Growth of bacterium in the presence of xylose as inducer.
A: Wild strain, W1= Control; W2 = xylose supplemented at 24 h; W3= xylose supplemented at 48 h. B: Recombinant strain, S1=Control; S2=xylose supplemented at 24 h; S3=xylose supplemented at 48 h.

Table 4.3: PHA production by self-disrupting recombinant *B. flexus* cells cultivated for 72h.

B. flexus strain	Biomass (g/l)	PHA (g/l)		Total PHA (%, of dry biomass)
		A	B	
Wild strain (Control)	3.8 ± 0.98	0.40 ± 0.20	1.7 ± 0.14	45
Recombinant (Control)	3.6 ± 1.00	0.40 ± 0.02	1.4 ± 0.04	39
Wild strain [a]	3.9 ± 0.90	0.40 ± 0.11	1.8 ± 0.42	46
Recombinant [a]	2.6 ± 0.81	0.20 ± 0.07	0.4 ± 0.20	15
Wild strain [b]	3.4 ± 0.87	0.30 ± 0.05	1.5 ± 0.14	44
Recombinant [b]	3.6 ± 1.21	0.77 ± 0.10	1.1± 0.07	31

Supplementation of xylose during growth: [a] 24 h; [b] 48 h.
Isolation of PHA: A, PHA extracted extracellularly from autolysed cells;
B, Total PHA concentration (Extracellular and intracellular).

4.3.6. Recovery of PHA by self-disruption

PHA released after cell disruption of recombinant *B. flexus* was confirmed by gravimetric quantification. The cells were cultivated for 72 h in PHA production medium supplemented with 20 g/l of sucrose. Lysis was induced by the addition of 10 g/l of xylose into the medium after 24 and 48 h of incubation (Table 4.3). Xylose addition did not affect biomass production in the wild strain. In recombinant strain, supplementation of xylose at initial stages of growth (24 h) resulted in decrease in biomass and PHA concentration of 2.6 g/l and 0.4 g/l (15% of biomass), respectively. Decreased growth was due to initiation of autolysis in the initial growth phase. Corresponding yields obtained after 48 h of supplementation was 3.6 g/l and 1.1 g/l (31% of biomass). Highest concentration of PHA (70% of total PHA) was recovered as extra cellular component from this culture compared to control wild strain (20% PHA).

It is very important to optimize the harvesting time of PHA, prior to which phage lytic system could be induced. Resch *et al.,* (1998) used lytic gene of bacteriophage ϕX174 under the control of thermo sensitive expression system, $MgSO_4$ was used as an

inhibitor, in which before lysis induction cells were harvested and resuspended in water to remove magnesium sulphate. Hori *et al*., (2002) proposed the self-disruption of *B. megaterium* in response to substrate exhaustion for PHA release. Glucose (20 g/l) and xylose (8 g/l) were added together in the medium where glucose acted as an inhibitor for the cell lysis and after complete utilization of glucose, xylose present in the medium induced lysis of the cells. This may cause the cell lysis even before the accumulation of PHA or presence of glucose may inhibit the induction of lysis.

Conclusion

In the present study, in order to establish a novel recovery system for PHA, a self-disruptive *B. flexus* strain was developed by introducing a gene cassette carrying the lytic system of lambda phage. Lambda phage lytic gene of 1.2 kb which is specific for Gram negative bacteria has been used for the first time to lyse the *B. flexus* cell successfully. The recombinant strain accumulated PHA and cell lysis was induced by supplementation of xylose after the accumulation of PHA. PHA granules were released to extra cellular environment after lysing the cell wall.

Summary and conclusions

Summary

Polyhydroxyalkanoate (PHA) is a complex class of storage polymer, which is composed of hydroxy fatty acids and synthesized by various bacteria and deposited as water insoluble intracellular organic inclusions. The granules are composed of polyester core which is surrounded by phospholipids and proteins. The key enzyme of polyester biosynthesis is the polyester synthases, which catalyzes the selective polymerization of (R)-hydroxyacyl-CoA thioesters to polyesters. Various metabolic pathways are involved in the PHA biosynthesis.

So far, biopolyester research has been carried out to understand the production of these biopolymers and to investigate the isolation, characterization of the polymer and its properties and to know its potential applications. Various microorganisms are capable of synthesizing a variety of PHAs that serve as reserve energy source upon carbon starvation. Currently more than 90 types of PHA have been identified. The type of PHA produced depends on the microorganism and the substrate utilized by the organism.

In the present work different *Bacillus* spp. were isolated from the local soil samples. The isolates were screened for PHA production by sudan black staining. Among 25 different *Bacillus* spp. tested for PHA production, *Bacillus* sp. isolate 9 produced highest amount of the polymer (62% of biomass) and hence it was selected for further studies.

The selected isolate was characterized by morphological, biochemical and molecular methods. The bacterium was gram positive, rod shaped and motile. Colonies were smooth, circular and off-white in colour. It gave positive reaction for catalase, urease, oxidase and indole tests. It could hydrolyze starch and gelatin. The results of morphological studies suggested that the isolated bacterium belonged to the genus *Bacillus.* The strain showed similarities with *B. flexus* because: it had ellipsoidal spore situated at terminal position, cells were motile, lacked anaerobic growth, Voges-Proskauer negative, oxidase positive, capable of hydrolyzing gelatin and starch; produced acid from lactose, arabinose, glucose, manitol, rhamnose, xylose and cellobiose. The bacterium was further examined by16SrRNA gene to determine its relationship at the genomic level. 16SrRNA gene was amplified from the genomic DNA and it was cloned and sequenced. The DNA sequence was found to be conserved and it was analysed using

various online softwares. The sequence was aligned with known *Bacillus* related 16S rRNA gene sequences. Sequence of 16SrRNA gene was similar to that of *Bacillus flexus* (99%). The literature data on phylogenetic tree indicated that *B. megaterium* is closer to *B. flexus* in the evolutionary position. Based on these factors the isolated culture has been tentatively identified as *B. flexus*. This is the first report which shows that *B. flexus* can synthesise optimum amounts of PHA.

In the present work, in order to obtain high yield and quality of PHA, various carbon and nitrogen sources were assessed as media supplements. Higher concentrations of biomass of 2.5, 2 and 1.95 g/l were obtained with ammonium acetate, ammonium sulphate and ammonium phosphate as N-sources, respectively. Corresponding yields of PHA obtained were: 2.2, 1.1 and 1.0 g/l. Utilization of acetate is activated by the enzyme acetate-CoA ligase (EC 6.2.1.1). Initially it was found to serve as a carbon source for growth, and when the nitrogen concentration in the medium was depleted then its utilization was channelised towards PHA synthesis. Due to this dual activity higher growth and PHA synthesis were obtained when ammonium acetate was used as a nitrogen source in the medium. Carbon source has profound influence on the PHA content of the cell. Optimal amount of PHA (1g/l; 50% of biomass weight) as well as biomass (2 g/l) were obtained in medium containing sucrose. *B. flexus* was also cultivated in media containing different plant oils, free fatty acids, economic media components such as palm oil effluent, molasses, corn starch, whey, rice and wheat bran extracts as sources of carbon/nitrogen/nutrients to enhance growth and PHA copolymer synthesis. At the end of cultivation period, cells were harvested; biomass and PHA were estimated and characterization of PHA was carried out by FTIR/GC/GC-MS/NMR spectroscopy/DSC. The bacterium was able to utilize various economic substrates optimally for growth and PHA production.

