STUDIES ON THE PRODUCTION OF BIOPOLYMERS BY *RHIZOBIUM SP*S. THEIR ISOLATION AND CHARACTERIZATION

A Thesis

By

Kshama Lakshman, M.Sc.,

Submitted to the

University of Mysore

For the award of Degree of

Doctor of Philosophy

In

Microbiology

Under the guidance of

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September 2004

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CERTIFICATE

It is certified that this thesis entitled **"STUDIES ON THE PRODUCTION OF BIOPOLYMERS BY *RHIZOBIUM SP*S. THEIR ISOLATION AND CHARACTERIZATION"** which is submitted to the University of Mysore, Mysore, for the award of the Degree of Doctor of Philosophy in Microbiology is the result of research work carried out by **Miss. Kshama Lakshman**, under my guidance and supervision during the period from October 1999 to August 2004 in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore.

T.R.Shamala
Guide

DECLARATION

I, **Miss Kshama Lakshman**, hereby declare that the data presented in this thesis entitled **"STUDIES ON THE PRODUCTION OF BIOPOLYMERS BY *RHIZOBIUM SP*S. THEIR ISOLATION AND CHARACTERIZATION"** which is submitted to the University of Mysore, Mysore, for the award of the Degree of Doctor of Philosophy in Microbiology is the result of research work carried out by me under the guidance and supervision of **Dr.T.R.Shamala** during the period from October 1999 to August 2004 in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore.

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

Place: Mysore
Date:

Kshama Lakshman
Research Fellow

At the feet of the

Holy Mother

To my Parents

Acknowledgments

At the outset I wish to express my sincere gratitude to my guide and mentor ***Dr T.R. Shamala****, Scientist, Department of Food Microbiology, CFTRI Mysore, for her constant encouragement, supervision and cooperation. It was a privilege to work under her inspiring presence. The stupendous task of research would not have been possible but for the guidance, direction and intellectual inputs I received from my guide during the period of research.*

I express my sincere gratitude to ***Padmashri Dr. V. Prakash****, Director CFTRI Mysore, for giving me the opportunity to work at CFTRI and utilize the excellent facilities at the institute. I am highly thankful to him for the all the help extended during my stay as a research fellow.*

I sincerely thank ***Dr. Umesh Kumar****, Head, Department of Food Microbiology, CFTRI Mysore, for his support in all my pursuits during the course of my work.*

I am indebted to, ***Dr.Krishnanand*** *and* ***Dr M S Prasad****, former heads of the department of Food Microbiology, CFTRI, for their encouragement and affectionate concern throughout the course of my work.*

My heartfelt gratitude and thanks to ***Dr.P.Srinivas****,* ***Dr.N.K.Rastogi*** *and* ***Dr. Baldev Raj*** *for their help, valuable suggestions, intellectual inputs and guidance given at crucial periods in my research work. I owe my sincere gratitude to* ***Dr. Arun Chandrashekar*** *and* ***Mr. Prakash Halami*** *for their constant help, guidance and support throughout my investigation.*

I wish to acknowledge Dr.H.K.Manonmani, Dr S.V.N.Vijayendra and Dr.A.Ramesh for their help at times of need. I sincerely thank Dr.M.C.Varadaraj, Dr.E.Rati Rao, Dr.G.Vijayalakshmi, Dr Renu Agarwal, Dr.G.Venkateshwaran, Mrs Vanajakshi and Mr G J Joshi for their help and kind cooperation during the course of my work. I also *acknowledge the help extended by Dr.Paramahans Salimath, Dr.M.C.Misra and Dr Eugene Raj.*

My sincere thanks to Mr.S.V.Basavaiah for his constant help and support in the lab. I also thank Mr. Abdul Khayoum and Mr. C.V.Venkatesh for their help during my stay in the department.

I sincerely thank ***Dr. Gurudutt****, Head of the Department of Central instrumentation facility and services, CFTRI, Ms. Asha, Mr. Mukund, Mr. Anbalgan, Mr. Krishnamurthy, Mr. Shivaswamy and Mr. Prakash for their kind cooperation. Ms Suma, Department of Food Packaging deserves special thanks for her kind help and cooperation.*

I am grateful to staff of administration, glass blowing, photography section, pilot plant and library, CFTRI, for their valuable help throughout my research work.

I am indebted to ***Dr. Rangaswamy****, Professor, Department of Chemistry, University of Mysore, for his support and timely help.*

My sincere gratitude to ***Dr. Suresh Kattera*** *and Mrs Vasudha, Raman Research Institute, Bangalore, for the valuable help rendered during my investigation. I wish to thank all the staff of Sophisticated Instrument facility, Indian Institute of Science, Bangalore.*

I sincerely acknowledge the ***Council of Scientific and Industrial research (CSIR****), New Delhi, for supporting me with a senior research fellowship, which enabled me to undertake this research. I also acknowledge with gratitude the* ***University of Mysore*** *for providing me an opportunity to submit the thesis to the University for Doctoral Degree in Food Microbiology*

My sincere thanks are due to each and every author and scientists whose works have been referred, cited and quoted in this thesis.

Special thanks to my dear friends, Mr. Kiran Kumar G.R, Mr.Harish Prashanth and Mr. Nagendra Prasad for their moral support and encouragement. I owe my sincere gratitude to my dear friends Ms. Divyashree and Mrs. Usha Rani for their genuine concern and help during the finalization of my thesis. I am indebted to their friendship and affection.

I also thank my friends, Sri Jyothi, Sudhamani, Bharati, Padmapriya, Jagannath, Ravi MS, Anand, Ravi K, Venkatesh, Anil, Rajeshwari, Vageeshwari, Sowmya, Roopa and Gunashree. I cherish the company of Rajni, Chetna, Jessy, Madhvi, Ritu, Indu and Kanika who made my stay at the hostel a memorable one.

My sincere and heartfelt thanks to my father Dr. UN Lakshman, and my Mother, Smt Jayanthi Lakshman, who have stood with me as my pillars of strength and support throughout my research work. Their love and spiritual guidance has helped me in fulfilling this work.

Kshama Lakshman

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(On thesis submission)

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September 2004

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Introduction

There is a worldwide concern regarding the development of biodegradable plastic materials as a remedy towards harmful effects caused by plastic wastes on the environment. These materials, which are synthesized chemically by polymerization, contribute towards air pollution and waste management problems. The fear of depletion of wood resources has established plastics as the material of choice in many applications. In food sector, various characteristics of plastics such as low density, ready sealability, resistant to break, appearance, impermeability to oxygen and water vapor, low temperature and flexibility have contributed for its large usage. Unfortunately, these highly durable, versatile and extremely useful material led to adverse effects on the environment after its use. Moreover, as over 99% of plastics are fossil fuel origin, their rapid increase will put further pressure on the already limited non-renewable resources on earth. Plastics are not biodegradable and there is no concerted effort for plastic waste management.

All this has promoted worldwide research to develop new biodegradable alternatives to plastics. Amidst the pro plastic arguments, there still is a strong need for the look out for alternatives for synthetic plastics and to replace them steadily. Biodegradable polymers or bioplastics are important and interesting areas that are being looked out as alternatives for synthetic plastics. These are a new generation of materials able to significantly reduce the environmental impact in terms of energy consumption and green house effect.

Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates are microbial polyesters. These polymers have properties similar to synthetic plastics and in addition are biodegradable and biocompatible. Polyhydroxyalkanoates in particular are attractive substitutes for conventional petrochemical plastics because of their similar material properties to various thermoplastics and elastomers and their complete degradability upon disposal in various environments. Polyhydroxyalkanoates are synthesized by numerous bacteria as intracellular carbon and energy source. These are accumulated in the cytoplasm of cells as granules under conditions of nutrient imbalance. Accumulation usually occurs when carbon is in excess and if atleast one other nutrient, which is essential for growth, is depleted.

These polyesters are used in a number of applications and have attracted considerable industrial attention. Hence these polymers are gaining attention as alternatives to synthetic plastics. PHA being thermoplastic polyester has the

potential to replace petrochemical plastics in a majority of applications. The extensive range of physical properties and broadened performance obtained by compounding and blending is exploited in such applications. Various applications for PHAs have been envisaged which includes molded containers, backsheet of hygiene articles such as diapers, coating agents, packaging materials etc. It is exploited in bulk applications such as coatings, low strength packing, medium strength structural materials, medical temporary implants (such as scaffolding for the regeneration of arteries and nerve axons), water based latex paints etc.

A wide variety of bacteria both gram positive and gram negative, aerobic, anaerobic, photosynthetic, lithotrophs and organotrophs are known to accumulate PHA intracellularly as carbon and energy source. Even though numerous bacteria are known to produce PHA during their growth, only a few are reported to produce high concentrations of PHA in their biomass. These organisms have limitations and hence the potential of other microorganisms in this regard needs to be explored. There is a lot of diversity among bacteria with regard to quantity and quality of PHA accumulated. Because of this diversity there is scope to discover better producers. Hence there is a need to explore indigenous bacterial cultures for PHA accumulating capability. The members of the family *Rhizobiaceae* differ in the mode of adaptations to stress and imbalanced nutrient conditions. They offer a variety of metabolic pathways directing carbon towards synthesis of biopolymers such as polysaccharides or polyhydroxyalkanoates. It is therefore interesting to study the intricacies of

carbon metabolic traffic being directed either towards polyhydroxyalkanoate synthesis or polysaccharide biosynthesis with respect to *Rhizobia*.

This study aims at studying the capability of a locally isolated bacterium, *Rhizobium meliloti* 14 for high production of biopolymer such as polyhydroxyalkanoates. This is an effort towards development of natural plastics from bacteria, which have potential to replace synthetic plastics and thereby in the long run eliminate the non-degradable plastics.

The details of the thesis are worked out based on the following objectives:

Objectives

1. Isolation and purification of *Rhizobia* from leguminous plants (such as root nodules). Screening of isolated cultures for the production of polyhydroxyalkanoates (PHA) and other similar biopolymers.
2. Identification of potent strain for the production of polyhydroxyalkanoates or other biopolymers by morphological and biochemical methods. Optimization of cultural and nutritional parameters for biopolymer production.
3. Improvement of potent strain for biopolymer production by mutation using physical or chemical methods.
4. Isolation of specific biopolymer such as polyhydroxyalkanoates from the culture using physical chemical or enzymatic methods.
5. Physicochemical properties of the isolated biopolymer (Melting point, solubility, molecular weight, composition etc).

Based on the above objectives the details of the materials, methods and results obtained in the present study are described in the thesis under the following chapters:

Materials and Methods (general)

The chapter on general material and methods, gives details about the general microbiological media and media components used in the study. This chapter forms the basis for all the experiments done in the future chapters. General cultivation methods, sterilization methods, staining methods and analytical methods used in this study are discussed. General staining techniques of PHA such as sudan black staining and nile blue staining, general methods of PHA extraction such as hypochlorite extraction, general media used such as nutrient agar, yeast mannitol agar and polyhydroxyalkanoate production medium and their preparation are dealt here.

Chapter 1: Isolation, screening and identification of polyhydroxyalkanoate producing *Rhizobia.*

The first chapter of the thesis deals with the isolation, screening and characterization of PHA producing *Rhizobia*. Here the different methods of isolation of polyhydroxyalkanoate producing bacteria have been dealt with. Various *Rhizobia* were isolated from soil, root nodules and other natural soil

samples. Isolation and screening of different PHA producing *Rhizobia* is dealt with by three different methods.

1. Traditional identification methods
2. Identification by polymerase chain reaction (PCR)
3. Identification by Fourier transform infrared spectroscopy (FTIR)

Rhizobia were isolated from various natural samples such as soil and root nodules of leguminous plants. Based on different staining techniques nearly 20 colonies were isolated as positive PHA producers from a batch of 150 purified strains. They were further grown in the Yeast Mannitol Agar medium along with standard strains and were examined for PHA production after hypochlorite extraction. Growth experiments and identification tests of *Rhizobia* have been discussed here. Growth of the isolated culture on different carbohydrate sources and on different amino acids was also studied. Microscopical observations, which were done using light, phase contrast and scanning electron microscope has also been explained in this chapter.

In the present study amount of PHA produced by the tested cultures varied from 5 - 65%. The standard strains produced PHA in the range of 11-47%. Amongst all the cultures that were tested culture R14 showed promising PHA yields of about 65%. This was 10% higher than previously reported data and hence this culture was further investigated. Based on the morphological, biochemical and nodulation tests R 14 was identified as a strain of *Rhizobium meliloti* (*Sinorhizobium meliloti*) and designated as *Rhizobium meliloti* 14.

An alternative technique such as polymerase chain reaction was used to develop a rapid detection technique for PHA producing bacteria. A total of 22 strains, both PHA positive and PHA negative strains were tested by PCR using the designed primers. There was a significant difference in the banding pattern of PCR amplicons between producers and non-producers of PHA. The absence of any amplification by the PHA negative strains justified the use of these primers for effective identification of PHA producing *Rhizobia*. Identification of PHA producing organism was also carried out using Fourier transform infrared spectroscopy (FTIR).

Chapter 2: Characterization of cultural conditions for polyhydroxyalkanoate production

The second chapter deals with characterization of cultural conditions for polyhydroxyalkanoate production. The chapter examines the selected culture for polyhydroxyalkanoate production and details of optimizing media for its production. Biochemical and physiological requirements of the organism such as the nutrient requirements for growth of the organism and the limiting nutrient that favours polyhydroxyalkanoate production, aeration, pH and temperature requirements and also the growth kinetics of the organism is studied. This chapter gives an overall idea about the general requirements for the growth and polyhydroxyalkanoate production by the selected organism. It also gives a foundation for the next chapters wherein improvements are made to enhance

polyhydroxyalkanoate production as well as to design an optimal medium for its production. Growth kinetics of the isolated culture with yeast extract mannitol medium has also been discussed here. The chapter as a whole gives a clear idea about the biochemical and physiological requirements of the selected culture, *Rhizobium meliloti* 14 for growth and PHA production.

Rhizobium meliloti 14 required an optimum pH of 7 and temperature of 30^{o}C at 200 rpm for good growth as well as PHA production. The most favoured carbon sources for PHA production were mannitol and sucrose. The initial studies suggested that nitrogen deficiency is one of the prerequisites for PHA production. By studies on the growth kinetics (in shake flask experiments) of the organism growth pattern and PHA accumulation in *Rhizobium meliloti* 14 were ascertained. A carbon and nitrogen ratio of minimum 105 was necessary for high PHA yields. It was also observed that both PHA and extracellular polysaccharide were produced simultaneously. As the carbon source supplied would was also be utilized for polysaccharide production, it was necessary to obtain a mutant which would be a negative polysaccharide producer.

Chapter 3: Strain improvement for enhancement of polyhydroxyalkanoate synthesis

The third chapter deals with the strain improvement by mutation. The results of improvement in the yields of polyhydroxyalkanoate by the mutant are summarized in this chapter. The chapter also deals with comparison of the

plasmid profiles of the parent (*Rhizobium meliloti* 14) and mutant (*Rhizobium meliloti* 22) strains. Comparison of parent and mutant strains at different carbon and nitrogen ratios is also discussed in this chapter.

Mutation of *Rhizobium meliloti* 14 resulted in a strain that accumulated nearly 10 % more of PHA. The exopolysaccharide produced by the mutant was 50% less compared to the parent. Plasmid profiles of *Rhizobium meliloti* 14 and *Rhizobium meliloti* 22 showed the lack of a high molecular weight plasmid in the mutant. Carbon uptake was significantly higher in the mutant (*Rhizobium meliloti* 22).

Chapter 4: Optimization of medium and cultural parameters for polyhydroxyalkanoate production

The fourth chapter deals with the optimization of medium and cultural parameters for polyhydroxyalkanoate production. Here response surface methodology (RSM) has been used for the optimization of bacterial polyhydroxyalkanoate yield. The technique has been extended to assess the nutrient limitation conditions favorable for polyhydroxyalkanoate accumulation in bacterial cells and to compare the nutritional performance of the mutant strain with that of parent simultaneously with limited experiment using CCRD experiments. Details of adopting this technique for factors and interactions, which affect the desired response, and tests for their effectiveness for optimizing the

nutritional parameters required has been discussed in limited number of experimentation.