In a preliminary experiment it was observed that *B. flexus* produced PHA under N limitation conditions with excess carbon source. The optimal ratio of N:P:C for growth and PHA synthesis appeared to be 1:3.6:13. The growth kinetics of *B. flexus* under nutrient limitation was studied by using a simple model giving mathematical description of kinetics of microbial growth, substrate consumption and product formation. In this study, a modification of the logistic equation was also attempted for describing the

growth of the bacterium. The microbial growth was described by means of a simplification of Monod's model for low substrate concentration. A simplified Luedeking-Pirat type model represented the product kinetics. The kinetic constants were evaluated based on non-linear regression and the differential equations were solved by application of Runge-Kutta algorithm using MATLAB software. The model was able to predict microbial growth and PHA production and values obtained were comparable to experimental data.

Response surface methodology was used to optimize media components based on central composite rotatory design (CCRD). From the results it was observed that KH_2PO_4 enhanced PHA production and *B. flexus* growth was not inhibited even at 15-g/l concentration of this nutrient. Ammonium phosphate and sucrose enhanced growth and PHA production. Nitrogen concentration used in the experiment varied from 0.21 to 1.27 g/l, P from 0.22 to 3.4g/l and C from 2.1-16.6g/l. Maximum production of biomass (7 g/l) and PHA (2.2 g/l) were obtained with 11.6g/l of KH_2PO_4, 4.7 g/l of ammonium phosphate and 31 g/l of sucrose.

PHAs having 3, 4 and 5 hydroxyalkanoic acids have been produced by bacteria such as *Alcaligenes eutropha, Azotobacter vinelandii*, and PHAs with C4 to C12 carbon monomers are produced by *Pseudomonas* spp. Copolymers of PHAs with P(HB) are better suited for various practical uses. *Bacillus* spp. are reported to produce C4 to C8 monomers, from a large variety of carbon sources and fatty acids. The fatty acids are converted to acyl CoA derivatives that are metabolized through β-oxidation pathway. In our study overall results showed that the homopolymer of P(HB) was synthesized in *B. flexus* cells fed with only sucrose as main carbon source and PHA copolymer of polyhydroxy(butyrate-co-valerate-co-octanoate) was produced in palm oil effluent containing medium and polyhydroxy(butyrate-co-octanoate-co-decanoate) with rice bran oil as co carbon substrate. This study has shown that based on the fatty acid supplemented in the medium, medium chain length PHA (mcl-PHA) such as octanoate and decanoate can be synthesized by *B. flexus* also. PHA was characterized by FTIR, GC, GCMS and NMR methods.

Bacterial PHAs are synthesized mainly through two pathways: a) It is known that P(HB) is synthesized from acetyl coenzyme A wherein β-ketothiolase catalyses

condensation of two acetyl coenzyme A molecules to acetoacetyl–CoA that is subsequently reduced to hydroxybutryl CoA by acetoacetyl CoA reductase. P(HB) is then produced by the polymerization of hydroxybutryl CoA by the action of PHB synthase. Further it is reported that the hydroxyvaleric (HV) unit, which is found as one of the components of copolymer, is produced either from propionic acid or valeric acid through β-oxidation and deacetylation. In the absence of these typical HV precursors, HV most likely would be produced by some of the bacterial strains by the methylmalonyl-COA pathway where other carbon substrates other than propionic acid and valeric acid, can be utilized. b) PHA 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. c) 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. 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.

Synthesis of PHA in bacterial cell depends on the synthase activity and also precursors present in the cell. The precursor supply can be enhanced by supplementation of fatty acids, fatty acid synthesis and control of fatty acid degradation pathway. In *P. putida* it has been shown that both β-oxidation and fatty acid biosynthesis can function independently and simultaneously in generating precursors for PHA synthesis. Acrylic acid inhibits enzymes involved in β-oxidation such as acyl CoA synthase and 3-ketoacyl-CoA thiolase. In the present study decrease in PHA synthesis was observed due to acrylic acid addition when the culture was growing on sucrose alone as carbon source. Acrylic acid suppressed PHA production in medium containing oil or fatty acids as co carbon substrate along with sucrose as main carbon source. Higher fatty acids such as dodecanoic acid, tridecanoic acid, hexadecanoic acids and octadecanoic acids were present both in acrylic acid treated and untreated cells cultivated in sucrose by supplementation of fatty acid as co carbon source. Presence of these was observed by GC-MS. This confirmed that fatty acid degradation rather than synthesis was inhibited by acrylic acid and P(HB) synthesis partly occurred through β-oxidation pathway

irrespective of carbon source supplied for growth. Cell dry weights were considerably higher without acrylic acid, which indicates the involvement of fatty acid pathways in bacterial growth. From this it can be concluded that precursors for P(HB) synthesis are derived from: a) 50% through acetyl CoA-acetoacetyl CoA--PHA synthesis steps b) 50% through β-oxidation pathway. It has been reported that hydroxyacyl coenzyme A thioesters required for quantitative and qualitative productivity of PHA are supplied by fatty acid biosynthesis and degradation pathway. When fatty acids in the form of rice bran oil was fed to cells that were growing on sucrose, extra metabolic flux of fatty acid degradation appears to have channelised towards enhanced growth, P(HB) and some quantity of mcl-PHA production, compared to sucrose fed cells. Rice bran oil that was provided as co carbon substrate for example contained: Oleic (43%) C18:1; linoleic (35%) C18:2 and palmetic acid (15%) C16. The substrate contained up to C18:2, however carbons in the monomers found even though in small quantities were C8 and C10. Using *Pseudomonas* species it has been reported that the monomer present in the polymer is always lower than that present in the substrate.

PHAs are accumulated intracellularly and hence their extraction from the biomass is a critical step for economic production. Numerous separation processes are employed for the recovery of PHA. These involve extraction by organic solvents such as chloroform, dichloroethane, dichloromethane etc. The use of solvents to recover PHA is generally hazardous and explosive. The solvent extraction is more useful in lab-scale experiment rather than pilot plant. Sodium hypochlorite is used to digest the non-PHA cellular material, which is known to degrade the polymer.

Studies were carried out to find out the effect of organic and inorganic nutrients on cell wall composition of *B. flexus*. Total sugar concentration (mg /100 mg cell wall) of cell wall isolated from cells grown in inorganic nutrient medium and complex organic medium was 8 mg and 6 mg, respectively. Concentration of glucosamine was not affected by organic or inorganic nutrients (8.8 mg/100 mg of cell wall). The cell walls contained glutamic acid, alanine, arginine, valine, leucine, tyrosine, aspartic acid and isoleucine. Under peptone and yeast extract supplementation higher concentration of amino acids were observed in the cell walls compared to those cultivated in inorganic salts medium. Cells grown in the presence of peptone and yeast extract contained

detectable amounts of diaminopimilic acid in their cell walls, which was totally absent in those grown in the inorganic salts medium. The general features and composition of cell wall of *Bacillus* spp have been worked out. The mucopeptide contains diaminopimilic acid, glutamic acid, alanine, N-acetylglucosamine, N acetylmuramic acid and amide groups. The cell wall also contains N acetylgalactosamine, phosphorous, glycerol and glucose. The cell wall peptide has four amino acids and it is usually made up of alanine and glutamic acid. Different peptidoglycan structures are formed due to substitutions of diamino acids found at the third position of the peptide. The most common diamino acids are lysine and diaminopimilic acid (DAP). DAP containing peptide participates in cross bridge formation between DAP and alanine or between two DAP residues. Such cross bridge formations provide structural stability to the cell. It is difficult to disrupt the cell walls of gram-positive bacteria such as *Bacillus* spp. due to the presence of thicker peptidoglycan layer, which is cross-linked. The formation of peptidoglycan is dependent on the uptake of nitrogen and its metabolism to provide the required peptides for peptidoglycan synthesis. Variations are known to occur in the chemical composition of the cell wall of *B. subtilis* during growth in different media. Two or three fold increase in cell wall thickness has been found after incubation of *B. subtilis* cells in a glucose-amino acids-salts medium. Earlier it has been reported that the growth phase and the composition of the growth medium lead to the variation in the chemical composition of the cell wall. The composition and fine structure of the vegetative cell wall peptidoglycan from *Bacillus subtilis* were determined by analysis of its constituent muropeptides. The structures of 39 muropeptides, representing 97% of the total peptidoglycan, were elucidated. About 99% analyzed muropeptides in *B. subtilis* vegetative cell peptidoglycan have the free carboxylic group of diaminopimelic acid amidated. This amount is, however, dependent on the composition of the growth media. *B. subtilis* peptidoglycan is incompletely digested by lysozyme due to de-N-acetylation of glucosamine. In our study, the presence of DAP in the cell walls of cells grown in peptone and yeast extract containing medium confirmed that the cell wall is more stable compared to those grown in inorganic medium. Cells grown in inorganic salts medium was more labile and allowed easier extraction of PHA from the cells.