The results suggested that phosphate was the limiting nutrient favouring PHA production in parent (*Rhizobium meliloti* 14). Mutant showed requirement for both phosphorus and nitrogen unlike parent. The quantitative yield of PHA and carbon conversion efficiency of the mutant was consistently higher compared to parent when grown in medium containing urea as the nitrogen source. The carbon conversion efficiency of the strains into PHA could go as high as 0.6 PHA (g) /g of carbon utilized, subject to the fermentation conditions. The PHA yields in both the strains exceeded 80% and reached 85 to 89% depending on the cultural conditions. The highest PHA yield ranged between 5.5 g/l to 6.5 g/l. Biomass obtained ranged between 6.0 g/l to 9.0 g/l.

Chapter 5: Characterization of polyhydroxyalkanoates

The fifth chapter deals with the characterization of polyhydroxyalkanoates obtained from *Rhizobium meliloti* 14. Chemical and structural characterization of the polymer was done by using ultra violet spectroscopy, infrared spectroscopy, gas chromatography, GCMS (mass spectroscopy) and nuclear magnetic resonance (^{1}H and ^{13}C NMR) methods. Melting point of the extracted polymer has been tested by differential scanning calorimetry (DSC). Solution casting of the films, tensile strength, water vapor permeability and oxygen transmission rate of the films obtained are also discussed in this chapter.

The results confirm that the PHA extracted from the sample contains 3-hydroxy functional group and the presence of methyl esters of hydroxy butyrate and valerate, the copolymer was about 3% of the total polymer content. The extracted polymer could be easily casted into films. The solution cast films could be air-dried and they peeled off the glass plates into thin transparent clear films. The tensile strength of the film was comparable to polypropylene.

Chapter 6: Production and characterization of copolymers

The sixth chapter deals with the production of and characterization of copolymers such as polyhydroxyvalerate. Copolymer production is important because the polymer becomes more flexible and finds more applications if copolymers are present. Various plant oils, animal fats, fatty acids and other organic acids etc have been tried as carbon sources in order to enable the organism to produce copolymers. Analysis and characterization of the copolymers by gas chromatographic methods have been discussed in this chapter.

Rhizobium meliloti 14 did not require addition of extra carbon substrates to induce copolymer production. 3% of valerate was seen in the polymer extracted from the cells grown in medium containing sucrose as sole carbon source. However a PHB: PHV content of 81: 19 and 69.7% of PHA yield was seen when the medium was supplemented with rice bran oil. PHB: PHV content of 90:10, 95:5, and 98:2 was seen when *Rhizobium meliloti* 14 was grown in medium

containing pongamia oil, palm oil and stearic acid respectively. PHA yield was 72% with PHB: PHV content of 88:12 when pyruvic acid was added in the medium as a co-substrate. Supplementing sodium butyrate also increased the HV content to 5.3% in the cells. Overall the organism was capable of synthesizing PHA with high valerate content. This is highly significant, as increase in the valerate content will improve the material properties of the polymer.

Chapter 7: Extraction and purification of polyhydroxyalkanoates

The seventh chapter deals with the methods of extraction and isolation of polyhydroxyalkanoates. Polyhydroxyalkanoates are intracellular storage polymers. They have to be extracted by breaking the bacterial cells. Various methods of extraction were tried for extraction of polyhydroxyalkanoates. Efforts were towards easier down stream processing after fermentation. Extraction using solvent such as chloroform has been discussed. Chemical extraction using sodium hypochlorite for breaking the cell walls and use of surfactant and chelating agents in order to digest and chelate the non PHA cell material are discussed. Enzyme extraction methods are also done using commercial enzymes such as proteases. Isolation and identification of the actinomycete culture capable of digesting the *Rhizobium meliloti* 14 has been dealt in this chapter. Simple and efficient extraction of PHA from the lytic culture and also by the lytic enzyme obtained from the lytic culture has also been highlighted. Lytic

activity of the crude enzyme from actinomycete culture has also been explained here. Purity analysis of the enzyme-extracted samples is also been dealt here.

Extraction by the lytic culture proved to be a simple and effective microbial method for extraction of polyhydroxyalkanoates from bacterial cells. In this method lytic enzyme of an actinomycete (*Microbispora*) culture has been used to lyse *Rhizobium meliloti* 14 cells. The *Microbispora* culture grew directly on heat killed *Rhizobium meliloti* 14 cells and no extra nutrients were added for the growth of the lytic culture. The lytic culture formed pellets and it could be separated easily by filtration. PHA was released into the broth and was extracted by a minimum quantity of chloroform. PHA extracted by this method was 90-94% pure. Crude culture filtrate was also effective in lysis of *Rhizobium meliloti* 14 cells. The *Microbispora sp* produced lytic enzyme, which could solubilise, the bacterial cell material and hence release PHA. Scanning electron microscopic analysis of enzyme treated biomass of *Rhizobium meliloti* 14 cells have also been studied.

Summary and conclusions

This part of the thesis deals with the salient findings of the work carried out and important conclusions drawn on the basis of results obtained.

References

All references pertaining to bacterial polyhydroxyalkanotes, which includes publications in journals, books, reviews, internet search and patents with a stress on the recent literature are tabulated using standard format under this section.

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Introduction

Polymers are the backbone of our life. Both material and the biological matter are standing on the strong bonds that these amazing macromolecules hold together. Various polymers are used as food, clothing, shelter and fuel. They are the building blocks of all living systems i.e. plants, animals and microorganisms. The biological world is made up of a number of polymers such as proteins, polysaccharides, polynucleotides, polyamides and lignin. Some of the earliest polymers known to man were silk, wool, cotton, cellulose, gums and starch. All these polymers have contributed towards human civilization. The advent of plastics or man made polymers started a new era in the polymer world. The changes from the stone age to bronze age to iron age took several thousands of years. But plastic age developed rapidly after the steel age. These highly versatile polymers have gradually replaced the other polymers in a myriad number of applications. They have influenced our economy, standard of living and health conditions, transportation, pharmaceuticals, building and construction and power sector. Their influence on the economic sector is unparalleled. They make or break economies and have transformed the quality of our society. Infact they have permeated every facet of human life (Shivaram and Singh, 2003).

CLASSIFICATION OF POLYMERS

Polymers can be broadly classified into two groups.

1. Natural or Biopolymers
2. Synthetic or Man-made polymers

1. Natural polymers or Biopolymers

Natural polymers are referred to those polymers that are formed in nature during the growth cycles of living organisms. They are also called as biopolymers. Based on the origin biopolymers may be further classified into plant, animal and microbial-based (Fig 1).

Apart from this, polymers are generally classified into seven classes (Table 1). They are nucleic acids, proteins (polyaminoacids), polysaccharides, polyhydroxyalkanoates, polyphosphates, polyisoprenoids and lignin. An eighth type, polythioester has been recently included (Eversloh et al, 2001).

2. Synthetic or Man-made plastics

These polymers are made from hydrocarbons derived from petroleum. Depending on its ultimate form and use these synthetic polymers are classified as follows (Gowariker et al, 1996):

a. **Plastics:** Polymers, which can be molded or formed into any state of our choice.

b. **Elastomers:** Elastomers are popularly called as rubbers.

c. **Fibres or liquid resins:** Some of the well-known synthetic fibres are nylon, polyesters, polypropylene and acrylics.

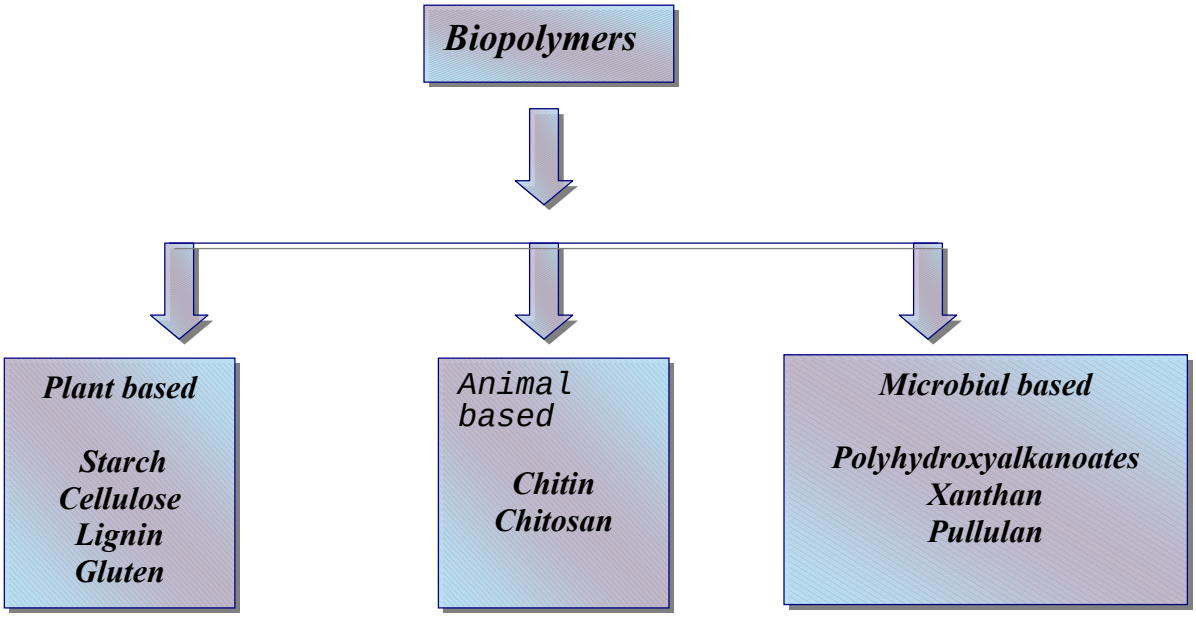


FIG 1: CLASSIFICATION OF BIOPOLYMERS BASED ON THEIR ORIGIN.

TABLE 1: CLASSES OF BIOPOLYMERS AND THEIR OCCURRENCE.

Class	Synthesis in	
	Prokaryotes	Eukaryotes
Nucleic acids	Yes	Yes
Proteins; Polyaminoacids	Yes	Yes
Polysaccharides	Yes	Yes
Polyhydroxyalkanoates	Yes	No
Polyphosphate	Yes	Yes
Polyisoprenoids	No	Plants, some fungi
Lignin	No	Only plants

Source: Modified from Eversloh et al, 2001

Synthetic polymers are further divided into two types,

a. Water-soluble

b. Water-insoluble

Water-soluble polymers are specialty polymers with functional groups that affect water solubility such as carboxyl, hydroxyl and amido group. Water-insoluble polymers are referred to as commodity plastics. They include polymers such as polyethylene, polypropylene, polystyrene, poly (vinyl chloride), poly (ethyleneterephthalate) and Nylon 6,6. Some synthetic polymers are biodegradable but most commodity plastics are non-biodegradable (Kawai, 1995).

GENERAL PROPERTIES OF POLYMERS

In Greek 'Poly' means 'many' and 'mer' means part. Polymers are a class of giant molecules (macromolecules) that are composed of smaller units. Simple units are referred to as monomers and more complicated building blocks are referred to as 'repeating units'. The process by which the monomers are assembled into polymers, either chemically or biologically is referred to as polymerization. When only one type of monomer is present, the polymer is referred to as a homopolymer. A copolymer is formed when two or more different monomers are linked together. Polymers can be linear or branched. They may be further classified as crystalline or amorphous. The following properties influence the physical property of a polymer:

1. Monomer composition.
2. Type of linkage.
3. Size or molecular weight.

The most important characteristic of a polymer molecule is their chain length, since that determines whether they have any usefulness at all. In general, characteristics such as melting temperature and glass transition are strongly dependent on the chain length up to degrees of polymerization of approximately 100. For example the viscosity of molten polymer varies as degree of polymerization, owing to entanglements between neighbouring chains in the melt. Copolymerization is used to modify properties such as crystallinity, solvent resistance, permeability and toughness, which generally vary with copolymer composition (Fourier et al, 1995). Some of the important properties and characteristics of polymers that should be understood are:

Melting point

Polymers exist only as solid or liquid and never as gas as they decompose before reaching their boiling point. The temperature at which there is a change in the polymer state is called the melting point.

Glass transition temperature

The glass transition temperature is an important parameter of a polymeric material. It is used as a measure for evaluating the flexibility of a polymer molecule and the type of response that would exhibit to mechanical stress. The temperature below which a polymer is hard and above which, it is soft, is called glass transition temperature.

Crystallinity

Crystallinity is expressed in terms of that fraction of the sample, which is crystalline. The density of the crystalline component is higher than the amorphous component. Other properties such as density, modulus, hardness, permeability and heat capacity will be affected by crystallinity.

Tensile strength

Tensile strength is a measure to withstand forces that tend to pull it apart to determine the extent to which the material stretches before breaking.

Biodegradability

Biodegradability is the ability of the polymer to be utilized as a nutrient source by microorganisms and converted into carbondioxide, biomass and water.

APPLICATIONS OF POLYMERS

Polymers have become an integral and indispensable part of life today. The natural polymers such as nucleic acids and proteins carry and manipulate essential biological information and polysaccharides provide fuel for cell activity. These polymers are integral part of the living systems and form the structural elements of life on earth. However, only plastics and plastic like materials are being discussed here.

Throughout history, man has relied extensively on biological materials such as wool, leather, silk and cellulose. Silk is a protein fibre whose mechanical properties were valued by textile industry long before the amide bond was used by the chemists for creation of nylon. Cellulose in the form of cotton is a major crop of the world and cellulose derivatives are used in polymer industries. With advances in chemistry and material science, a vast array of novel synthetic polymers generally known as 'Plastics' has been introduced. Plastics have many attractive properties compared to natural polymers. They are light in weight, high in strength, can be super tough, rigid as well as flexible. They can be molded to any designs, can be made transparent or opaque. Most important aspect of these versatile polymers is that they are extremely cost effective. Plastics have almost replaced materials such as metal, glass, wood, paper, fiber and ceramics, in packaging, automobiles, building constructions, biomedical fields, electronics, electrical equipment, appliances and furniture. From agriculture to transport and

from aerospace to food packaging the use of plastics has become an integral part of modern day living (Brown, 1991).

GROWTH OF PLASTICS AND ITS IMPLICATIONS ON THE SOCIETY

Plastics are made from oil, natural gas, coal and salt (Fig 2). The polymer / plastic growth worldwide has been steady around 6 per cent per annum which is much higher than the GDP (gross domestic product) growth rate of 3.3 per cent. The annual world production of polymer materials was around 150 million tons in 1996 with the average per capita consumption of plastics in developed countries ranging from 80-100 kg per year. The demand for polymers was poised to increase from 2.6 million metric tons per annum (MMTPA) to 4.2 MMTPA by the year 2002 and is expected to cross 7.7 MMTPA by the year 2007.

The plastic consumption in China was estimated to be 16 million tons in 2000, fifth in the world after USA, Japan, Germany and South Korea. In USA, the annual per capita consumption of plastics is 90 kg and in the European union it is 60 kg and the world average is 15 kg (Market assessment).

In India, the consumption of plastics has been trebling every decade. The present consumption has grown upto 4 million kilotons and is likely to go up to 12 million kilotons by 2010. Indian per capita consumption was only 1.9 kg, the lowest in the world but was projected to reach 3.2 kg by 2002 (Market assessment).

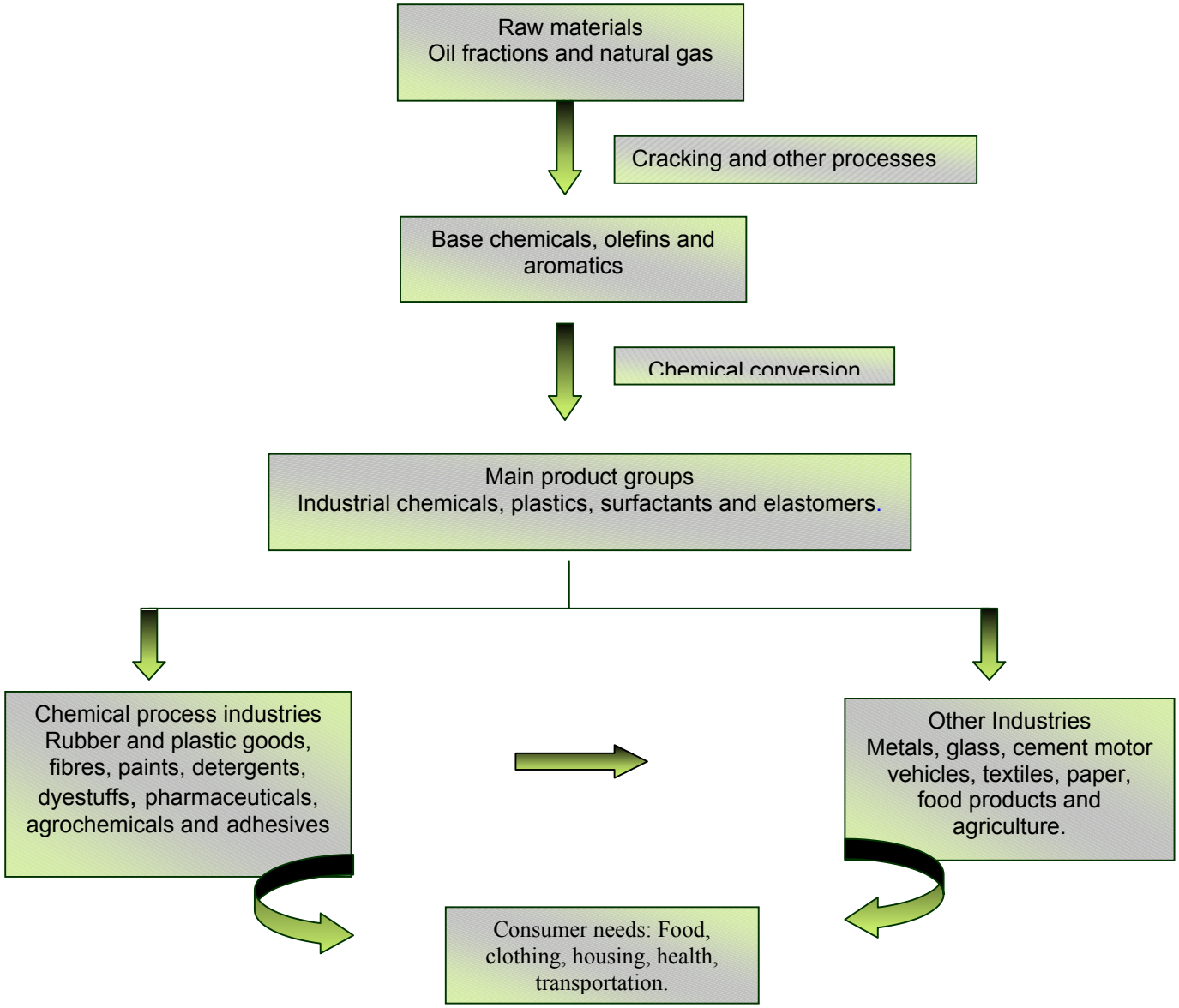


FIG 2: CONVERSION OF OIL TO POLYMER PRODUCTS (MISRA, 2003).

However demand for plastics reached 4.3 MT in the year 2001-2002 and is expected to reach 8 million tons by 2006-07 (Biodegradable plastics). Major end uses of plastics in India are for agricultural and water management 25%, construction 25%, electronics 17%, packaging 15%, transportation 15%, and others 3%. There are 8 major polymer producers in India. Indian petrochemical corporation Ltd. (IPCL), Haldia petrochemicals Ltd. (HPL), Reliance industries Ltd. (RIL), Gas authority of India Ltd. GAIL, Natural Organic Chemicals Industry Ltd. (NOCIL), Polyolefins Ltd., (PIL), Southern petrochemicals and Industrial Corporation (SPIC) and ICI/ Oswal industries are the major ones. Among the various polymers produced, LDPE is 1,80,000 MTPA, linear LDPE is 9,70,000 MTPA, HDPE is 3,60,000 MTPA and PP and PS is 9,00,000 MTPA (Market assessment). The present market in India is 25,000 crore (Biodegradable plastics).

The fear of depletion of wood resources has established plastics as the material of choice in many applications. In food sector, various characteristics of plastics such as low density, ready sealability, resistant to break, appearance, impermeability to oxygen and water vapour, low temperature and flexibility have contributed for its large usage (Levy, 1993). Nearly one fifth of (about 3.5 - 4 million tons) the total plastic consumption worldwide is used as packaging, of which half is foamed plastics such as expanded polystyrene (Ren, 2003). Plastic shopping bags are the most ubiquitous consumer item on earth. Their light weight, low cost and water vapour resistance make them very convenient

for carrying groceries, clothing or any other routine purchase that it is hard to imagine life without them. The first plastic bag was introduced in USA in 1957 for sandwiches, fruits and vegetables. Plastic trash bags appeared around the world around 1960 and they took off by 1970 as HDPE (Halweil, 2004).

"WHITE POLLUTION"- HAZARDS OF PLASTICS

Unfortunately, these highly durable, versatile and extremely useful materials cause adverse effects on the environment after its use. Plastics are not biodegradable. They accumulate in the environment and harm the ecosystem.

The United States of America discards approximately 25 million tons of plastic household waste every year and most of it is landfilled where it remains undegraded for years. In China, the annual plastic waste generation is 4 million tons with only 10% recovered or recycled and about 20-30% is incinerated or landfilled. (Hankermeyer and Tjeerdema, 1999). In Europe and Japan there are only a few sites left that can be landfilled (Risch, 1991).

Plastics lead to adverse affects on environment during and after use. These polymers which are synthesized chemically by polymerization contribute towards air pollution and waste management problems. Plastics threaten animal life. Some well-known cases that are reported are the death of cows due

to choking after consumption of plastics. Millions of marine animals are also killed every year due to toxicity caused by plastic debris or entanglement with them. The plastic packages are indiscriminately thrown away from houses, trains and shops. Some drain into drainage and block them. If plastics are incinerated they produce poisonous gases like carbon monoxide and chlorine for e.g. burning of PVC results in production of hydrogen chloride, a corrosive pollutant. Americans throw away 100 billion plastic grocery bags each year. These are becoming more and more common in poorer nations as well. Bags produced in Asia account for one quarter of those used in wealthy nations (Halweil, 2004). Accumulated plastic film residues in the soil have caused significant decrease in agricultural yield. Plastic wastes floating on rivers and lakes are increasingly threatening fishery, navigation, and operation of hydropower plants, irrigation and other public works. Moreover, as over 99% of plastics are fossil fuel origin, their rapid increase will put further pressure on the already limited non-renewable resources on earth (Ren, 2003).

Because plastics account for 20% of volume of solid municipal waste, there is increasing motivation for society to find alternate disposal methods for them. Overall there is no concerted effort done for plastic management.

PLASTIC WASTE MANAGEMENT

Domestic organic wastes including plastics are usually disposed off in landfills. However, burying of waste is no longer considered an ecologically acceptable method. Scott (2000) has suggested an alternative option for waste management. The method indicates the need for mechanical recycling, energy recovery and biological recycling of wastes (Fig 3).

The United Nations Environmental program (UNEP) defines waste management as taking all practical steps to ensure that wastes are managed in a manner which will protect human health and the environment against the adverse effects. The major principles considered in such waste management are (Ren, 2003):

1. Source reduction
2. Integrated life cycle
3. Integrated pollution control
4. Polluter pays
5. Standardization
6. Public participation.

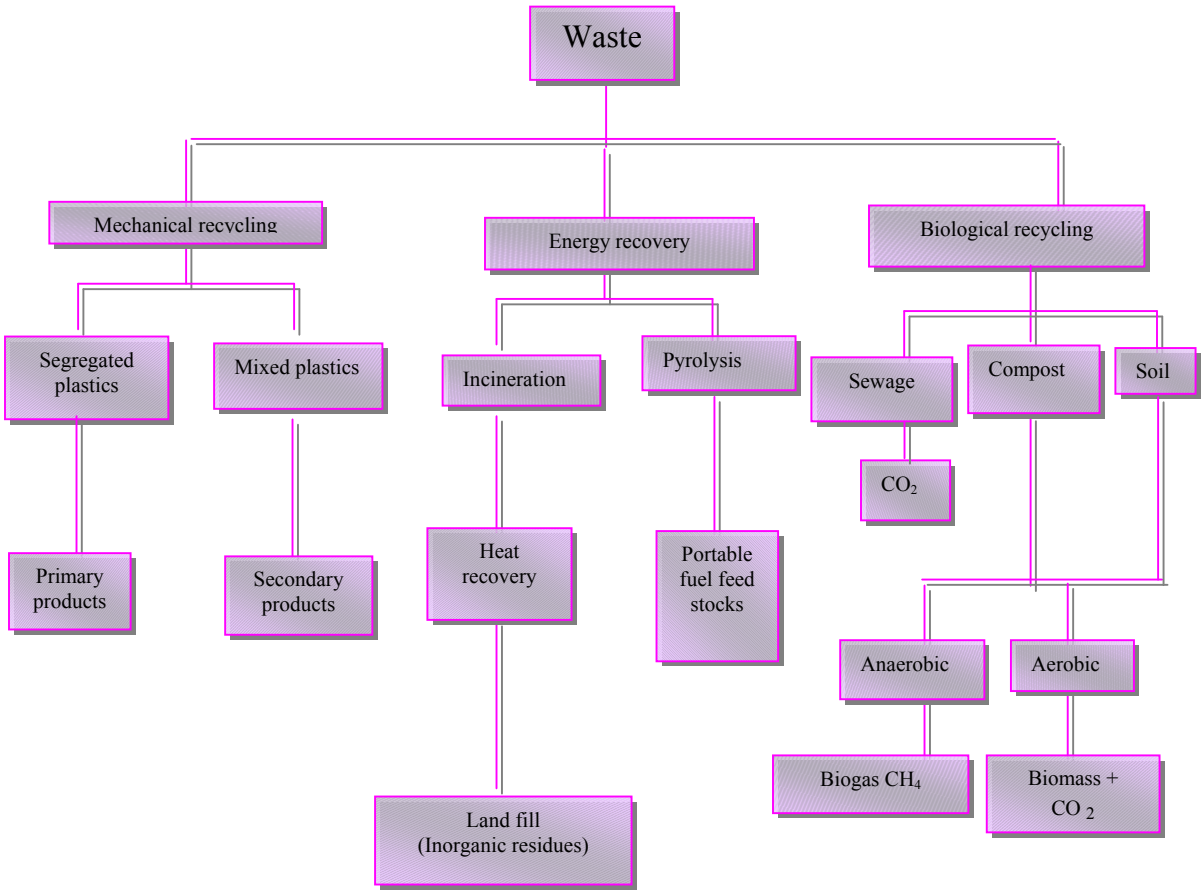


FIG 3: POLYMER WASTE MANAGEMENT OPTIONS (SCOTT, 2000).

Derived from these principles, a sound waste management follows the strategy of ***'Reduce, Reuse, Recycle and Recovery of energy'***.

In Europe as well as world wide, legal actions support environmentally sound waste management systems. European parliament and council of directive 94/62/EC of 20 December 1994 on packaging and packaging waste, states clearly that the prevention of waste should be 'a first priority', while reuse, recycling and other forms of recovery are 'additional fundamental principles' for the reduction of final disposal of such wastes. This packaging directive also highlights the key role played by public participation for the success of waste management (Ren, 2003) (Table 2). The concept of taxing plastic bags as means of curbing plastic waste is starting to become popular worldwide. The introduction of consumption tax of Euro 0.15 per bag on plastic bags by Irish government in 2002, led to a reduction of 90% in the first month of operation (Klemz, 2002). However the enthusiasm on tax in UK has waned (Bennet, 2003). Taiwan is the largest Asian country to drive restrictions and even ban on plastic disposables with environmental protection and administration banning disposable tableware, including polystyrene foam containers and plastic bags (Moore, 2003). In china, the law of solid waste pollution prevention and control (1996) defines the responsibility of producers, sellers and users in recovery and recycling. It says that producers and users should choose easily recyclable, disposable and environmentally safe

TABLE 2: PROS AND CONS OF MAJOR WASTE TREATMENT TECHNOLOGIES

Technology	Pros	Cons
Recycling	❖ Reduce amount of wastes for disposal ❖ Save resources and energy in virgin production ❖ Extend product's life time, conserve resources	❖ Not everything economically recyclable ❖ Recycling consumes energy and emits pollutants ❖ Recycled product inferior in quality, low-grade application, and limited market.
Composting	❖ Reduce load of landfill by digesting organics ❖ End product useful for soil amendment ❖ Need less energy than recycling, incineration.	❖ Economics still unfavorable ❖ Risk of odor and pest problem ❖ No reliable market for end product (Compost)
Incineration	❖ Reduce weight substantially by volume/weight ❖ Generate energy ❖ Need small space, reduce burden of landfill	❖ High capital and operational costs ❖ Emission of hazardous substances ❖ More stringent in operation and control
Landfilling	❖ Final and indispensable disposal of wastes, residues from recycling, incineration etc. ❖ Relatively easy to build and operate.	❖ Suitable sites become scarce worldwide ❖ Cost is increasing significantly due to higher economical and sanitary requirement ❖ Leachate and gas

		emission problems.

Source: Ren, 2003

materials as packaging (Ren, 2003). There are reports on ban on plastic bags in Bangladesh as well as in Bhutan (Moore, 2003).

Government of India (the Ministry of environment and forests) has issued a notification dated 2nd September 1999 under the title 'Recycled plastics manufacture and usage', prohibiting the use of recycled plastic carry bags for carrying, storage and packing of ready to eat food items. Some cities in India have seen limited success at banning plastic bags.

According to E S Stevens, "In ignoring nature's way of building strong materials we have for many applications over engineered our plastics for stability with little consideration of their recyclability or ultimate fate and ended up transforming irreplaceable resources to mountain of wastes" (Stevens, Green plastics).

In conclusion, the lack of biodegradability, growing water and land, surface litter problem, air and pollution of incinerated plastics has raised worldwide concern on use of plastics. Amidst the pro plastic arguments, there still is a strong need for the look out for alternatives for synthetic plastics and to replace them steadily. All this has promoted worldwide research to develop new biodegradable alternatives to plastics.

BIODEGRADABLE ALTERNATIVES

Biodegradable polymers or bioplastics are important and interesting areas that are being looked out as alternatives for synthetic plastics. These are a new generation of materials able to significantly reduce the environmental impact in terms of energy consumption and green house effect. ISO 472 -1988, a standard authority defines a biodegradable plastic as, 'a plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties that may vary as measured by standard test methods appropriate to the plastics and application in a period of time that determines its classification'. The change in chemical structure results from the action of naturally occurring microorganisms. Biodegradation is a process by which bacteria, fungi, yeasts and their enzymes consume a substance as food source so that its original form disappears (Chandra and Rustgi, 1998).

Biodegradable polymers are classified into two broad groups. They may be of natural origin or synthetic or man made (Table 3). Further biopolymers are classified based on their origin and method of production as follows:

TABLE 3: CLASSIFICATION OF BIOBASED POLYMERS BASED ON THEIR ORIGIN AND METHOD OF PRODUCTION

<table>
<tr><td colspan="8">Biobased Polymers</td></tr>
<tr><td colspan="6">Directly extracted from biomass</td><td colspan="1">Classically synthesized from bioderived monomers</td><td colspan="1">Polymers produced directly by organisms</td></tr>
<tr><td colspan="4">Polysaccharides</td><td colspan="2">Proteins</td><td>Lipids</td><td>Poly lactate</td><td>PHA</td></tr>
<tr><td>Starch</td><td>Cellulose</td><td>Gums</td><td>Chitosan/ Chitin</td><td>Animal</td><td>Plant</td><td>Cross linked triglyceride</td><td>Other Polyesters</td><td>(Polyhydroxyalkanoates)</td></tr>
<tr><td>Potato
Maize
Wheat/ rice derivatives</td><td>Cotton
Wood
Other derivatives</td><td>Guar
Locust bean
Alginates
Carrageenan
Pectins etc</td><td></td><td>Casein Whey
Collagen
Gelatin</td><td>Zein Soya
Gluten</td><td></td><td></td><td></td></tr>
</table>

Source: Paltani and Goyal, 2003

PLANT BASED POLYMERS

1. Starch

Starch is an inexpensive agricultural resource and is industrially produced in large quantities (7 million tons per year in Europe). Nearly 50% of starch is used for non-food applications and about 30% of the starch production are industrially used for making films. Starch, a polysaccharide has a mixture of linear and branched polysaccharide with the latter predominating. It is a repeating unit of mixture of two polymers, amylose and amylopectin. Amylose is a predominantly linear (to highly branched) polymer, comprised of (1- 4) α-D-linkages with number average molecular weight in the range of several thousands. Amylopectin is highly branched with intermittent (1–6) linkages. Its molecular weight is of the order of several millions and can be as high as 50 millions. Starch is a renewable degradable carbohydrate polymer that can be purified from various sources by environmentally sound processes. It is found in high amounts in plants like corn (maize) potato and wheat. Three generations of starch as bioplastics have come through history:

1. Starch as only a filling material along with synthetic plastics. Here the biodegradable part of plastic was only 5-20 %.