Cells grown in inorganic salts medium plate, when compared with those grown on complex organic medium-showed susceptibility or resistance to certain antibiotics. Antibiotic sensitivity or resistance involves various mechanisms. Changes in the sensitivity observed in the present study may be due to the drug inactivation through enzymes, altered uptake due to changes in the outer membrane and the channel through which the molecules move into the cell and altered target site or antibiotic binding proteins, which are based on the metabolic activity and nutritional status of the cell. The results demonstrate that the composition of cell wall of *B. flexus* is significantly influenced by the composition of the growth medium. Cells grown in inorganic medium lysed easily and this can be further exploited for easier recovery of the intracellular PHA.

Amongst various chemicals tested for isolation of PHA, utilization of alkali appeared to be easy and economical. Amongst various alkalis tested for hydrolysis, NH_4OH efficiently digested non-PHA cellular material at pH 11, to give 50% of PHA with 98% purity. The recovery was less with NaOH and KOH (30% and 20%, respectively), compared to NH_4OH (50%).

Bacteriolytic enzymes have been isolated from various microorganisms such as *Streptomyces, Bacillus subtilis, Flavobacterium, Myxobacter* sp. etc. Most of these lyse gram +ve bacterial cells. It has been reported that in the genus *Arthrobacter* some of the species are susceptible to lytic enzyme of *Myxobacter* sp and others are resistant to the enzyme attack. The difference in susceptibility is apparently due to macromolecular outer layer. Various mechanisms of cell lysis are reported based on peptidoglycan degrading specificity and are broadly classified as 1) glycosidases 2) N acetylmuramyoyl-l-alanine amidases and 3) endopeptidases. The preoteases (trypsin, chymotrypsin, papin and bromelain), β-glycosidases (cellulases) and lysozyme were the enzymes studied to cleave the peptidoglycan- β-1,4-glycosidic links located between N-acetyl glucosamine (NAG) and N acetylamine muramic acid (NAM) residues and peptide links of tetrapeptides that connect the polymeric chain of NAG and NAM which forms very rigid structure of bacterial cell wall.

In the present work Actinomycetous culture was used for cell hydrolysis. The isolate was identified as *Microbispora* sp. which produced white aerial mycelium, brownish-orange diffusible pigment. Earlier studies using the same isolate has shown that

the lytic enzyme produced was active in lysing gram –ve cells of *Rhizobium meliloti*. The enzyme produced by the organism was also used optimally to hydrolyse the gram+ve cells of *B. flexus* in the current study. Data reported in the literature indicate that the lytic enzyme produced by a specific strain is either active against gram+ve or gram -ve bacterium. However we have observed that both gram +ve and gram –ve cells can be hydrolysed by the enzymes secreted by a single strain of microorganism which indicated the presence of wide spectrum of activity and various substrate specifities. This may be because *R. meliloti* or *B. flexus* cells were used as substrates for the production of cell lytic enzyme. However the studies have shown that both the lytic enzymes were proteases.

Albertson first introduced aqueous two-phase system (ATPS) in 1950s for the partition of macromolecules and cell particles. Now a vast variety of methods currently exist for separation and characterization of biomolecules. The special feature of the aqueous two-phase system is that the solvent used in both the phases is water. This feature provides the suitable environment for biomolecule in both the phases. There is a diverse application of ATPS for biotechnological downstream process. In the present study, the effect of different extraction methods, pH and temperature on ATPS partitioning of bacterial cell lysate containing PHA was studied. PEG 8000/potassium phosphate was found to be the most suitable system for the separation and concentration of PHA and protease. Data obtained indicated that by using PHA containing cells of *B. flexus, Microbispora* sp culture filtrate containing protease in ATPS system, cell hydrolysis, partitioning of the polymer and recovery of protease to several fold purity can be achieved simultaneously in a single system. The results on the whole prove that ATPS is a potential and powerful separation step in the overall enzyme and PHA protocol.

Recently research has been focused on modifications of polymer for various applications. Polymer modification by ionizing radiation has advantages over chemical modifications. The radiation modification includes: radiation cross-linking, radiation induced polymerization and also degradation. Radiation affects the properties of polymeric materials, depending on the intensity and type of radiation, nature of the polymer and mechanism of reaction. Radiation has been used to modify a few polymeric materials. The objective of the present work was to use *B. flexus* for PHA production, to

improve polymer properties by using plant oil as a co carbon substrate for fermentation and by exposing PHA containing cells to gamma irradiation. Usefulness of gamma irradiation of whole wet cells for improvement of polymer properties and also cell lysis, which leads to easier isolation of the polymer, has been worked out. PHA recovery of 18-20% from whole unirradiated cells was achieved by high temperature solvent extraction for longer periods, whereas mere homogenization of irradiated cells (10kGy) with chloroform resulted in maximum isolation of the polymer (54% of biomass weight). This was due to cell damage that occurred during irradiation. Exposure of cells to higher dosages of irradiation did not result in additional yields. However it was observed that after 5-kGy of gamma-exposure, PHA extractability from the cells cultivated on sucrose and sucrose with castor oil was 25 and 27% of biomass weight, respectively. There was an increase in molecular weight and a better tensile strength of film obtained by extraction of PHA from irradiated cells (10kGy) (2.3×10^5 and 35 MPa, respectively) compared to unirradiated sample (1.7×10^5 and 20 MPa, respectively). Molecular weight of PHA decreased at higher intensities of irradiation (40 kGy), which was due to the depolymerization of PHA. Polymer degradation may be due to the chain-scission reaction of irradiation. From the data it appears that there may be predominant cross linkage of the polymer at low doses and chain scission at higher doses. The polymerization and the degradation depend on the structure of the polymer.

Utilization of phage lytic genes to disrupt recombinant *Escherichia coli* cells that produce PHA has been reported. In this method, the ability of the bacteria to self-disrupt to release PHA without the addition of any solvents, hypochlorite and enzymes is conferred on *E coli* cells. In the present work, in order to develop a self-disruptive strain, the primer was designed for the phage lytic gene. The vector selected was Vector-pSG1154 (7.6kb), screening of lytic gene in different bacterial genome through PCR was carried out. Ultimately the lytic gene (1.2kb) was isolated from lambda phage DNA. The amplified DNA was purified by using PCR product purification kit and cloned into PTZ57 (2.8kb) and transformed into *E. coli*. Plasmid was obtained in multiple copies. Plasmid isolated was subjected to restriction digestion and fragment was cloned into pSG1154 and transformed into *B. flexus.* The transformant was analysed for the gene

expression in the presence of xylose. PHA was quantified gravimetrically after self-disruption of the cells that was induced by xylose.

Conclusions

- Among 35 different *Bacillus* spp isolated from local soil samples and tested for PHA production, *Bacillus flexus* produced highest amount of the polymer (61% of biomass). This is the first report on PHA synthesis by *B. flexus*.
- The 16SrRNA gene sequence of *B. flexus* showed it shared the same cluster along with *B. megaterium* and *B. simplex*.
- PHA production was optimized and the bacterium was found to produce polyhydroxybutyrate on sucrose as carbon source and copolymer of polyhydroxybutyrate-co-octanoate-co-decanoate on saponified rice bran oil as co carbon source. FTIR, GC, GC-MS, ^{1}H NMR and ^{13}C NMR were used for characterization of PHA.
- Molar percentage of PHA copolymer varied, depending on the fatty acid/oil supplemented to the sucrose-containing medium as a co-substrate. This is the first report on the production of scl-co-mcl PHA copolymer synthesis on rice bran oil by *Bacillus* sp.
- Acrylic acid suppressed PHA production in medium containing oil or fatty acids as co carbon substrate along with sucrose as main carbon source. Supplementation of acrylic acid an inhibitor of fatty acid degradation pathway indicated that P(HB) synthesis partly occurs through β-oxidation pathway irrespective of carbon source supplied for growth.
- Diaminopimilic acid was found in only cell walls of cells grown in peptone and yeast extract containing medium and it was absent in those cultivated in inorganic nutrient medium. Cells grown in inorganic medium also had lower concentrations of amino acids in the cell wall, allowed easier extractability of the polymer, compared to peptone/yeast extract grown cells. The results demonstrate that composition of cell wall of *B. flexus* is significantly influenced by the composition of the growth medium. Cells grown in

inorganic medium lysed easily and this can be further exploited for easier recovery of the intracellular PHA.

- Amongst different alkalis tested for cell hydrolysis, NH_4OH efficiently digested non-PHA cellular material at pH 11, to give 50% of PHA with 98% purity.
- The enzyme produced by *Microbispora* sp. hydrolysed the cells of *B. flexus* to release the intracellular PHA. The enzyme was identified as protease and it was purified to homogeneity. The enzyme involved in cell hydrolysis was 40kDa.
- Results have indicated that aqueous two-phase system can be successfully employed as a non-organic solvent method for the isolation of PHA.
- Exposure of cells to gamma irradiation indicated that irradiation resulted in cell lysis leading to easy PHA extractability, low degree of cross-linking, and improvement in molecular weight as well as tensile strength of the polymer.
- Lambda lytic gene was integrated into the amylase gene (amyE) locus of the chromosomal DNA of *B. flexus*. This caused autolysis of the recombinant cells when xylose was supplemented into the medium.

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Published papers

1. P. K. Anil Kumar, T. R. Shamala, L. Kshama, M. H. Prakash, G. J. Joshi, A. Chandrashekar, K. S. Latha Kumari and **M. S. Divyashree**. Bacterial synthesis of poly(hydroxybutyrate-co-hydroxyvalerate) using carbohydrate rich mahua (*Madhuca* sp.) flowers. Journal of Applied Microbiology, 2007; 103: 204-209.

2. **M. S. Divyashree,** T. R. Shamala. Effect of gamma irradiation on cell lysis and polyhydroxyalkanoate produced by *Bacillus flexus.* Radiation physics and chemistry. 2009; 78: 147-152.

3. **M. S. Divyashree**, T. R. Shamala, and N. K. Rastogi. Isolation of polyhydroxyalkanoate from hydrolyzed cells of *Bacillus flexus* using aqueous two-phase system containing polyethylene glycol and phosphate. (Accepted by Biotechnology and Bioprocess Engineering, 2008).

4. T. R. Shamala, **M. S. Divyashree,** Reeta Davis, K. S. Latha Kumari,· S. V. N. Vijayendra and Baldev Raj. Production and characterization of bacterial polyhydroxyalkanoate copolymers and evaluation of their blends by fourier transform infrared spectroscopy and scanning electron microscopy. (Accepted by Indian Journal of Microbiology, 2008).

Papers presented in symposia

1. Biosynthesis of polyhydroxybutyrate-co-polyhydroxyvalerate in bacteria from palm oil effluent: **Divyashree M. S.,** Kshama Lakshman, Shamala T. R, Usha Rani M, and Joshi G. J. 5th International Food Convention on "Innovative food technologies and quality systems-strategies for global competitiveness", 5-8 Dec 2003, held at CFTRI, Mysore.
2. Utilization of mixed organic acids by *Bacillus licheniformis* for PHA copolymer production: P. K. Anil Kumar, N. K. Rastogi, T. R Shamala, **M. S**. **Divyashree** and A. Chandrashekar. 73rd annual meeting of society of biological chemists, Challenges

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3. Simplified recovery of bacterial polyhydroxyalkanoates: **Divyashree M. S.**, Joshi, G. J. and Shamala T. R. 16th Convention of Association of Food Scientists and Technologists of India, Food Technology: Rural outreach-vision 2020, Held at DFRL, Mysore, 9-10 December 2004.
4. Optimized production of polyhydroxyalkanoate by *Rhizobium meliloti*: A biodegradable polymer: Anil Kumar S. L., **Divyashree M. S.**, Rastogi, N. K. and Shamala T. R. 16th Convention of Association of Food Scientists and Technologists of India, Food Technology: Rural outreach-vision 2020, Held at DFRL, Mysore, 9-10 December 2004.
5. Optimized production of polyhydroxyalkanoate by *Bacillus* sp. (HP1): Latha Kumari K. S., Lakshmi M. C., **Divyashrre M. S.,** Rastogi N. K. and Shamala T. R. 74th Annual conference of Society of Biological Chemists (India) held at CDRI, and Lucknow University, Lucknow, 7-10 November 2005.
6. Production of polyhydroxyalkanoate co polymer by *Rhizobium meliloti* and *Bacillus* sp. using plant oils as co substrates. **Divyashree M. S.**, Lakshmi M. C., Latha Kumari K. S. and Shamala T. R. 74th Annual conference of Society of Biological Chemists (India) held at CDRI, and Lucknow University, Lucknow, 7-10 November 2005.
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10. High cell density cultivation of *Rhizobium meliloti* for the production of polyhydroxyalkanoate copolymers. Y. S. Rohinishree, **M. S. Divyashree** and T. R. Shamala. 18th Annual conference of Food Scientists and technologists on " Heritage foods: opportunities and challenges", organized by the Association of Food Scientists and Technologists (India), held at Acharya N. G. Ranga Agricultural University, Hyderabad, 16-17 November 2006.
11. Production of polyhydroxybutyrate-co- hydroxyvalerate by *Bacillus flexus* and effect of gamma irradiation on the polymer cross-linking. **M. S. Divyashree**, T. R. Shamala and N. K. Rastogi. 77th annual session and symposium of the national academy of sciences, India on "Novel approaches for food and nutritional security" held at CFTRI, Mysore, 6-8 December 2007.
12. A simple kinetic model for growth and synthesis of polyhydroxyalkanoate in *Rhizobium meliloti.* **Divyashree M. S.**, Rastogi N. K., Rohinishree Y. S. and Shamala T.R. 19th Indian Convention of food scientists and technologists on "Health foods" held at IIT, Khargpur, 31-12-2007 to 2-1-2008.

Polyhydroxyalkanoate from *Bacillus* sp.: its production, isolation and characterization

Final synopsis of the thesis

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Introduction

Synthetic plastics have been used in several applications including food packaging. This is because they can be easily molded into almost any desired shape including fibers and thin films and they resist environmental stress. They have high chemical resistance and are more or less elastic; hence they are used in the manufacture of popular durable goods. Plastics being xenobiotic are recalcitrant to microbial degradation (Flechter, 1993) and they persist in the soil for a long time leading to environment pollution. Accumulation of plastic in the environment has become a global problem. The rapid increase in demand of plastics has increased the plastic waste called 'white pollution' (Ren, 2003).

To overcome the problem of plastic pollution, attempts are being made to replace synthetic plastics by various biopolymers. This includes starch, pullulan, polylactic acid, chitin, polyesters etc. Amongst polyesters, various organisms are known to produce biopolyesters namely polyhydroxyalkanoates (PHAs) (Sasikala and Ramana, 1996). Many microorganisms are capable of producing PHAs under stress conditions. They serve as carbon and energy sources of the cell. They have several properties that are similar to synthetic plastic such as polypropylene and hence are considered as "bioplastics of future". This is one of the frontier areas in research and development and the thesis deals with various basic and production aspects of bacterial PHA from one of the bacterial strains, which is so far not utilized for polymer production.