2. Hydrophilic synthetic polymer base filled with 50-80% starch.

3. Starch as plastics with 100% starch.

Starch itself has a severe limitation. It is water-soluble, hence will swell and deform upon exposure to moisture. Therefore blending with hydrophobic synthetic polymers chemically modifies them. Starch only plastics are highly suitable in cushioning electrical goods where the packages are kept dry. Thermoplastic starch (TPS®) has been developed by BIOTEC®, Germany. TPS® derivatives, starch esters, bioplastic granules (BIOPLAST) and films (BIOFLEX) are being produced (Lorcks, 1998).

2. Cellulose derivatives

Cellulose is a structural polysaccharide and is the constituent of higher plants. It is the most abundant natural polymer on earth and is a linear polymer of anhydroglucose. It is highly crystalline, fibrous and insoluble in water in its native form (Petersen et al, 1999). It can either be used in its original form or modified into cellulose acetate, cellulose butyrate or cellulose nitrate. Cellophane a regenerated form of cellulose was the first transparent, flexible packaging film that was developed. Since the introduction of synthetic thermoplastic packaging films in 1950's cellophane sales have dropped by 90% inspite of having good tensile strength, elongation, excellent printability and good machinability (Krock, 1997). Moreover for film production cellulose is dissolved in an aggressive toxic mixture of sodium hydroxide and carbon disulfide (Xanthation) and then recast into sulphuric acid to produce a cellophane film. These films are sensitive to moisture, not heat sealable,

manufactured at the cost of trees and are highly inflammable. In order to improve its moisture barrier properties blends such as cellulose acetate films, cellulose ether and ethyl cellulose films are manufactured. These have better moisture, oil, gas and grease resistant properties. But modification of cellulose films is costly and difficult. Also gas and moisture barrier properties of cellulose acetate are not optimal with respect to food packaging (Petersen et al, 1999).

Apart from these, corn zein, wheat gluten, soy protein have been exploited as packaging materials. The barrier, vitamin adhesion and anti microbial carrier properties of zein film coatings have been used on a variety of foods. Zein is also used on pharmaceuticals and for production of water-soluble pouches for dried foods. Wheat gluten and soy protein is used in sausage casings.

ANIMAL BASED

1. Chitin and Chitosan

Chitin is the main structural component of shells of crustaceans, molluscs, and insects. It also forms parts of jaws and body spines of certain worms, and is found in cell walls of fungi and in some algae. It is almost as common as cellulose and this ubiquity has made it useful in some potential uses. Chitosan is a derivative of chitin that is deacetylated. Chitin is a poly- β-(1-4)

linked N-acetyl-D-glucosamine widely found in nature. It is produced commercially by deacetylating chitin obtained from shellfish waste. Chitosan films are clear, tough, flexible and have good oxygen barrier properties. They can be obtained by casting from aqueous solution. Chitosan-based coatings can protect foods from fungal decay and modify the atmospheres of fresh fruits.

2. Collagen

Collagen is a fibrous, structural protein in animal tissue that can be converted into edible and biodegradable films. These are not very strong and tough, but have reasonably good mechanical properties. They do not have good moisture barrier properties. These are commercially successful as edible films.

Gelatin, casein and whey proteins are also used as biodegradable packaging materials.

MICROBIAL BASED

1. Microbial extracellular Polysaccharides

Polysaccharides are condensation polymers of monosaccharides resulting in the formation of glycosidic linkages by elimination of water. Microorganisms (Table 4) produce several polysaccharides.

TABLE 4: POLYSACCHARIDES OF MICROBIAL ORIGIN

Polysaccharide produced	Microorganisms (source)
Xanthan gum	*Xanthomonas campestris, X oryzae, X phaseoli*
Dextran	*Leuconostoc mesenteroides*
Pullulan	*Aureobasidium pullulan*
Curdlan	*Alcaligenes faecalis var. myxogenes*
Scleroglucan	*Sclerotium rolfsii*
Succinoglucan	*Alcaligenes faecalis var. myxogenes, Agrobacterium, Rhizobium*
Gellan	*Pseudomonas elodea*
Elsinan	*Elsinoe leucospila*
Welan	*Alcaligenes sps*
Rhamsan	*Alcaligenes sps*
Levan	*Acetobacter levanicum, Bacillus subtilis*
Alginate	*Pseudomonas aeruginosa, Azotobacter vinelandii*
PS 7	*Beijerinkia indica var myxogenes*
S-130	*Alcaligenes ATCC31555*
S-194	*Alcaligenes ATCC3196*
S-198	*Alcaligenes ATCC 31853*

Source: (compiled) Kawai, 1995; Triveni, 2000.

The first microbial polysaccharide to be commercialized was dextran. Dextran is a α-1, 6-glucopyranoside polymer.

Bacteria such as *Alcaligenes faecalis var. Myxogenes*, *Azotobacter vinelandii* and *Beijerinkia indica* produce curdlan, bioalgin, and PS-7 respectively.

The only microbial polysaccharide that has reached the maximum commercialization is xanthan gum; an exopolysaccharide produced from bacterium *Xanthomonas campestris.* Xanthan has a cellulose type main chain and trisaccharide graft chains containing glucuronic acid.

Pullulan is a microbially produced polysaccharide; this water-soluble extract is a neutral glucan and is synthesized by a fungus *Aureobasidium pullulans*. Pullulan is composed of 1,6-linked maltotriose units. It is a α D-glucan in which maltotriose or maltotetrose units (linked) are coupled through α 1,6 bonds to form a polymer whose molecular weight is dependent on the cultural conditions. Although resistant to amylases these can be degraded by pullulanase from *Enterobacter areogenes* strains. The main use is because of its capacity in film formation. Films formed by 5-10% solutions have low permeability to oxygen especially when compared to other commercially available materials such as cellophane or polypropylene. Plasticizers are necessary for further processing.

All the above are being used as biodegradable alternatives to synthetic plastics in various applications but subject to their limitation of water solubility.

2. Polylactic acid (PLA)

Polylactic acid is derived from lactic acid for which carbohydrates in sugar beets, potatoes, wheat, maize and milk are the source. Polylactic acid is a substance familiar to the human body, as we ourselves produce it by every muscle contraction. The body can break it down. PLA can be processed through for example injection moulding, foil blowing and deep drawing. PLA may be applied as a coating. PLA is water-resistant but cannot withstand high temperatures (>55°C). In comparison to starch biopolymer the degradation process is very slow. However, within a composting facility it can be broken down in 3 to 4 weeks. PLA is commercially available as LACEA (polylactic acid) (Hiraishi and Khan, 2003).

3. Polyhydroxyalkanoates (PHA)

PHAs are a group of microbial polyesters formed intracellularly in a number of bacteria. These polymers have properties similar to synthetic plastics and have gained much attention and importance as future biodegradable plastics (Lee, 1996). Many of these are also commercially available as BIOPOL (PHBV) and BIOGREEN (PHB) (Hiraishi and Khan, 2003).

OTHER POLYMERS

Apart from these polyesters, the only other polyester existing in living organism are polymalic acid which is water soluble polyester occurring in lower eukaryotes. Cutin and suberin are water insoluble polyesters occurring in plants (Kolattukudy, 2001).

Other important biopolymers known are alginates, carrageenan, pectin, lipid films and protein films. Among these microbial polymers are gaining much importance as bioplastics.

CHALLENGES FOR BIOPLASTICS

Use of these biodegradable alternatives brings additional challenges, which needs to be addressed with at most importance. Biodegradability is not a favourable property in many of the applications. The more biodegradable a polymer is the less useful it is in many applications. Development of a biodegradable plastic is challenging because it has to perform better than synthetic plastics in all aspects and also be biodegradable. The issues of energy consumption air and water pollution and production of waste during the entire lifecycle of the product is questioned. Apart from this the economical aspect of the process from raw material to end products and the land utilized for the process is seriously addressed (Scott, 2000). Hence to achieve the benefits of biodegradable plastics there is a need to address the potential areas where it

can be implemented. To date the applications of biodegradable polymers have focussed on three major areas (Chandra and Rustgi, 1998):

1. **Medical**
2. **Agricultural**
3. **Packaging**

Biodegradable plastics have been developed as surgical implants and as drug delivery agents for controlled and long-term release of drugs to targeted sites. These biomaterials are also being used as absorbable surgical sutures, for use in the eye, bone fixation devices, vascular grafts, and artificial skin. Biopolymers are also used in controlled release of pesticides, nutrients, agricultural mulches and planting containers. Most of the biopolymers are developed into packaging materials especially as food packaging materials (Chandra and Rustgi, 1998). Biodegradable plastic packaging is slowly being adopted by food service companies for use as films for sandwich wraps or for packaging fresh products such as salads, pasta or bakery goods (Pierce, 2004).

Bioplastics are being considered not necessarily for biodegradable aspects but also because of their performance. Environmental friendliness is considered as an additional benefit and hence there is a lot of investment in the area. An interaction between research and implementation is necessary to make a significant impact on the food-packaging sector. Cargill Dow LLC, which

manufactures polylactic acid (PLA) is producing more than 300 million pounds and economically competing with the costs of polyethylene terepthalate (PET). Wilkinson Manufacturing and Wild oats Markets Inc are manufacturing thermoformed food containers and plastic containers (Pierce, 2004). Companies that are currently involved in the production of these polymers are listed in table 5.

An effort to understand and analyze microorganisms that are capable of producing bioplastics such as polyhydroxyalkanoates is the main effort of this study. This research unfolds ‘New Hope’ in the field of biodegradable alternatives to plastics.

TABLE 5: LIST OF COMPANIES INVOLVED IN PRODUCING BIODEGRADABLE PACKAGING MATERIAL		
Trade Name of the product	**Raw materials**	**Manufacturing company**
	STARCH BASED	
	PLA from whey and potato waste	Argonne Natl Laboratory, Argonne Illinois USA.
BioBag	Starch (Materbi)	Polargruppen Saltdal, Norway
Swirl	Starch Polycaprolactone (PCL)	Milleta (Biotech division Germany)
Bionolle1000	Bionolle & starch (Corn Starch) PLA & PE/ Butylene succinate	Showa high Polymer Co. Japan
Biopar	Starch	BIOP Biopolymer GmbH Germany
BIOPLAST	Starch cellulose synthetic polyester	Biotec GmbH, Wentus, Germany
BIOSKG	Starch/PVA Corn	PlastirollOY
Clean green	Starch (Wheat)	Clean green packages Minneapolis USA
Cohpol	Starch	Starch tech USA UTT Chemical technology Finland
Farthshell	Starch potato & limestone	Earth Shell Corp of Santa Barbara California
Ecopla	PLA	Cargill Dow polymers

Continued

Ecoware	Starch	Nisser, Japan
Envirofil	Starch/PVA	Enpac (Dupont &Con Agra joint venture)
EverCorn	Starch (Corn)	Michigan Biotechnology Institute and Japan Corn Starch Co.,Ltd., &Grand rines technology Joint venture
FLO-PAK BIO 8	Starch (corn or wheat)	Marfred Industries USA
Green fill	Starch (wheat) / PVA	Green light products ltd., London,UK
Green Pol	Aliphatic polyester and starch	Green pol.co.Dacjeon, Korea
Lacea	Poly LA from fermented glucose	Mitsui Chemicals, Japan
Mater-Bi	Starch (40-85% corn or potato/cellulose poly caprolactone PCL/PVA)	Novamount, Novara Italy
WOOD BASED		
Cellotherm T	Regenerated cellulose film	UCB films
Enviro plastic	Cell acetate and polyethylene succinate	Planet polymer technologies San Diego, USA
Lignopol	Lignin	Lignopol Borregaurd Norway
MICROBIAL POLYMERS		
Biomer	PHBs	Biomer Germany
Biogreen	PHB	Mitsubishi Gas Chemical Japan

Continued

Biopol	PHB/PHV	Metabolix, Cambridge Massachusetts USA
	Curdlan	Takeda Chemical Industries
Nodax	Aliphatic polyesters principally PHA	Proctor & Gamble USA
	Pullulan	Hyashibara co
SYNTHETIC BIODEGRADABLE POLYMERS		
Biomax	PET	Dupont USA
Cell green	PCL and acetyl cellulose resin	Daicel Kagaku, Japan
Poval	PVA	Shiu Etsu chemicals

(Compiled from: Materials list, Friendly packaging)

Review of Literature

POLYHYDROXYALKANOATES (PHA)

Polyhydroxyalkanoates are microbial polyesters. These polymers have properties similar to synthetic plastics and in addition are biodegradable and biocompatible. These polyesters are used in a number of applications and have attracted considerable industrial attention (Madison and Huisman, 1999). Hence these polymers are gaining attention as alternatives to synthetic plastics.

A wide variety of both gram +ve and gram –ve, aerobic, anaerobic, photosynthetic, lithotrophic and organotrophic bacteria accumulate PHA intracellularly as carbon and energy source (Dawes and Senior, 1973).

HISTORY OF POLYHYDROXYALKANOATES

The first polyhydroxyalkanoate to be discovered was from *Bacillus megaterium* by Lemoigne in 1926. Lemoinge along with Roukhelman in 1940 also suggested the physiological role of these polyesters (Dawes and Senior, 1973). Macrae and Wilkinson in 1958 observed that PHB was accumulated in response to imbalance of growth brought about by a nutrient limitation. In 1973, Dawes and Senior reviewed on storage polymers and emphasized the role of polyhydroxyalkanoates as carbon storage compounds in microorganisms during nutrient depletion or limiting conditions.

Amongst various PHAs only polyhydroxybutyrate was known until the sixties and seventies. In early eighties, 3 hydroxyvaleric acid (3HV) and 3 hydroxyhexanoic acid (3HHX) was incidentally detected as constituents of PHA (Findlay and White, 1983). Later elaborate reviews on PHA concerning production, characterizations and applications were dealt with (Anderson and Dawes, 1990; Byrom, 1992; Eggink et al, 1994; Lee, 1996; Madison and Huisman, 1999,). Since then extensive work has been carried out on biochemical aspects of microbial synthesis of PHA, fermentation strategies for PHA production, genetic engineering aspects and development of recombinants for PHA production.

TERMINOLOGY (Sasikala and Ramana, 1996)

1. Polyhydroxyalkanoates (PHAs) is a general terminology

2. Polyhydroxybutyrate is a homopolymer and is abbreviated as P (3HB) or PHB. Similarly other homopolymers are named according to their monomer composition.

Eg: Polyhydroxyvalerate (5-carbon monomer) (PHV)

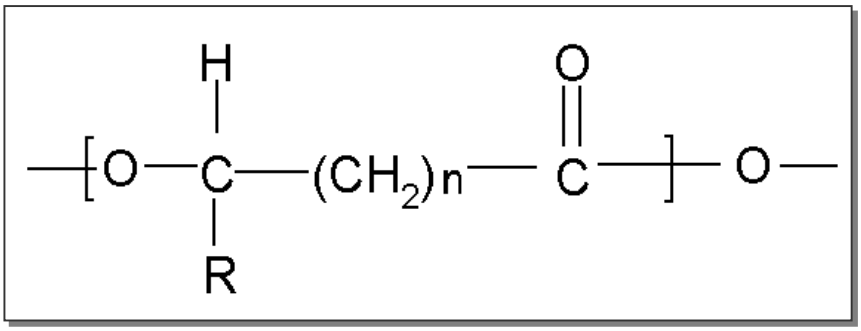
Polyhydroxyoctanoate (8-carbon monomer) (PHO)

3. Copolymers of hydroxybutyrate and hydroxyvalerate are abbreviated as P (3HB-co-HV) or PHV. Similarly other copolymers are named based on their monomer units.

4. Short chain length (SCL), medium chain length (MCL), and long chain length (LCL) refer to those hydroxyalkanoates that consist 3-5, 6 -14, and >14 carbon respectively. These are referred to as PHA_{SCL}, PHA_{MCL}, and PHA_{LCL}.

STRUCTURE OF PHA

PHAs are primarily linear, head to tail polyesters composed of 3-hydroxyfatty acid monomers. In these polymers, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the next monomer (Fig 4). In all PHA that has been characterized so far the hydroxyl substituted carbon atom is of the R configuration, except in special cases where there is no chirality. At the same C-3 or β– position, an alkyl group, which can vary, from methyl to tridecyl is positioned. As many as 91 different hydroxyalkanoates has been detected as constituents of biosynthetic PHA (Steinbuchel and Valentin, 1995). Apart from these, aromatic, unsaturated, halogenated, epoxised and branched monomers are reported. Specialized unnatural monomers such as 4 - cyanophenyl valerate with special properties has also been reported.



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

FIG 4:GENERAL STRUCTURE OF POLYHYDROXYALKANOATES (STEINBUCHEL AND VALENTIN, 1995)

The position of hydroxyl group is somewhat variable and 4 - , 5 - and 6 - hydroxy acids have been incorporated (Madison and Huisman, 1999).

CLASSIFICATION OF PHA (Kim and Lenz, 2001)

Polyhydroxyalkanoates can be classified into three major groups.

I Short chain length (SCL) PHAs.

Short chain length PHAs contain only 3HB and 3HV units. Apart from these 4HB and 5HV units have also been reported.

II Medium Chain Length (MCL) PHAs

Medium chain length PHAs contain 3hydroxyalkanoates (3HA) and is longer than 3HV. The MCL PHAs are further divided into two types. Those that contain only 3 hydroxyalkanoates and those that contain substituted 3hydroxyalkanoates.

III A third group forms the hybrid of the above two. PHAs containing repeating units of both SCL and MCL PHA

Eg: Polymer containing Poly 3HB, 3HHX, 3HO, 3HD.

This classification is based on the size of the repeating units of the polymer.

Apart from the above types, PHAs are studied based on their alkyl substituents.

i. PHAs containing unsaturated units

Eg: PHA containing carbon-carbon double bond

ii. PHAs containing aromatic groups

Eg: 5-phenylvaleric acid polymer (5PVA)

iii. PHAs containing halogen groups

Eg: 3-hydroxy-9-fluoronanoate.

PHA AS A STORAGE POLYMER

An important development in an evolving life form is the acquisition of characteristics, which bestow upon the organism the ability to survive the adverse conditions imposed by nutrient starvation. Once these characteristics are acquired, the life form then has a more secure basis for further development and diversification. In general, protection against starvation has been achieved by the organism accumulating reserve compounds during periods of nutritional plenty and then degrading these storage materials during times of famine, thereby maintaining viability. Even in conditions where there is absence of

growth, energy is required for osmotic regulation, maintenance of intracellular pH, for motility, for turnover of proteins and nucleic acids and for the specialized phenomena of sporulation, encystment and luminescence. The value of an intracellular store of carbon and energy can be readily appreciated when the plight of a nitrogen fixing organism is considered, for the reduction of dinitrogen is energetically expensive. When new enzymes have to be synthesized after a period of starvation, some degree of nitrogen fixation will probably be necessary, a process which requires both carbon skeletons and energy. In the absence of a readily metabolizable exogenous source of carbon and energy, enzymes must be induced to deal with the novel substrates and the necessary reactions would be virtually impossible in the absence of substantial intracellular reserves.

PHA is produced in bacterial cells as discrete granules and is an intracellular energy reserve. Bacterial cells with a high content of PHB survive better than those with low PHB accumulation capabilities. The capability of synthesizing P (3HB) is wide spread in prokaryotes. This capability is neither a taxon-related property nor an ecophysiological peculiarity. It is not an essential constitutive component of bacteria or living beings and hence is not seen in all circumstances. Imbalanced supply of nutrients or deviation from optimum levels of physical factor triggers its synthesis. Synthesis of PHA is genotypically determined and is related to overflow metabolism or nutrient imbalance (Babel et al, 2001). But microorganisms have to cope up with external shortcomings and

environmental fluctuations of widely varying frequency and intensity. Energy storage compounds are accumulated during the following conditions:

1. When the energy from exogenous sources is in excess of that required by the cell for growth and related processes at that particular moment in time.

2. The compound is utilized when the supply of energy from exogenous source is no longer sufficient for the optimal maintenance of the cell either for growth and division or for viability.

3. The compound is degraded to produce energy in a form utilizable by the cell.

PHA is generally overproduced and intracellularly accumulated. When more reducing power is being generated than can be productively consumed due to limitation for other synthesis. This can been seen as an investment for the future, as P (3HB) can be mobilized under conditions of external energy starvation. It is also advantageous for the bacterium to polymerize soluble intermediates into insoluble molecules because the cell does not undergo alterations of its osmotic state and leakage of these valuable compounds out of the cells are prevented. Consequently the nutrient stores will remain available at a relatively low maintenance cost and with a secure return on investment. The synthesis of PHA is unique amongst energy storage compounds in not requiring the direct participation of ATP. PHB is formed when reoxidation of reduced nicotinamide nucleotides has to occur under conditions where there is restriction of it, either because of oxygen limitation or cessation of protein synthesis when

there is nitrogen limitation. PHB is thus a highly reduced carbon and energy storage compound which according to organism may additionally play role in spore or cyst formation for prolonging survival under adverse conditions. In case of nitrogen fixing organisms in the soil the possession of PHB will afford respiratory protection when readily oxidisable exogenous substrates are not available.

Understanding the role played by PHAs as internal storage polymers is of fundamental importance in microbial ecology. The accumulation, degradation and utilization of PHAs by bacteria under stress conditions constitute a mechanism that may favor the establishment, proliferation, survival and completion of the life cycle of organisms especially in the competitive environments in which carbon and energy sources are limiting such as those encountered in soil. PHB accumulating cells exhibit increased stress endurance. For example, *Azospirillum brasilense* accumulating PHA endure variety of stress such as UV irradiation, heat, osmotic shock, osmotic pressure, desiccation, and to grow in the presence of hydrogen peroxide (Kadouri et al 2003). High content of PHB delays the autolysis and death of nitrogen deficient *Bacillus megaterium.*

PHA GRANULES

PHA accumulates as discrete granules within the bacterial cells. The granules are spherical and range in diameter from 0.2 to 0.7μm; each granule contains atleast several thousand PHA molecules. A membrane of 4.5-nm thickness surrounds the granules. The chemical composition of PHB is as shown in the table 6.

Heating the granules for 5 minutes at 100^{o}C destroys over 50% of it as substrate to depolymerase. A 30% loss was obtained after freezing and thawing, 40% loss was seen after four-repeated centrifugation. Extensive digestion of the granules with trypsin destroys the capacity of the granules completely as substrate to depolymerase (Griebel et al, 1968). PHB granules have been observed to coalesce during cell growth. Treatment of the granules with acetone causes dehydration. Granules stored at 4^{o}C for 3 weeks shrink physically as well as lose activity. Native granules are effectively stained by sudan black as the stain directly dissolves in the mobile PHB phase (Barnard and Sanders 1989). High-resolution ^{13}C NMR spectroscopy of live cells shows that PHB is predominantly in a mobile phase in the storage granules. Water acts as a plasticizer within the granules in the cell. It is involved in hydrogen bonding or dipole-dipole interactions with the ester groups, thereby inhibiting chain–chain proximity (Gerngross et al, 1993). A model of PHB granule formation in *Alcaligenes eutrophus* has also been proposed by

TABLE 6: CHEMICAL COMPOSITION OF PURIFIED PHB GRANULES

Granule composition	Dry weight (%)
PHB	97.7
Protein	1.87
Lipid a. Acetone extractable b. Alcohol-ether and ether extractable	 0.21 0.25

Source: Griebel et al, 1968

Gerngross et al (1993). According to the model the granule formation starts when the cell metabolism reaches a stage where there is no free Coenzyme A and the increased acetyl-CoA results in the production of hydroxybutyryl-CoA. The polymer chains are then produced by synthase. Increased chain length of the polymer will make the polymer hydrophobic. The hydrophobic polymer chains form a micelle –like structure in which the synthase enzyme forms a skin around the granule effectively separating the hydrophobic polymer from the aqueous cytoplasm. Polymerization continues by effectively taking the substrate from cytoplasm and extruding the growing polymer chain into increasingly densely packed amorphous hydrophobic center of the granule. Granules are forced to fuse as the polymer continues to accumulate and synthase is sent to the periphery.

The number of granules per cell is fixed at the earliest stages of polymer accumulation. For example, polymer production in *Alcaligenes eutrophus* ceases when a PHB content of about 80% is attained, although PHB synthase activity remains high, suggesting that physical constraint operate and the cell is unable to accommodate more polymer within the fixed existing amount of cell wall material despite the availability of substrate and active synthase. On an average each polymer molecule contains a minimum of 1000 polymer chains.

PHYSICAL CHARACTERISTICS

PHB is the most common polymer accumulated in the organisms and is also the only polymer that has been studied extensively. PHB is a compact right-handed helix with a two-fold screw axis and a fibre repeat of 0.596 nm. It is optically active with the chiral centre of the monomer unit always in the R absolute configuration D (-) in the traditional nomenclature. This is similar to polypropylene, which has a compact helical configuration (Table 7).

Polyhydroxyvalerate and other higher polymers or copolymers of PHB are less stiff and brittle and can be used to prepare excellent films with excellent water and gas barrier properties. They can also be processed at a lower temperature. The values of melting temperature of P (3HB-co-HV) decreased from 180- 123^{o}C with an increase in the 3HV content in the copolymer content from 0 to 20 mol% (Savenkova et al, 2000). The crystalline chain conformation of poly (3-hydroxyvalerate) is very similar to that of PHB. It is also a α 2 helix with a two repeating unit volume of 0.13 nm^3 and a fiber repeat of 0.556 nm compared with 0.11 nm^3 and 0.596 nm respectively, for PHB. Copolymers of 3HB and 3HV are isodimorphic. It was observed that the rate of crystallization decreased with increase in 3HV content; the rate could be significantly increased by the addition of nucleating agents, a procedure which simultaneously reduces brittleness and increases elongation at break. The molecular mass of PHA's vary but is generally in the order of 50,000 to 1000,000 Da.

TABLE 7: COMPARISON OF POLYHYDROXYBUTYRATE (PHB) AND POLYPROPYLENE (PP)

Parameter	PP	PHB
Melting point Tm (^{o} C)	171-186	171-182
Glass transition temperature Tg (^{o} C)	-15	5-10
Crystallinity (%)	65-70	65-80
Density (g cm^{-3})	0.905-0.940	1.23-1.25
Molecular weight M_w ($x10^{-5}$)	2.2-7	1-8
Molecular weight distribution	5-12	2.2-3
Flexural Modulus (gPa)	1.7	3.5-4
Tensile strength (mPa)	39	40
Extension to break (%)	400	6-8
U V Resistance	Poor	Good
Solvent resistance	Good	Poor
Oxygen Permeability (cm^3 m^{-2} atm^{-1} d^{-1})	1700	45
Biodegradability	-	+

Source: Brown, 1991

Polymers consisting of only MCL PHA are semicrystalline thermoplastic elastomers and their mechanical properties might have to be enhanced by reinforcement with non-composite materials and fillers. Where as SCL-MCL PHA copolymers can have a wide range of physical properties depending on the percentage composition of the monomers. SCL-MCL PHA copolymers are currently being exploited in a wide range of applications (Nomura et al 2004).

DETECTION, ISOLATION AND ANALYSIS OF PHA

Microbiologists have traditionally used various staining techniques for detection of PHA in bacteria. Various stains such as sudan black (Gerhardt et al, 1981), nile blue (Ostle and Holt, 1982), nile Red (Gorenflo et al, 1999) stains have been used to identify bacteria which have accumulated PHA intracellularly. Spectrophotometric method of Law and Slepecky is generally used for quantitative analysis of PHA (Law and Slepecky, 1961). Gas chromatography and HPLC are also being used for analysis of PHA (Brandl et al, 1988).

MICROORGANISMS THAT PRODUCE PHA

A wide variety of bacterial species are known to accumulate PHA (Dawes and Senior, 1973; Andersen and Dawes, 1990). PHA has been reported from various environments such as soil, sewage sludge (Findlay and white, 1983), marine sediments, ponds, mangrove environment (Rawte et al, 2002) and Gas field soil (Tajima et al, 2003).

Microorganisms that produce PHA are discussed with respect to two aspects viz. microbiological and basic genetic make up of the organism and the economic aspects for PHA production.

Many microorganisms are known to store PHA as their intracellular storage of energy. Some of the well-known organisms and the amount of PHA produced are listed in table 8. In most of the bacteria, PHA is synthesized and intracellularly accumulated under unfavorable conditions such as limitation of nitrogen, phosphorus, magnesium, oxygen etc in the presence of excess carbon (Andersen and Dawes, 1990; Babel et al, 2001). Table 9 summarizes the organisms and their corresponding limiting compounds leading to PHA production.

Synthesis and intracellular accumulation of poly (3HB) are promoted when bacteria are subjected to limitations. Bacteria respond to all limitations (ammonium, oxygen, phosphate, sulfate, potassium, magnesium and iron) in similar way though the physiological roles of these limitations are different (Table 10).

TABLE 8: THE ACCUMULATION OF PHAs IN VARIOUS MICROORGANISMS

Genus	**PHA (Wt % of dry biomass)**	**Substrate from which PHA is produced**
Acinetobacter	<1	Glucose
Aphanothecae	<1	Nil
Azospirillum	57	3-hydroxybutyrate
Axobacter	73	Glucose
Bacillus	25	Glucose
Beggiatoa	57	Acetate
Beijerinkia	38	Glucose
Caulobacter	36	Glucose / glutamate / Yeast extract
Chloroflexus	<1	Yeast extract / glycylglycine
Chlorogloea	10	Acetate / CO_2
Chromatium	20	Acetate
Chromobacterium	37	Glucose / Peptone
Clostridium	13	Tryptone / peptone / glucose
Derxia	26	Glucose
Halobacterium	38	Glucose
Leptothrix	67	Pyruvate
Methylobacterium	47	Methanol
Methylocystis	70	Methane
Methylosinus	25	Methane
Micrococcus	28	Peptone / tryptone
Nocardia	14	Butane
Pseudomonas	67	Methanol
Ralstonia	96	Glucose
Rhodobacter	60	Acetate
Rhodospirillum	47	Acetate
Sphaerotilus	45	Glucose / peptone
Spirillum	40	Lactate
Spirulina	6	CO_2
Streptomyces	4	Glucose
Syntrophomonas	30	Crotonate

Source: Kim and Lenz, 2001

TABLE 9: LIST OF LIMITING COMPOUNDS LEADING TO PHA FORMATION

Compound	Organism
Ammonium	*Alcaligenes eutrophus, A latus, Azospirillium brasiliense, Pseudomonas oleovorans, P cepacia, Rhodspirillum rubrum, R sphaeroides, Methylocystis parvus, Rhizobium ORS 571.*
Carbon	*Spirillum sp, Hyphomicrobium sp,*
Iron	*Pseudomonas sp K*
Magnesium	*Pseudomonas sp K, Pseudomonas oleovorans, Rhizobium ORS 571.*
Manganese	*Pseudomonas sp K*
Oxygen	*Azospirillium brasiliense, Azotobacter vinelandii, A beijerinkii, Rhizobium ORS 571.*
Phosphate	*Rhodospirillum rubrum, Rhodobacter spheroides, Caulobacter cresentus, Pseudomonas oleovorans.*
Potassium, Sulfate	*Bacillus thuringensis, Pseudomonas sp K, Pseudomonas oleovorans, Rhodospirillum rubrum, Rhodobacter spheroides.*

Source: (Compiled) Sasikala and Ramana 1996; Kim and Lenz, 2001

TABLE 10: ROLE OF IMPORTANT LIMITING COMPOUNDS ON PHA SYNTHESIS

Limitations	Reactions
Nitrogen	No amino acids synthesis. Acetyl-CoA and NAD (P) H restricted. Acetyl-CoA accumulates. Citrate synthase inhibited. TCA cycle stops. Acetyl-CoA becomes available for β-ketothiolase and PHA is synthesized.
Oxygen	2/H generated are not oxidized via electron transport phosphorylation. TCA cycle stops. Acetyl-CoA becomes available for β-ketothiolase and PHA is synthesized.
Phosphorus	ATP is not generated. No phosphorylation of ADP in the absence of phosphate. PHA synthesis acts as store of available Acetyl-CoA and reducing equivalents.