Objectives of the thesis:

Based on the literature survey, envisaged objectives of the thesis were:

- **Isolation and characterization of PHA producing bacterium and optimization of polymer yields.**
- **Comparison of physical, chemical and biological methods for the isolation of polymer from the cells.**

The thesis is divided into 4 chapters and the salient features are given below:

Introduction and review of literature

PHAs are mainly classified into short-chain-length PHA (scl-PHA) and medium-chain-length PHA (mcl-PHA). Scl-PHA has up to 5 carbon containing monomers and mcl PHAs are made of 6-14 carbon monomers. Various bacteria commonly produce polyhydroxybutyrate (PHB), which is an scl-PHA. This homopolymer is brittle; copolymer of P(HB) with other scl-PHA or mcl-PHA are known to posses' better mechanical properties. Attempts have been made to produce copolymers of scl-PHA or scl-PHA-co-mcl-PHA in bacteria. PHA is intracellular and hence cell wall hydrolysis is essential prior to isolation of the polymer. This chapter deals with literature survey on structure of PHA, classification, various biosynthetic pathways and genes involved, PHA production in bacteria, various methods of PHA analysis and characterization, methods of polymer isolation, properties and applications. These are supported by details of work carried out by various workers world over and relevant references.

Materials and Methods (Common to chapters)

This chapter deals with details of chemicals, equipments, media, methods of staining, sterilization, and cultivation conditions used generally in various experiments. It also includes details on analysis and characterization of PHA and molecular biology experimental methods.

Objectives defined earlier, were worked out and relevant introduction, materials and methods, results, discussion and conclusions are presented in the following chapters:

Chapter 1: Isolation, identification and characterization of PHA producing *Bacillus* sp.

The genus *Bacillus* is widely distributed in nature and most commonly found in soil. It comprises of gram-positive, endospore forming, aerobic bacteria. Many bacteria have been screened for the production of PHA. PHA synthases of *B. megaterium* is known to be distinctly different from all known PHA synthases (McCool and Cannon,

1999) and hence it appeared feasible to isolate *Bacillus* sp., which is not reported so far as a PHA producer. Culture used in this study was isolated from the soil collected locally near municipal waste disposal yard. Characterization was carried out by: gram staining, measurement of cell size, by various biochemical tests such as catalase, oxidase, growth on various carbon sources; indole, methyl red, Voges Proskauer, citrate and nitrate reduction; gelatin and starch hydrolysis. The selected strain of *Bacillus* sp. was observed to be a Gram positive, motile, endospore forming, rod shaped, aerobic bacterium. It showed positive tests for urease, catalase, oxidase, starch and gelatin hydrolysis and produced acid from various carbon sources. In addition to these tests, 16SrRNA analysis was carried out. 1.2kb of entire 16SrRNA gene from the selected isolate was sequenced which involved, isolation and purification of chromosomal and plasmid DNA, PCR, preparation of competent cells, transformation of *E. coli* and DNA cloning by standard procedures. The sequence was compared by using the BLAST program from the National Center for Biotechnological information (NCBI) gene bank for the identification of the species. Based on 16SrRNA, the position of the isolated strain within the same group of species was analyzed by the tree constructed using maximum homology obtained from NCBI blast tool. The bacterium was identified as *B. flexus*. The sequence similarity within the species was 99%, other closely related species of *Bacillus flexus* was found to be *B. megaterium* having a homology of 97% and it shared the same cluster with *B. simplex*.

In order to find out the ability of the isolated strain of *B. flexus* to produce PHA, growth and PHA production were compared with that of standard *Bacillius* spp. The strain was found to produce maximum PHA compared to other standard strains and it gave 52% polymer yield on biomass weight. Based on ecological diversities, multiplicity of substrate degradation and production of industrially important molecules such as enzymes, antibiotics, the genus may be called as “potential biocell”. This study has led to a finding of a new unexplored *Bacillus flexus* strain, which has the ability to produce PHA at 50-60% level. Optimization of culture conditions, characterization of polymer synthesized may lead to a possible turning point for the use of *Bacillus* sp. as a commercial PHA producer.

Chapter 2: Production of polyhydroxyalkanoate by *B. flexus* using different substrates, optimization of growth and characterization of the polymer

Many bacteria are capable of accumulating P(HB) within the cell during stationary phase, when essential nutrient is limiting, but carbon concentration is in excess. Research is being conducted for the efficient production of PHA using inexpensive carbon sources because production cost is still high compared to synthetic petrochemical based plastics. Using sucrose as inexpensive carbon source, high productivity has been achieved in an optimized fermentation process (Lee and Choi, 1998).

In the present work, in order to obtain high yield and quality of PHA, various carbon and nitrogen sources were assessed as media supplements. *B. flexus* was cultivated in media containing different salts of nitrogen, amino acids, carbon sources, plant oils, free fatty acids, economic media components such as palm oil effluent, molasses, corn starch, whey, rice and wheat bran extracts as sources of carbon/nitrogen/nutrients to enhance growth and PHA copolymer synthesis. At the end of cultivation period, cells were harvested; biomass and PHA were estimated and characterization of PHA was carried out by FTIR/GC/GC-MS/NMR spectroscopy/DSC. Higher concentrations of biomass of 2.5, 2 and 1.95 g/l were obtained with ammonium acetate, ammonium sulphate and ammonium phosphate as N-sources, respectively. Corresponding yields of PHA obtained were 2.2, 1.1 and 1.0 g/l. In the biosynthesis of PHA, HB monomer is produced through acetyl CoA (Hassan *et al.,* 1997). Utilization of acetate is activated by the enzyme acetate-CoA ligase (EC 6.2.1.1), which is the initial step in its metabolism. Acetate utilization leads to cell growth as well as PHA synthesis. Initially it serves as carbon source for growth, and when the nitrogen concentration in the medium is depleted then it is channelised towards PHA synthesis. Due to this dual activity higher growth and PHA synthesis may be expected when ammonium acetate is used as a nitrogen source in the medium. Carbon source has profound influence on the PHA content of the cell. Optimal amount of PHA (1g/l; 50% of biomass weight) as well as biomass (2 g/l) were obtained in medium containing sucrose.

Response surface methodology was used to optimize media components based on central composite rotatory design (CCRD). From the results it was observed that KH_2PO_4 enhanced PHA production and bacterial growth was not inhibited even at 15 g/l

concentration of this nutrient. Ammonium phosphate and sucrose enhanced growth and PHA production. Nitrogen concentration used in the experiment varied from 0.21 to 1.27 g/l, P from 0.22 to 3.4 g/l and C from 2.1-16.6 g/l. Maximum production of biomass (7 g/l) and PHA (2.2 g/l) were obtained with 11.6 g/l of KH_2PO_4, 4.7 g/l of ammonium phosphate and 31 g/l of sucrose.

In a preliminary experiment it was observed that *B. flexus* produced PHA under N limitation conditions with excess carbon source. The optimal ratio of N:P:C for growth and PHA synthesis observed was 1:4:13. The growth kinetics of *B. flexus* under nutrient limitation was studied by using a simple model giving mathematical description of kinetics of microbial growth, substrate consumption and product formation. The correlation of PHA synthesis with cell growth and the effect of nutrients were studied by applying mathematical model. In this study, a modification of the logistic equation has also been attempted for describing the growth of the bacterium. The microbial growth was described by means of a simplification of Monod's model for low substrate concentration. A simplified Luedeking-Pirat type model represented the product kinetics. The kinetic constants were evaluated based on non-linear regression and the differential equations were solved using Runge-Kutta algorithm using MATLAB software. The model was able to predict microbial growth and PHA production and values obtained were comparable to experimental data.