Source: (Compiled) Madison and Huisman; 1999, Kim and Lenz, 2001

Oxygen limitation

During oxygen limitation nicotinamide nucleotides are not oxidized. This will result in decrease of effectiveness of TCA cycle and activity of citrate synthase and isocitrate dehydrogenase is decreased by NADH. Thus acetyl-Co A accumulates and there is a low intracellular concentration of free CoA. Increase in acetyl-CoA / CoASH ratio partially relieves the inhibition on β-ketothiolases and favours PHB formation.

Phosphorus and magnesium limitation

During phosphorus and magnesium limitation, PHA accumulates from the beginning of the cell growth. Magnesium ions are known to increase the growth rate and phosphorus is an essential nutrient for the living organism, which is required for the regulation of the physiological state and energy metabolism. It would not be possible to achieve high mass and PHB content at low levels of phosphorus. As phosphate does not participate directly in protein composition, its limitation will allow residual cell growth for some time (Squio et al, 2003). Phosphorus is involved in recycling of energetic intermediates i.e ATP /ADP. In its deficiency, there is a demand from NADP and hence will lead to PHA production.

Nitrogen limitation

If nitrogen is limiting then acetyl-CoA and NAD (P) H are restricted. Liberated NAD (P) H cannot be consumed for reductive synthases, i.e. for amino acid synthesis. As there is absence of protein synthesis, acetyl-CoA will be channeled into PHB synthesis (Oeding and Schlegel, 1973; Senior and Dawes, 1973; Anderson and Dawes, 1990).

For effective synthesis of PHA, Acetyl-CoA and NADPH are necessary. The intracellular concentration of acetyl-CoA should be high. The flux of acetyl-CoA either towards the TCA cycle for cell growth or PHB biosynthesis pathway for energy storage has been controlled by NADPH / NADP ratio. Elevated levels of NADPH significantly enhances PHB accumulation (Choi et al, 2003).

METABOLISM OF PHA IN MICROORGANISMS

A lot of research has been done on the metabolism and biochemistry of PHA production in microorganisms (Steinbuchel and Hein, 2001; Babel et al, 2001). Numerous genes encoding enzymes involved in the PHA formation and degradation have been cloned and characterized from a variety of organisms. From these studies it is clear that nature has evolved several different pathways for PHA formation, each optimized for the ecological niche of PHA producing organism. Genetic studies have given further insight into the regulation of PHA formation with respect to growth conditions. Similarly PHB is also degraded

intracellularly in the organisms. Some of the accumulated PHB is always being degraded internally but degradation is not just the reversal of synthesis.

Metabolically, three conditions are necessary for PHB synthesis.

1. High NAD(P)H
2. High Acetyl-Co A
3. Low free Co A

Polyhydroxybutyrate (PHB) is the best known member of the polyhydroxyalkanoate series of polyesters. PHA is synthesized by diverting either the central intermediates of carbon metabolism or derivatives from precursor substrates, which are provided as carbon sources for the growth of bacteria, to hydroxyl-CoA thioesters. The later are then polymerized by PHA synthases that become bound to surface of PHA granules together with proteins. First the condensation of two molecules of acetyl-CoA by a β- ketothiolase and the subsequent reduction of acetoacetyl-Co A to D- (-) 3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase, which is NADPH dependent in most bacteria is the most widespread pathway. A schematic representation of the overall mechanism of biosynthesis is given in fig 5.

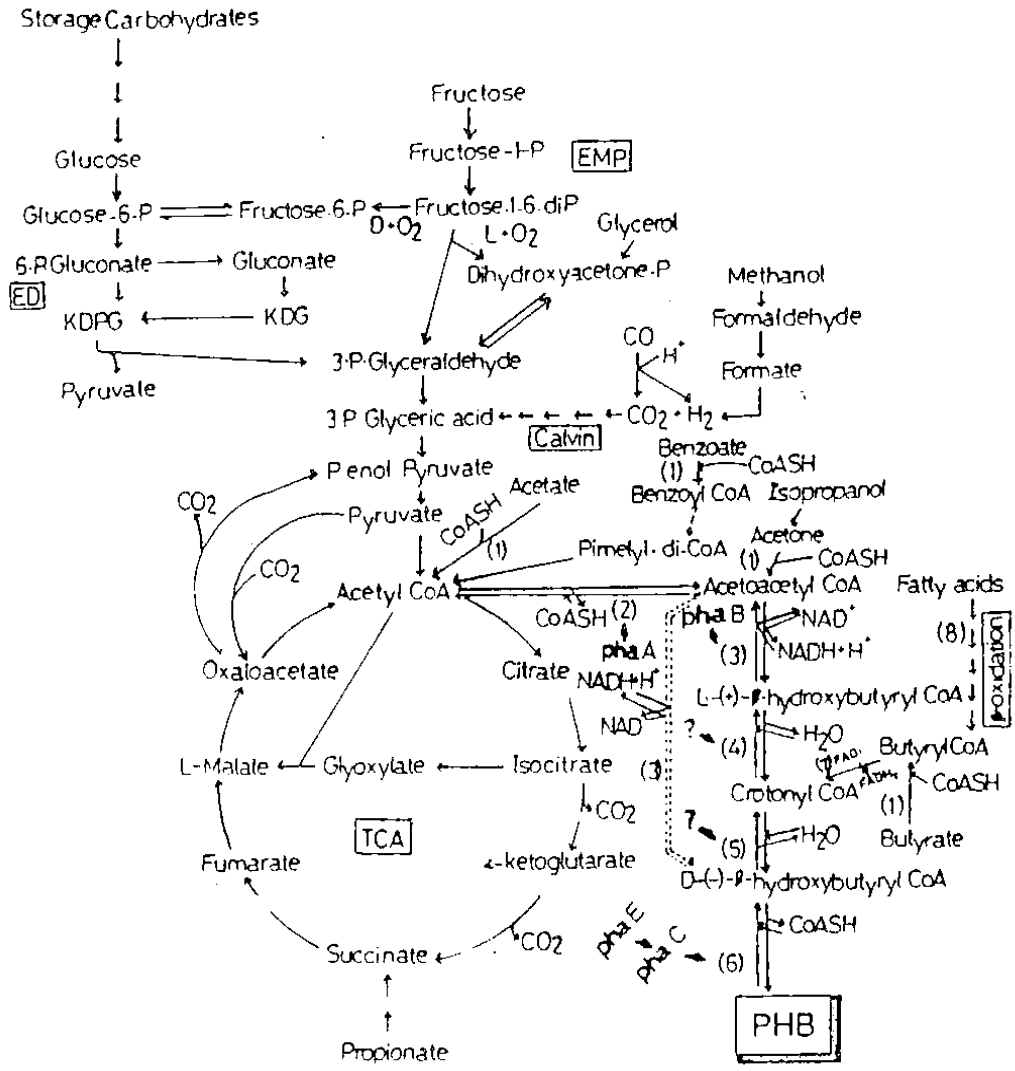


FIG 5: SCHEMATIC REPRESENTATION OF DIFFERENT BIOCHEMICAL PATHWAYS FOR PHA SYNTHESIS (*Sasikala and Ramana, 1996)*

ED: Entner doudoroff pathway, EMP: Embdem meyerhof pathway, Calvin: Calvin reductive pathway, TCA: Tricarboxylic cycle, 1: Acyl-CoA synthase, 2: β-Ketothiolase, 3: NADH dependent acetoacetyl CoA reductase, 4: Enoyl-CoA hydratase (forming butyryl-CoA, 5: Enoyl-CoA hydratase forming β-hydroxybutyryl-CoA, 6: PHB synthase, 7: butyryl-CoA dehydrogenase, 8: Enzymes involved in the β- oxidation pathway.

Secondly a modified pathway where acetyl-Co A is first reduced to L- (+)-3-hydroxybutyryl-CoA by an NADH dependent acetoacetyl-CoA reductase which is subsequently converted into D- (-)-3-hydroxybutyryl-CoA by stereospecific enoyl-CoA hydratases. In phototrophic bacteria and synthrotrophic bacteria two stereospecific enol-CoA hydratases catalyse the conversion of L (+)-3-hydroxybutyryl-CoA via crotonyl-CoA to D (-)- 3-hydroxybutyryl-CoA which is then polymerized to PHB (Sasikala and Ramana, 1996). Metabolic demand for large amounts of acetyl-CoA and NADPH results in increased synthesis of PHB within the cells. For example, a phosphoglucose isomerase mutant shifts its glucose metabolism to hexose monophosphate shunt thus enhancing PHB synthesis (Kabir and Shimizu, 2003) (Fig 5A).

The production of PHA copolymer (PHB-co-HV) is usually done by feeding propionic acid or valeric acid (Page et al, 1992; Aldor and Keasling, 2001), butyric or pentanoic acids (Doi et al 1988) or acetate or oleate (Yim et al, 1996) as a secondary carbon source. Copolymer is formed when β-ketothiolase mediates the condensation of propionyl-CoA and acetyl-CoA and then there is reduction and polymerization of the β-hydroxyacyl-CoA subunits. P (3HB-Co-HV) was produced efficiently from propionate by recombinant *E coli* by inducing with acetate, propionate or oleate (Yim et al, 1996) (Fig 5B).

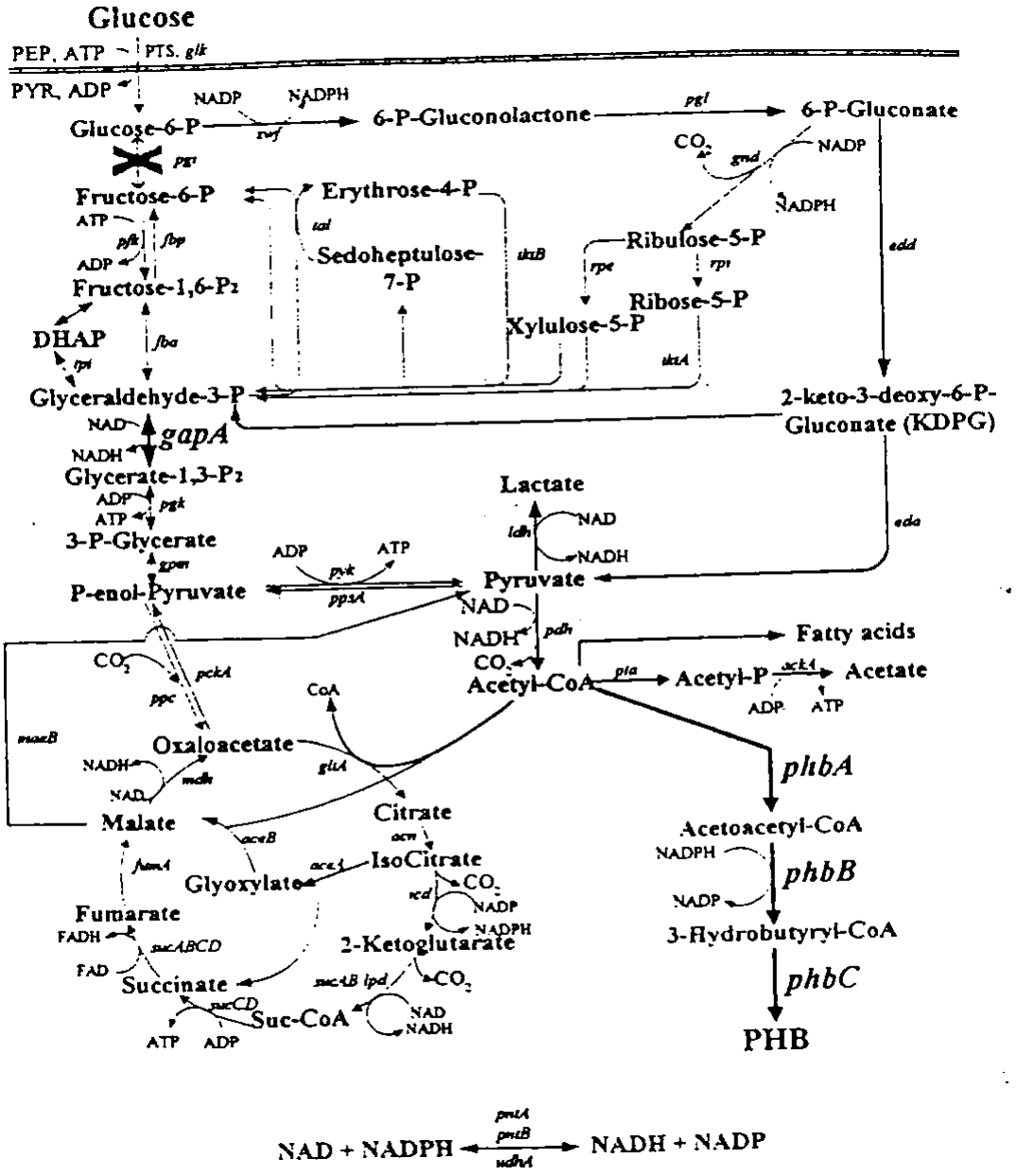


FIG 5A: CENTRAL METABOLIC REACTION IN RECOMBINANT *E COLI* INVOLVED IN PHB PRODUCTION (*Kabir and Shimizu, 2003*)

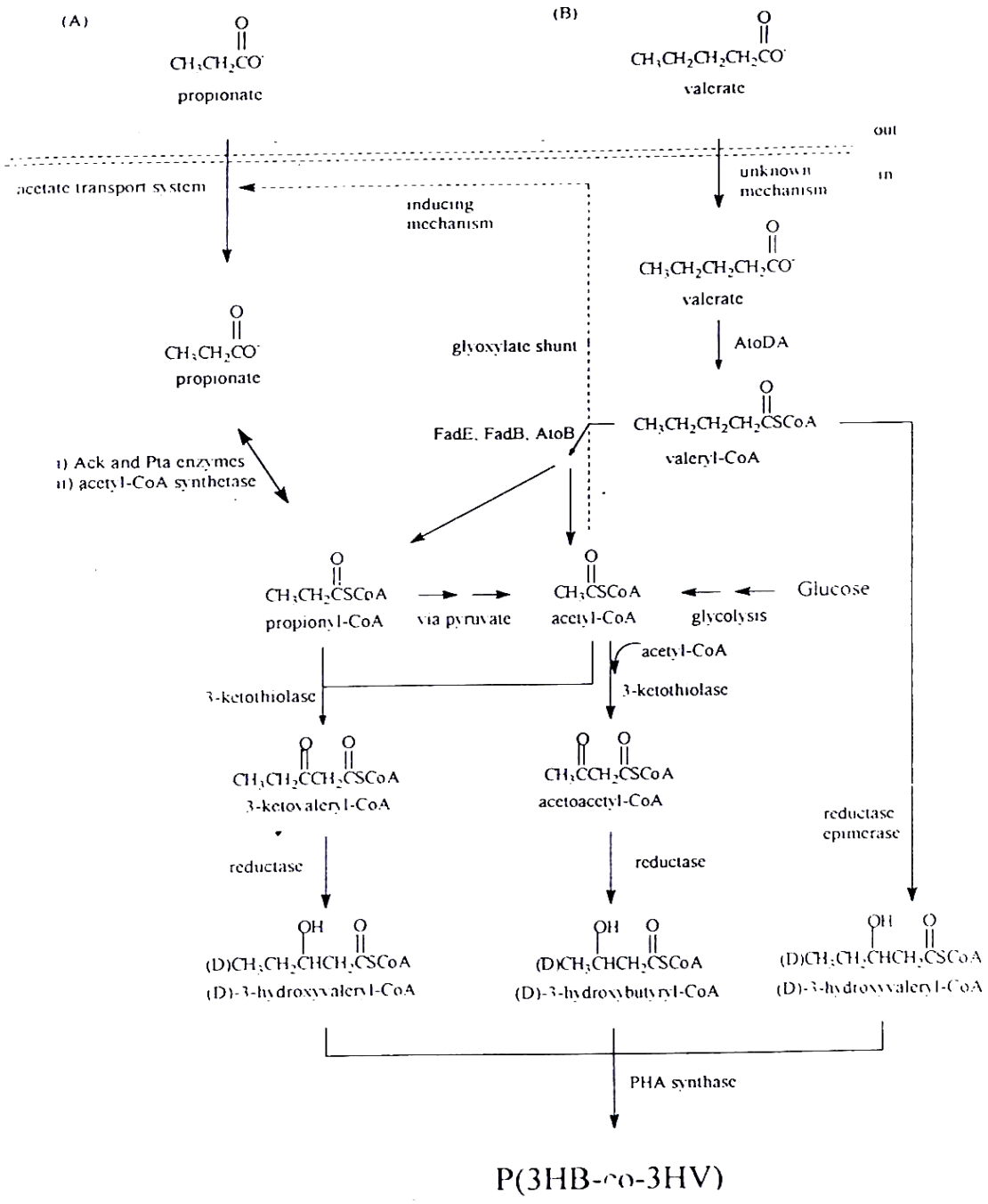


FIG 5B: PATHWAY FOR THE SYNTHESIS OF P(HB-CO-HV) FROM PROPIONATE AND VALERATE IN RECOMBINANT *E COLI* (*Yim et al, 1996*)

Apart from these PHB-co-HV is also produced in the absence of typical 3HV precursors such as propionate or valerate (Lee et al, 1995a; Valentin and Dennis, 1996; Aldor et al, 2002). Here the HV monomer is derived from acetyl – CoA and propionyl-CoA where the latter is a product of the methyl malonyl-CoA pathway. In this pathway succinyl-CoA is converted to methyl malonyl-CoA, which is decarboxylated to propionyl-CoA (Madison and Huisman, 1999). Here succinate, an intermediate of the TCA cycle, is abstructed and then converted to propionyl-CoA via methyl malonyl-CoA pathway by reactions catalyzed by methylmalonyl-CoA mutase and methylmalonyl-CoA decarboxylase. Alternatively the reactions are catalyzed by methylmalonyl-CoA oxaloacetate transcarboxylase. Succinyl-CoA is also converted to propionyl-CoA by succinyl- CoA decarboxylase (Fig 5C). In recombinant *E coli*, a novel pathway has been described for PHV production from unrelated carbon source. A mutase catalyses the conversion of succinyl-CoA to methylmalonyl-CoA. A methylmalonyl-CoA decarboxylase catalyses the conversion of methylmalonyl- CoA to propionyl-CoA. Finally propionyl-CoA is converted to succinyl-CoA by a transferase. The TCA cycle and 2-methyl citric acid cycle compete for acetyl-CoA and propionyl-CoA. A block in the expression of 2-methylcitric acid cycle is said to enhance HV production (Bramer and Steinbuchel, 2001; Aldor et al, 2002; Bramer et al, 2002) (Fig 5D).

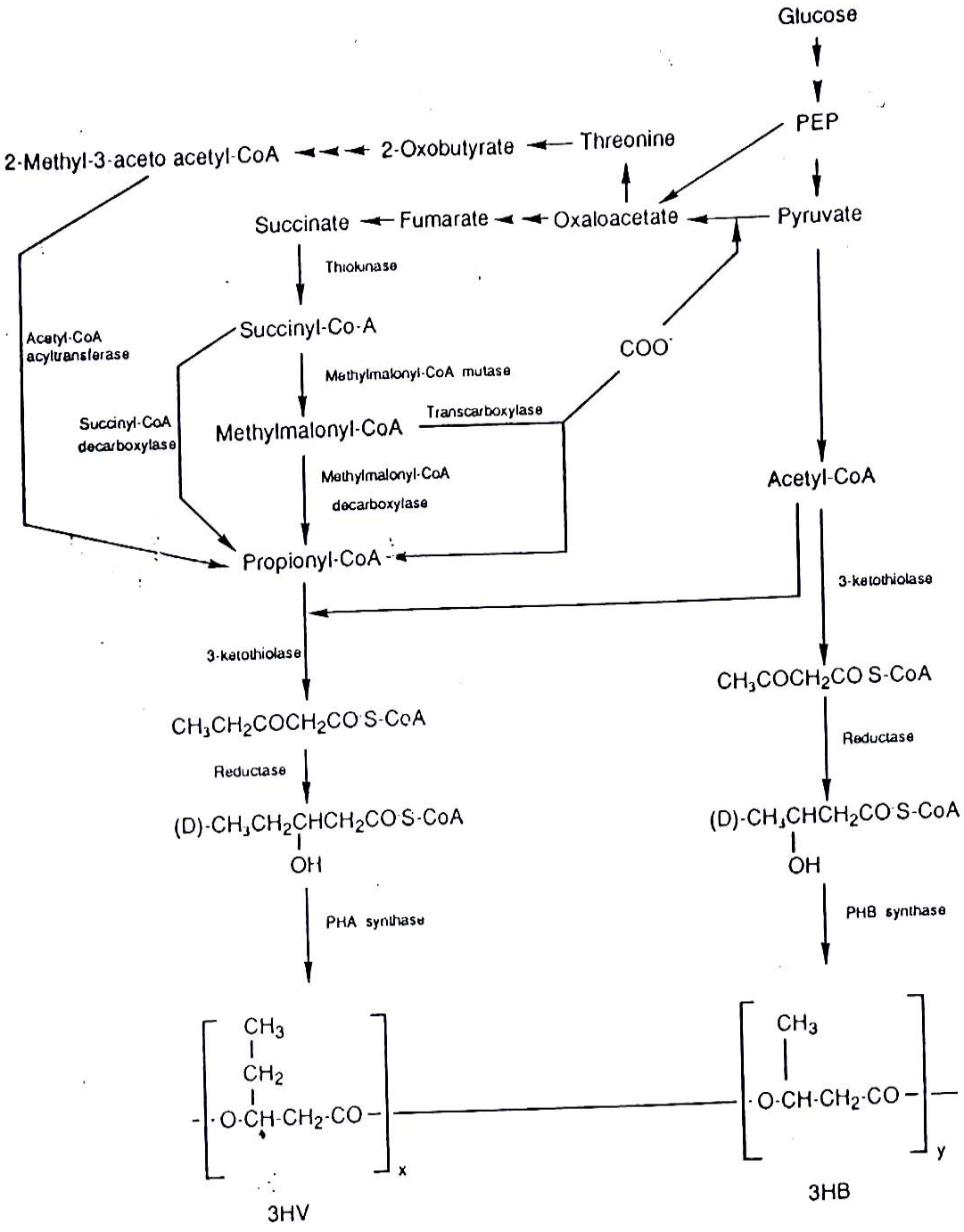


FIG 5C: BIOSYNTHETIC PATHWAY FOR P (HB-CO-HV) FROM GLUCOSE (*Lee et al, 1995a*)

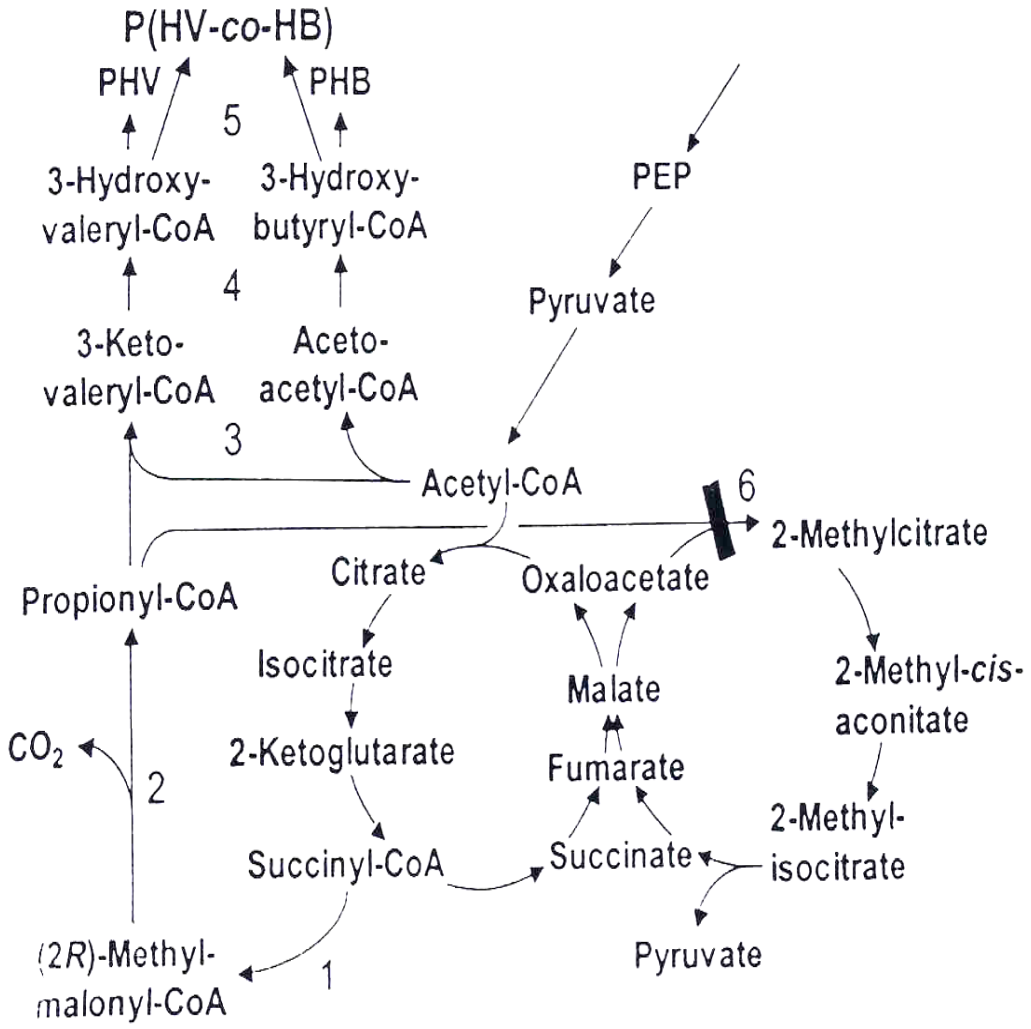


FIG 5D: METABOLIC PATHWAY FOR P (HB-CO-HV) PRODUCTION FROM UNRELATED CARBON SOURCE (*Aldor et al, 2002*).

1: Sbm: Sleeping beauty mutase, 2:methylmalonyl-CoA decarboxylase, 3: 3-Ketothiolase (PhaA), 4:Acetoacetyl-CoA reductase (PhaB), 5:PHA synthase, 6: 2- methylcitric acid synthase.

Alternatively PHB-Co-HV is also produced by altered anabolism in branched-chain amino acids. 2-keto–3 methylbutyric acid, 2-Keto-4-methylvaleric acid are presumably overproduced if the amino acids valine, leucine and isoleucine respectively are not synthesized due to ammonium deficiency. Otherwise, these intermediates may be formed from the corresponding amino acids if an other nutrient rather than ammonium depletion limits the growth of cells. These methyl branched 2-ketoalkanoates are oxidatively decarboxylated to the corresponding CoA thioesters by the branched chain 2-ketoacid dehydrogenase complex, and the latter are further degraded via various other CoA thioesters to intermediates of the central metabolism with propionyl-CoA as the intermediate of the valine and isoleucine catabolic pathways (Fig 5E). Therefore, it is obvious that propionyl-CoA is synthesized by the cells themselves from intermediates of amino acid metabolism and no precursors such as propionate have to be supplied as an additional carbon source for the production of PHB-Co-HV (Steinbuchel and Pieper, 1992). Other culture strategies have been applied to increase synthesis of HV content by modifying amino acid metabolic pathways. Addition of amino acids such as threonine, isoleucine and valine into culture medium and construction of mutant strains which produce excessive amounts of the above amino acids for production of high quantity of HV in the polymer has been reported (Yoon et al, 1995).

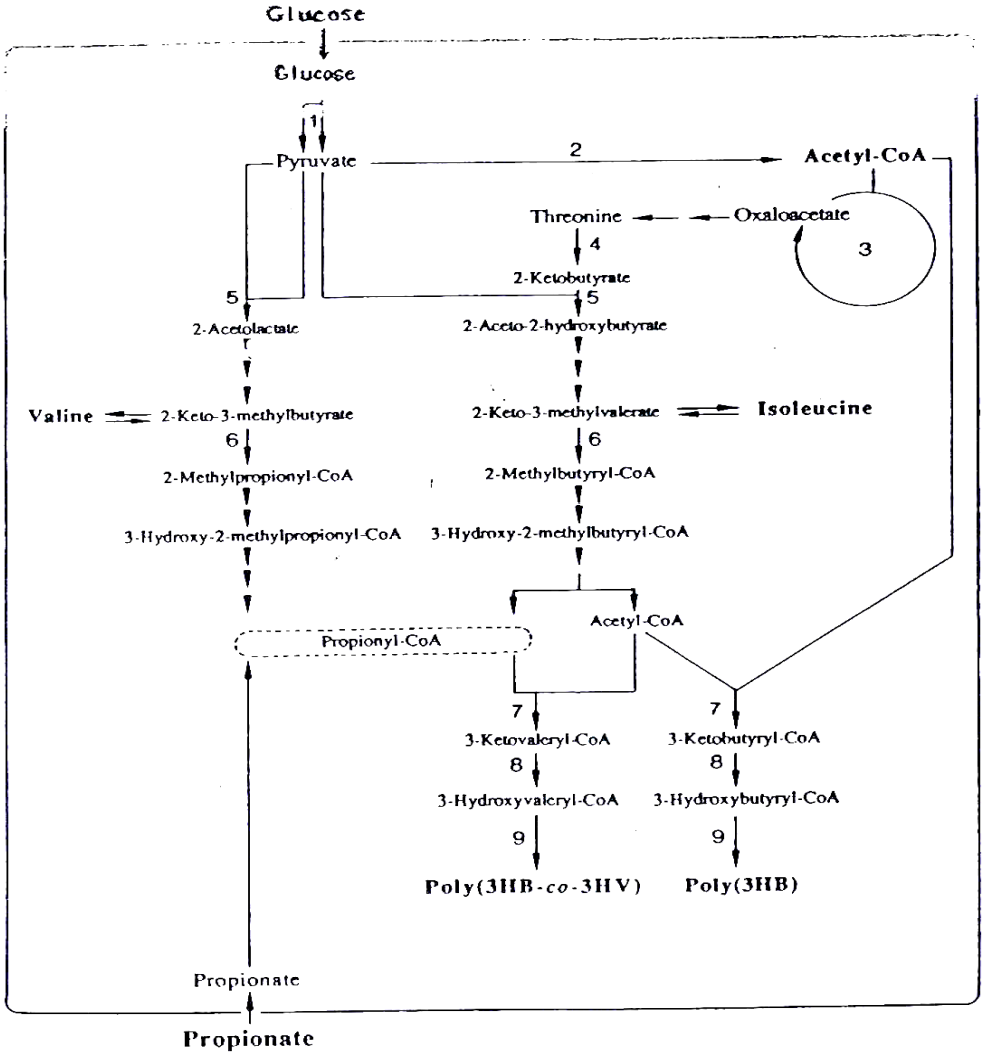


FIG 5E:METABOLISM OF BRANCHED CHAIN AMINO ACIDS AND SYNTHESIS OF POLY (3HB-CO-HV) (*Steinbuchel and Pieper, 1992*)

1.Enter-doudoroff pathway, 2.Pyruvate dehydrogenase complex, 3.Citric acid cycle, 4.Threonine dehydratase, 5.α–acetolactase synthase, 6.Branched chain α- ketoacid dehydrogenase complex, 7. β-ketothiolase, 8. Acetoacetyl-CoA reductase, 9. PHA synthase.