PHAs having 3, 4 and 5 hydroxyalkanoic acids have been produced by bacteria such as *Alcaligenes eutropha*, *Azotobacter vinelandii,* and PHAs with C4 to C12 carbon monomers are produced mainly by *Pseudomonas* spp (Brandl *et al.,* 1990). Copolymers of PHAs with P(HB) are better suited for various practical uses. *Bacillus* spp. are reported to produce C4 to C8 monomers, from a large variety of carbon sources and fatty acids (Brandl *et al.,* 1990, Caballero *et al.,* 1995; Valappil *et al.,* 2007; Shamala *et al.,* 2003; Anil Kumar *et al.,* 2007). The fatty acids are converted to acyl CoA derivatives that are metabolized through β-oxidation pathway (Kato *et al.,* 2001). In our study overall results showed that homopolymer of P(HB) was synthesized in *B. flexus* cells fed with only sucrose as main carbon source and PHA copolymer of polyhydroxy(butyrate-co-valerate-co-octanoate) was produced in palm oil effluent containing medium and polyhydroxy(butyrate-co-octanoate-co-decanoate) with rice bran oil as co carbon

substrate. This study has shown that based on the fatty acid supplemented in the medium, mcl PHA such as octanoate and decanoate can be synthesized by *Bacillus* spp. also. FTIR, GC, GCMS and NMR methods were used for characterization of the polymer. GC-MS and NMR analysis confirmed the presence of mcl-PHA in the copolymer.

Bacterial PHA are synthesized mainly through two pathways: a) It is known that P(HB) is synthesized from acetyl coenzyme A wherein β-ketothiolase catalyses condensation of two acetyl coenzyme A molecules to acetoacetyl–CoA that is subsequently reduced to hydroxybutryl CoA by acetoacetyl CoA reductase. P(HB) is then produced by the polymerization of hydroxybutryl CoA by the action of PHB synthase (Madison and Huisman, 1999; Tsuge, 2002). Further these authors have reported that the hydroxyvaleric (HV) unit, which is found as one of the components of copolymer, is produced either from propionic acid or valeric acid through β-oxidation and deacetylation. In the absence of these typical HV precursors, HV most likely would be produced by some of the bacterial strains by the methylmalonyl-COA pathway where other carbon substrates other than propionic acid and valeric acid, can be utilized. b) PHA 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. c) 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 (Huijberts *et al.,* 1994). 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.

Synthesis of PHA in bacterial cell depends on the synthase activity and also precursors present in the cell. The precursor supply can be enhanced by supplementation of fatty acids, fatty acid synthesis and control of fatty acid degradation pathway. In *P. putida* it has been shown that both β-oxidation and fatty acid biosynthesis can function independently and simultaneously in generating precursors for PHA synthesis (Huijberts *et al.,* 1994). Acrylic acid inhibits enzymes involved in β-oxidation such as acyl CoA synthase and 3-ketoacyl-CoA thiolase (Huijberts *et al.,* 1994). In recombinant *E. coli* having *phaC* and *phaJ*, PHA accumulation increased due to blocking of β-oxidation path

way by acrylic acid which resulted in more enoyl CoA leading to channelisation of this towards PHA synthesis. While recombinant harbouring *phaC, phaJ* and *yafH* (acyl CoA dehydrogenase gene), polyhydroxybutyrate-co-hydroxyhexanoate content decreased when acrylic acid was added to the culture suggesting that acrylic acid inhibited over expression of *yafH* (Lu *et al.,* 2003). In the present study decrease in PHA synthesis was also observed due to acrylic acid addition when the culture was growing on sucrose alone as carbon source. Acrylic acid suppressed PHA production in medium containing oil or fatty acids as co carbon substrate along with sucrose as main carbon source. Free fatty acids such as dodecanoic acid, tridecanoic acid, hexadecanoic acids and octadecanoic acids were present both in acrylic acid treated and untreated cells cultivated in sucrose by supplementation of fatty acid as co carbon source. Presence of these was observed by GC-MS. This confirmed that fatty acid degradation rather than synthesis was inhibited by acrylic acid and PHB synthesis partly occurred through β-oxidation pathway irrespective of carbon source supplied for growth. Cell dry weights were considerably higher without acrylic acid, which indicated the involvement of fatty acid pathways in bacterial growth. From this it can be concluded that precursors for P(HB) synthesis are derived from: a) 50% through actyl CoA-acetoacetyl CoA--PHA synthesis steps b) 50% through β-oxidation pathway. It has been reported that hydroxyacyl coenzyme A thioesters required for quantitative and qualitative productivity of PHA are supplied by fatty acid biosynthesis and degradation pathway. When fatty acids in the form of rice bran oil was fed to cells that were growing on sucrose, extra metabolic flux of fatty acid degradation appears to have channelised towards enhanced growth, P(HB) and some quantity of mcl - PHA production, compared to sucrose fed cells. Rice bran oil that was provided as co carbon substrate for example contained: Oleic (43%) C18:1; linoleic (35%) C18:2 and palmetic acid (15%) C16. The substrate contained up to C18:2, however carbons in the monomers found were C8 and C10. Using *Pseudomonas* species it has been reported that the monomer present in the polymer is always lower than that present in the substrate (Bassas *et al.,* 2008).

Chapter 3: Isolation of PHA from bacterial cells

PHAs are accumulated intracellularly and hence their extraction from the biomass is a critical step for economic production. PHA being intracellular, isolation and purification are major steps in the downstream process of the polymer. Numerous separation processes are employed for the recovery of PHA. These involve extraction by organic solvents such as chloroform, methylene chloride, dichloroethane, dichloromethane (Ramsay *et al.,* 1994). The use of solvents to recover PHA is generally hazardous and explosive. The solvent extraction is more useful in lab-scale experiment rather than pilot plant. Sodium hypochlorite is used to digest the non-PHA cellular material, which is known to degrade the polymer.

In the present study novel physical, chemical and biological methods have been assessed for isolation of the intracellular PHA and a summary is presented below:

Effect of nutrients on cell wall synthesis

Degradability of cell wall is based on the nature of cell wall present in the organism. Gram-positive cells are known to be tough and not easily amenable for degradation. However nature of cell wall is dependant on the nutritional status of the cell. Studies were carried out to find out the effect of organic and inorganic nutrients on cell wall composition of *B. flexus*. Total sugar concentration (mg /100 mg cell wall) of cell wall isolated from cells grown in inorganic nutrient medium and complex organic medium was 8 mg and 6 mg, respectively. Concentration of glucosamine was not affected by organic or inorganic nutrients (8.8 mg/100 mg of cell wall). The cell walls contained glutamic acid, alanine, arginine, valine, leucine, tyrosine, aspartic acid and isoleucine. Under peptone and yeast extract supplementation higher concentration of amino acids were observed in the cell walls compared to those cultivated in inorganic salts medium. Cells grown in the presence of peptone and yeast extract contained detectable amounts of diaminopimilic acid in their cell walls, which was totally absent in those grown in the inorganic salts medium. The general features and composition of cell wall of *Bacillus* spp have been worked out (Hughes *et al.,* 1970). The mucopeptide contains diaminopimilic acid, glutamic acid, alanine, N-acetylglucosamine, N

acetylmuramic acid and amide groups. The cell wall also contains N acetylgalactosamine, phosphorous, glycerol and glucose. The cell wall peptide has four amino acids and it is usually made up of alanine and glutamic acid. Different peptidoglycan structures are formed due to substitutions of diamino acids found at the third position of the peptide. The most common diamino acids are lysine and diaminopimilic acid (DAP). DAP containing peptide participates in cross bridge formation between DAP and alanine or between two DAP residues. Such cross bridge formations provide structural stability to the cell. It is difficult to disrupt the cell walls of gram-positive bacteria such as *Bacillus* spp due to the presence of thicker peptidoglycan layer, which is cross-linked. The formation of peptidoglycan is dependent on the uptake of nitrogen and its metabolism to provide the required peptides for peptidoglycan synthesis. Variations are known to occur in the chemical composition of the cell wall of *B. subtilis* during growth in different media (Young, 1965). Two or three fold increase in cell wall thickness has been found after incubation of *B. subtilis* cells in a glucose-amino acids-salts medium (Hughes *et al.,* 1970). Earlier it has been reported that the growth phase and the composition of the growth medium lead to the variation in the chemical composition of the cell wall. The composition and fine structure of the vegetative cell wall peptidoglycan from *B. subtilis* were determined by analysis of its constituent muropeptides. The structures of 39 muropeptides, representing 97% of the total peptidoglycan, were elucidated. About 99% of analyzed muropeptides in *B. subtilis* vegetative cell peptidoglycan had the free carboxylic group of diaminopimelic acid amidated. This amount is, however, dependent on the composition of the growth media. *B. subtilis* peptidoglycan is incompletely digested by lysozyme due to de-N-acetylation of glucosamine.

In our study, the presence of DAP in the cell walls of cells grown in peptone and yeast extract containing medium confirmed that the cell wall is more stable compared to those grown in inorganic medium. Complex organic medium which contained ammonium sulphate as one of the nitrogen sources also lacked DAP, had lower concentrations of amino acids in the cell wall, allowed easier extractability of the polymer, compared to peptone/yeast extract grown cells, which may be due to changes in the metabolic activities in the presence of inorganic nitrogen source.

Cells grown in inorganic salts medium plate, when compared with those grown on complex organic medium-showed susceptibility or resistant to certain antibiotics. Antibiotic sensitivity or resistance involves various mechanisms (Mims *et al.,* 1998). Changes in the sensitivity observed in the present study may be due to the drug inactivation through enzymes, altered uptake due to changes in the outer membrane and the channel through which the molecules move into the cell and altered target site or antibiotic binding proteins, which are based on the metabolic activity and nutritional status of the cell. The results demonstrate that composition of cell wall of *B. flexus* is significantly influenced by the composition of the growth medium. Cells grown in inorganic medium lysed easily and this can be further exploited for easier recovery of the intracellular PHA.

Chemical method of PHA isolation

Amongst various chemicals tested for isolation of PHA, utilization of alkali appeared to be easy and economical. Amongst various alkalis tested for hydrolysis, NH_4OH efficiently digested non-PHA cellular material at pH 11, to give 50% of PHA with 98% purity The recovery was less with NaOH and KOH (30% and 20%, respectively), compared to NH_4OH (50%).

Biological method for PHA isolation

Bacteriolytic enzymes have been isolated from various microorganisms such as *Streptomyces, B. subtilis, Flavobacterium, Myxobacter* sp. etc. Most of these lyse gram +ve bacterial cells (Ensign and Wolfe, 1965). It has been reported that in the genus *Arthrobacter* some of the species are susceptible to lytic enzyme of *Myxobacter* sp. and others are resistant to the enzyme attack. The difference in susceptibility is apparently due to macromolecular outer layer. Various mechanisms of cell lysis are reported based on peptidoglycan degrading specificity and are broadly classified as 1) glycosidases 2) N acetylmuramyoyl-l-alanine amidases and 3) endopeptidases. The preoteases (trypsin, chymotrypsin, papin and bromelain), β-glycosidases (cellulases) and lysozyme were the enzymes studied to cleave the peptidoglycan- β-1,4-glycosidic links located between N-acetyl glucosamine (NAG) and N acetylamine muramic acid (NAM) residues and peptide

links of tetrapeptides that connect the polymeric chain of NAG and NAM which forms very rigid structure of bacterial cell wall (Bugg, 1997).

In the present work Actinomycetous culture was used for cell hydrolysis. The isolate was identified as *Microbispora* sp., which produced white aerial mycelium, brownish-orange diffusible pigment. Earlier studies using the same isolate has shown that the lytic enzyme produced was active in lysing gram –ve cells of *Rhizobium meliloti* (Kshama and Shamala, 2006). The enzyme produced by the organism was also used optimally to hydrolyse the gram+ve cells of *B. flexus* in the current study. Data reported in the literature indicate that the lytic enzyme produced by a specific strain is either active against gram+ve or gram -ve bacterium (Ensign and Wolfe, 1965). However we have observed that both gram +ve and gram –ve cells can be hydrolysed by the enzymes secreted by a single strain of microorganism which indicated the presence of wide spectrum of activity and various substrate specificities. This was mainly due to utilization of *R. meliloti* or *B. flexus* cells as substrates for the production of cell lytic enzyme by *Microbispora* sp. However the studies have shown that both the lytic enzymes were proteases. The enzyme was purified to homogeneity by ammoniun sulphate precipitation followed by column chromatography on Sephadex G100. Molecular size of the purified protein was ~40kDa.

Physical method of PHA isolation

Albertson first introduced aqueous two-phase system (ATPS) in 1950s for the partition of macromolecules and cell particles. Now a vast variety of methods currently exist for separation and characterization of biomolecules. The special feature of the aqueous two-phase system is that the solvent used in both the phases is water. This feature provides the suitable environment for biomolecule in both the phases. There is a diverse application of ATPS for biotechnological downstream process. But the use of this system has so far been limited probably due to the poor predictability and analysis of the parameters affecting the partitioning (Bensch *et al.,* 2007). In the present study, the effect of different extraction methods, pH and temperature on ATPS partitioning of bacterial cell lysate containing PHA was studied. PEG 8000/potassium phosphate was found to be the most suitable system for the separation and concentration of PHA and

protease. Data obtained indicated that PHA containing cells of *B. flexus* can be simultaneously hydrolysed by using *Microbispora* sp. culture filtrate containing protease, the released polymer can be partitioned by aqueous two phase system and protease used can also be recovered to get several fold purity, from the cell hydrolysate in the same system. The results on the whole prove that ATPS is a potential and powerful separation step in the overall enzyme and PHA protocol.

Effect of gamma radiation on PHA isolation from bacterial cell

Recently research has been focused on modifications of polymer for various applications. Polymer modification by ionizing radiation has advantages over chemical modifications. The radiation modification includes: radiation cross-linking, radiation induced polymerization and also degradation. Radiation affects the properties of polymeric materials, depending on the intensity and type of radiation, nature of the polymer and mechanism of reaction. Radiation has been used to modify a few polymeric materials (Chmeilewski *et al.,* 2005; Bassas *et al.,* 2008; Dufresne *et al.,* 2001). The objective of the present work was to use *B. flexus* for PHA production, to improve polymer properties by using plant oil as a co carbon substrate for fermentation and by exposing PHA containing cells to gamma irradiation. Usefulness of gamma irradiation of whole wet cells for improvement of polymer properties and also cell lysis, which leads to easier isolation of the polymer, has been worked out. PHA recovery of 18-20% from whole unirradiated cells was achieved by high temperature solvent extraction for longer periods, whereas mere homogenization of irradiated cells (10kGy) with chloroform resulted in maximum isolation of the polymer (54% of biomass weight). This was due to cell damage that occurred during irradiation. Exposure of cells to higher dosages of irradiation did not result in additional yields. However it was observed that after 5-kGy of gamma-exposure, PHA extractability from the cells cultivated on sucrose and sucrose with castor oil was 25 and 27% of biomass weight, respectively. There was an increase in molecular weight and a better tensile strength of film obtained by extraction of PHA from irradiated cells (10kGy) (2.3×10^5 and 35 MPa, respectively) compared to unirradiated sample (1.7×10^5 and 20 MPa, respectively). Molecular weight of PHA decreased at higher intensities of irradiation (40 kGy), which was due to the

depolymerization of the PHA. Polymer degradation may be due to the chain-scission reaction of irradiation. From the data it appears that there may be predominant cross linkage of the polymer at low doses and chain scission at higher doses. The polymerization and the degradation depend on the structure of the polymer (Chmeilewski *et al.,* 2005).

Chapter 4: Cloning and expression of lytic gene in *Bacillus flexus*

Utilization of phage lysis genes to disrupt recombinant *Escherichia coli* cells that produce PHA has been reported (Fidler and Dennis, 1992). In this method, the ability of the bacteria to self-disrupt to release PHA without the addition of any solvents, hypochlorite and enzymes is conferred on *E coli* cells. Resch *et al.,* (1998) used the lytic gene of bacteriophage ϕX174 under the control of a thermo sensitive expression system. Hori *et al.,* (2002) have developed a self-disruptive strain of *Bacillus megaterium* by using lysis system of *Bacillus amyloliquefaciens* phage holin and endolysin that responds to substrate exhaustion.

In the present work, in order to develop a self-disruptive strain, the primer was designed for the phage lytic gene. The vector selected was Vector-pSG1154 (7.6kb), screening of lytic gene in different bacterial genome through PCR was carried out. Ultimately the lytic gene (1.2kb) was isolated from lambda phage DNA. The amplified DNA was purified by using PCR product purification kit and cloned into PTZ57 (2.8kb) and transformed into *E. coli*. Plasmid was obtained in multiple copies. Plasmid isolated was subjected to restriction digestion and fragment was cloned into pSG1154 and transformed into *B. flexus.* PHA was quantified gravimetrically after self-disruption of the cells that was induced by xylose. Highest concentration of PHA (70% of total PHA) was recovered as extra cellular component from this culture compared to control wild strain (20% PHA).

Summary and conclusions:

In the present study *B. flexus* was isolated from local soil sample for PHA production and following are the important findings:

- Among 35 different *Bacillus* spp, isolated from local soil samples and tested for PHA production, one of the isolate was found to produce highest amount of the polymer (61% of biomass). The strain was characterized by morphological, biochemical and molecular methods. **The 16SrRNA gene sequence of this bacterium indicated that the strain was *B. flexus.* This bacterium shared the same cluster along with *B. megaterium* and *B. simplex*.** The phylogenetic tree of *Bacillus* spp, was constructed by clustalW program and the test strain was closely related to *B. megaterium* (97% homology). **This is the first report on PHA synthesis by *B. flexus*.**
- In shake flask cultures, maximum biomass (2.5 g/l) and PHA production (1.8 g/l; 72% of biomass weight) was observed in medium containing ammonium acetate as nitrogen source. The isolated polymer was highly viscous in nature.
- The bacterium grew optimally on various economic substrates such as starch, molasses, mahua (*Madhuca indica*) flower extract, corn steep liquor etc and produced P(HB) or PHA copolymer.
- Amongst various plant oils tested as co-substrate for copolymer synthesis, castor oil, palm oil and rice bran oil were found to be better suited for PHA production by *B. flexus*. Biomass obtained was 1.6, 1.4 and 1.5 g/l with PHA concentration of 1, 0.6 and 1.1 g/l, respectively. Corresponding values for PHA concentration in the biomass was 62, 43 and 73%. PHA production was optimized and the bacterium produced P(HB) on sucrose as carbon source and copolymer of polyhydroxy(butyrate-co-octanoate-co-decanoate) on saponified rice bran oil as co carbon source. FTIR, GC, GC-MS, 1H NMR and ^{13}C NMR were used for characterization of PHA.
- Molar percentage of PHA copolymer varied, depending on the fatty acid/oil supplemented to the sucrose-containing medium as a co-substrate. PHA formed was a copolymer P(HB-co-HV) of 97:3 mol% in valeric and hexanoic acid and propionic acid medium. Scl-PHA-co-mcl-PHA of 98:8 mol% was obtained under supplementation of rice bran oil. **This is the first report on the production of scl-co-mcl PHA copolymer synthesis on rice bran oil by *Bacillus* sp.**

- Acrylic acid suppressed PHA production in medium containing oil or fatty acids as co-carbon substrate along with sucrose as main carbon source. Free fatty acids such as dodecanoic acid, tridecanoic acid, hexadecanoic acids and octadecanoic acids were present both in acrylic acid treated and untreated cells cultivated in sucrose by supplementation of fatty acid as co carbon source. Presence of these was observed by GC-MS. **This confirmed that fatty acid degradation rather than synthesis was inhibited by acrylic acid and P(HB) synthesis partly occurred through β-oxidation pathway irrespective of carbon source supplied for growth. The monomer present in the polymer was always lower than that present in the substrate.**
- The cell wall of bacterium grown in organic medium was more stable compared to those grown in inorganic medium. Diaminopimilic acid was found only in the cell walls isolated from cells cultivated in organic nutrient medium. This component was absent in the cells cultivated in inorganic nutrient medium. Cells grown in inorganic medium also had lower concentrations of amino acids in the cell wall. The results demonstrate that the composition of cell wall of *B. flexus* is significantly influenced by the composition of the growth medium. **Cells grown in inorganic medium lysed easily and this can be further exploited for easier recovery of the intracellular PHA.**
- **Amongst different alkalis tested for cell hydrolysis, NH_4OH efficiently digested non-PHA cellular material at pH 11, to give 50% of PHA with 98% purity.**
- The enzyme produced by *Microbispora* sp., hydrolysed the cells of *B. flexus* to release the intracellular PHA. The enzyme was identified as protease and it was purified to homogeneity. The enzyme involved in cell hydrolysis was ~40kDa.
- Aqueous two phase system (ATPS) having polyethylene glycol (12%, w/v) and potassium phosphate (9.7%, pH 8.0) containing cell lysate obtained by sonication or hypochlorite treatment of *B. flexus* biomass, was held at 28^0 C for 30 min, which partitioned PHA into top PEG phase and residual cell

materials into bottom phase. PHA recovered by centrifugation was 19-51% of cell dry weight, depending on the mode of cells disruption. For enzymatic cell hydrolysis, *Microbispora* sp., culture filtrate having protease as cell lytic activity was mixed with *B. flexus* biomass and ATPS, incubated at 37^{o} C for 2 h prior to phase separation. Protease was recovered along with PHA in the PEG phase and showed 7-fold increase in activity. **Results indicated that ATPS could be successfully employed as a non-organic solvent method for the isolation of PHA. PHA was isolated from protease hydrolyzed bacterial cells and purified protease was also recovered as a by-product, in a single defined experiment.**

- *B. flexus* cells containing PHA was exposed to different dosages of gamma radiation (5-40kGy). PHA recovery of 18-20% from whole unirradiated cells was achieved by high temperature solvent extraction for longer periods, whereas mere homogenization of irradiated cells (10kGy) with chloroform resulted in maximum isolation of the polymer (54% of biomass weight). This was due to cell damage that occurred during irradiation. **Overall results indicated that exposing of PHA containing cells to low degree of gamma irradiation resulted in cell lysis leading to easy PHA extractability, low degree of cross-linking, and improvement in molecular weight as well as tensile strength of the polymer.**
- *B. flexus* was transformed with pSG1154 vector having lambda lytic gene. The target gene integrated into the amylase gene (*amyE)* locus of the chromosomal DNA of *B. flexus.* The introduced lytic system caused autolysis of the recombinant cells when xylose was supplemented into the medium. The integration of the target gene in the amylase locus was analysed by starch degradation. This system resulted in the timely disruption of the cell to release the intracellular PHA, when it reached maximum level within the cell.

The present work has indicated that *B. flexus* can be utilized for PHA copolymer production. The study has focused on economic production of PHA, newer aspects of PHA isolation methods such as aqueous two-phase extraction, gamma irradiation for improvement of polymer extraction and properties.

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