Medium chain length PHA biosynthetic pathway is by β-oxidation. 3-hydroxyacyl Co A with a chain length of 6-14 carbon atoms are used as substrate by the PHA synthases. C_2 units are removed from fatty acids as acetyl-CoA. The acyl CoA's are oxidised to 3-ketoacyl CoA's via 3-hydroxyacyl CoA intermediates. When medium (C4 - C11) and long chain (C12 - C18) fatty acids are used as co-substrates (R) specific enoyl-CoA hydratase (Pha J) converts the intermediates of β- oxidation pathway to (R)-3hydroxyacyl-CoAs which are then linked to growing PHA chain by PHA synthase (Fig 5F) (Park et al, 2001). Fatty acid de novo synthesis is the main route during growth on carbon sources such as gluconate, acetate or ethanol, which are metabolized to acetyl-Co A. Chain elongation reaction, in which acetyl-CoA moieties are condensed to 3-hydroxyacyl-CoA, is involved in the PHA synthesis. When non-related carbon sources such as gluconate are used the R-3- hydroxyacyl-ACP intermediate of fatty acid de novo synthesis must be converted to the corresponding Co A derivative (Hoffman et al, 2000). To serve as a substrate for PHA synthase, hydroxyacyl-ACP, an intermediate of fatty acid de novo synthesis must be converted to corresponding Co A derivative. A transacylase Pha G directly links the fatty acid de novo synthesis with PHA biosynthesis (Fig 5G) (Fiedler et al, 2000). Various strategies for altering the monomer composition of MCL PHAs have been worked out by varying the activities of enzymes in the β-oxidation as well as fatty acid biosynthesis pathways (Park et al, 2003; Nomura et al, 2004).

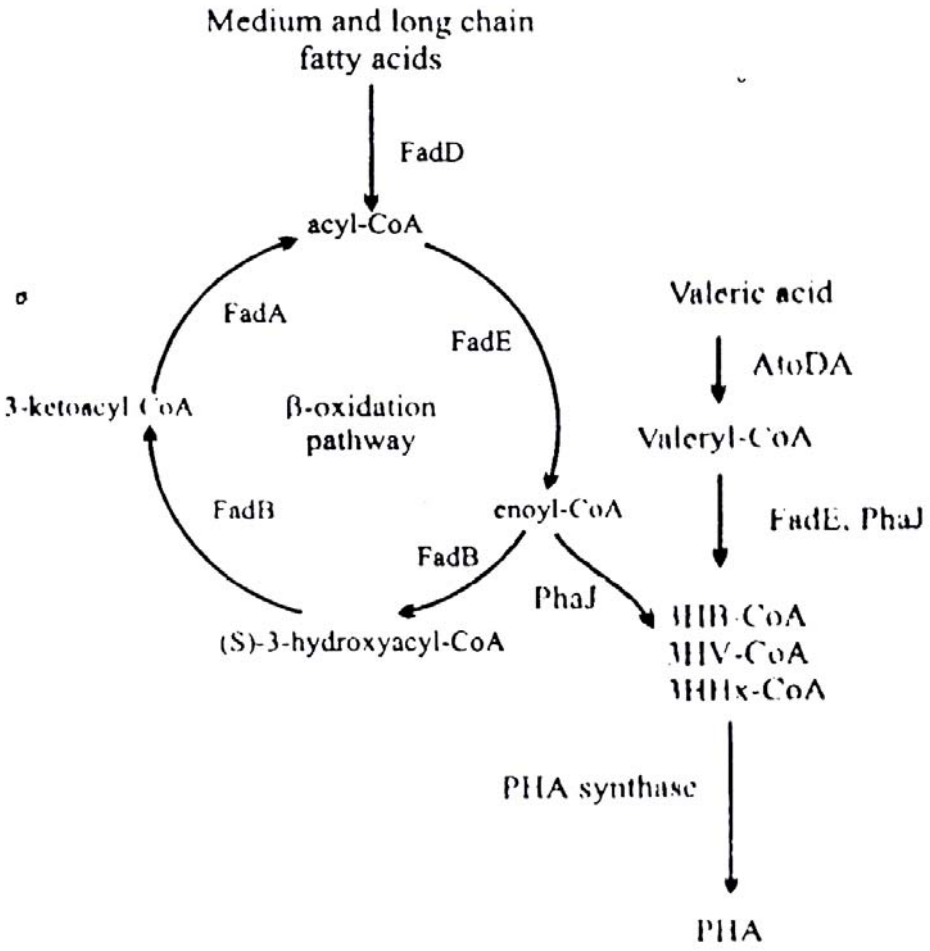


FIG 5F: THE METABOLIC PATHWAY FOR THE SYNTHESIS OF P(HB-CO-HV) FROM FATTY ACIDS (*Park et al, 2001*)

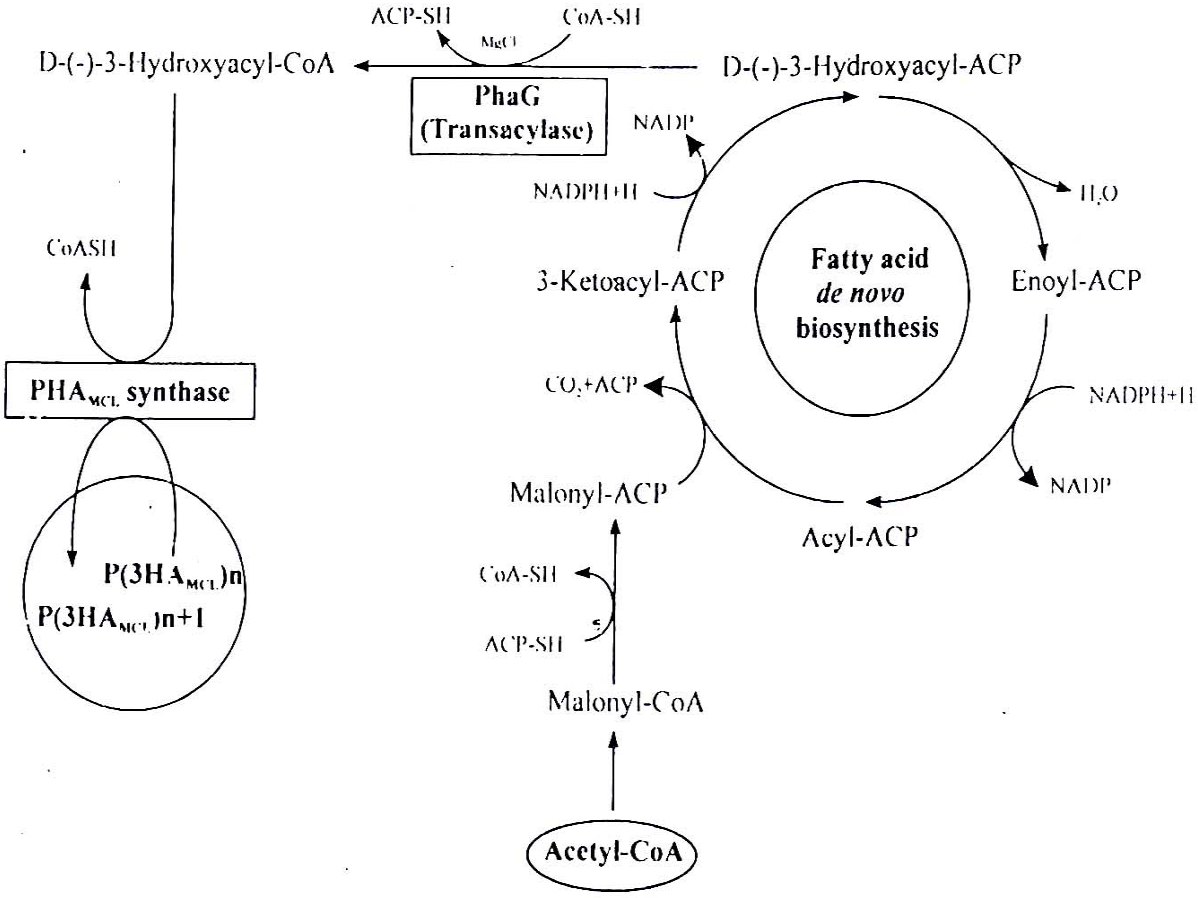


FIG 5G: TRANSACYLASE (PhaG) MEDIATED LINK BETWEEN FATTY ACID DE NOVO SYNTHESIS AND BIOSYNTHESIS OF PHA (*Fiedler et al, 2000*)

FERMENTATION STRATEGIES FOR PHA PRODUCTION

The suitability of the organism for PHA production depends on the following factors:

a) Stability

b) Safety of the organism

c) Growth rate

d) Accumulation rates

e) Achievable cell densities

f) Extractability of the polymer

g) Molecular weights of the accumulated polymer

h) Range of utilizable carbon sources

i) Cost of the carbon source and other components of the medium.

j) Occurrence of the by-products.

Among the numerous bacteria that are known to synthesize P (3HB), only a few have been employed for the production of P (3HB). These include *Ralstonia eutropha*, *Alcaligenes latus*, *Azotobacter vinelandii*, several strains of methylotrophs, and recombinant *Escherichia coli*. Although these bacteria can be cultivated efficiently to high cell densities with a moderately high P (3HB) content in a relatively short period of time, much effort has been devoted to develop fermentation strategies for the production of P (3HB) with higher P (3HB) content and productivity. Various fermentation strategies have been adopted for PHA production.

1. Optimization of nutritional parameters for PHA production

The use of PHAs as substitutes for conventional plastics in a wide range of applications has been hampered due to the high production cost of PHAs compared with petrochemical-based polymers. Much effort has been devoted to reduce the cost of PHA by the isolation of better bacterial strains and development of more efficient fermentation strategies. In the growth experiments performed, the cultures were harvested when the cells reached the early stationary phase and the polymer composition was determined. Nothing is known about the time dependent change of the monomer composition of PHA during cell growth. Since the composition of the medium changes during bacterial growth because of buildup and subsequent utilization of metabolic intermediates, one would expect a change in the monomer composition of the PHA as the culture ages. The growth time, therefore is a controlling factor in the production of biodegradable polymer with specifically defined properties (Brandl et al 1988). There are very few reports as to statistical optimization of nutrition for PHA production. Designing of a growth medium that is optimal for the parameter under study such as high product yield, higher biomass or copolymer production is necessary for large-scale production.

2. Use of cheaper carbon sources or alternative substrates for PHA copolymer production

Strategies for the production of P (3HB) with high productivity, and production of P (3HB) from inexpensive substrate are emphasized. Sugars such as glucose and sucrose are the most common carbon sources used in usual fermentations. For the production of P (3HB-co-HV) copolymer, propionate or pentanoate is usually added to the medium as a co substrate for providing the precursors for HV synthesis. Other C sources used belong to either agricultural or food industrial wastes or plant oils or fatty acids.

Saponified palm kernel oil and its major fatty acids as carbon substrates for the production of polyhydroxyalkanoates in *Pseudomonas putida* PGA1 has been worked by Tan et al (1997). Whey and inverted sugar along with propionic acid feeding for poly (3-hydroxybutyrate-co-valerate) production by *Ralstonia eutropha* has been studied by Marangoni et al (2002). *Ralstonia eutropha* also uses mixed volatile and fatty acids for PHA production (Yu et al, 2002). Xylose has also been used as carbon source by *Alcaligenes eutrophus* and *Lactococcus lactis* in a 2 stage culture method (Tanaka et al, 1993). Whey a major byproduct in the manufacture of cheese is regarded as a pollutant due to its high biological oxygen demand and hence its disposal is costly. Whey has been used as a carbon source for PHA production. PHB is produced from whey by cell recycle fed batch culture by recombinant *Escherichia coli* harbouring *Alcaligenes latus* genes (Ahn et al, 2001). *A latus* has been considered a good candidate for the

production of P (3HB) since it grows quickly, accumulates P (3HB) during growth, and can utilize sucrose and inexpensive raw sugar and beet / cane molasses.

Two strains of *Burkholderia sp* that synthesized and accumulated a copolyester of 3-hydroxybutyric acid and 3-hydroxypentanoic acid from single, unrelated carbon source such as sucrose, glucose or gluconate has been studied (Rodrigues et al, 1995). Strains of bacteria known to accumulate PHAs using n-alkanes, n-alcohols, n-acids, etc have been chosen and studied (Haywood et al, 1989).

Poly (3Hydroxybutyrate) tercopolymers (3HB, 3HV, 3HHX) were synthesized by *Bacillus cereus* UW85 when grown on ε-caprolactone (Labuzek and Redecka, 2001). *Pseudomonas oleovorans* is reported to produce 3-hydroxyalkanoates on octanoate (Durner et al, 2000). *Pseudomonas sp* A33 is also known to accumulate a complex copolyester containing 3HB and various medium chain length 3-hydroxyalkanoic acids $3HA_{MCL}$ from 3-hydroxybutyric acid or from 1,3 butanediol (Lee et al, 1995). Production of PHA by *Pseudomonas citronellolis* on various carbon sources such as C_2-C_{10} monocarboxylic acids, C_3-C_{10} dicarboxylic acids, saccharides, α,ω –diols, hydrocarbons, 3-methyl branched substrates etc have been studied. Novel copolyester, poly (3-hydroxy-7methyl-6-octeneoate-co-3-hydroxy-5methyl hexonoate) was synthesized when grown on citronellol (Choi and Yoon, 1994). Simultaneous production of PHA as well as rhamnolipids to reduce cost of PHA production has been worked out in *Pseudomonas aeruginosa* (Hori et al, 2002a). When pentanoic acid was used as

a sole carbon source of copolyester of unusually high 3HV content of 90 mol% was produced in *Alcaligenes eutrophus.* Other substrates such as butyrate and acetate have also been worked out (Doi et al, 1988). Production of PHA from activated sludge treating municipal wastewater is another alternating method for PHA production from cheaper sources (Adeline et al, 2003).

3. Development of competent organisms for PHA production (recombinant and mutants)

Recent advances in biochemistry and molecular biology of PHA biosynthesis and cloning of the PHA biosynthesis genes from a number of different bacteria has allowed development of recombinant bacteria with superior PHA producing capacity. Recombinant strains have been studied for the development of various blends of PHA. Recombinant strains of *Pseudomonas oleovorans*, which harboured PHB biosynthetic genes of *Alcaligenes eutrophus* accumulated PHA composed of 3HB, 3HHx,and 3HO, upto 70% of the cell dry weight when grown on octanoate. (Timm et al, 1990). Engineering of metabolic pathways can optimize the formation of a product and improves the efficiency in the utilization of nutrients during fermentation. An algK::Tn5 mutation impairing alginate production in *Azotobacter vinelandii* led to a 50% increase in poly-β-hydroxybutyrate production to 75% of the dry cell wt. (Martinez et al,1997). PHA is also obtained by growing *Pseudomonas oleovorans* on a mineral medium containing l-fluorononane and sodium glutamate. *Pseudomonas oleovorans* is

also capable of producing P (3HA) containing various functional groups such as olefins, phenyl, fluoride, bromide and cyanide. These unnatural PHA are expected not only to show new physical properties but also to be used for synthesis of new functional polymers by chemical modifications (Hori et al, 1994).

ENZYMOLOGY OF POLYHYDROXYALKANOATE SYNTHESIS

Since the cloning of PHA operon in *Ralstonia eutropha* more than 50 PHA synthesizing structural genes from various bacteria have been cloned. These genes comprise the structural genes for

a. β-ketothiolases
b. Acetoacetyl-CoA reductases
c. PHA synthases.
d. Granule associated proteins.

The availability of these genes and detailed knowledge of the biochemistry of the enzymes and the regulatory proteins involved in the organism is necessary for understanding the PHA synthesis (Fig 6).

β-ketothiolases

β-ketoacetyl-CoA thiolase catalyses the first step in the PHA synthesis. This belongs to a member of a family of enzymes involved in the thiolytic cleavage of substrates into acyl-CoA and acetyl-CoA. These are found thorough

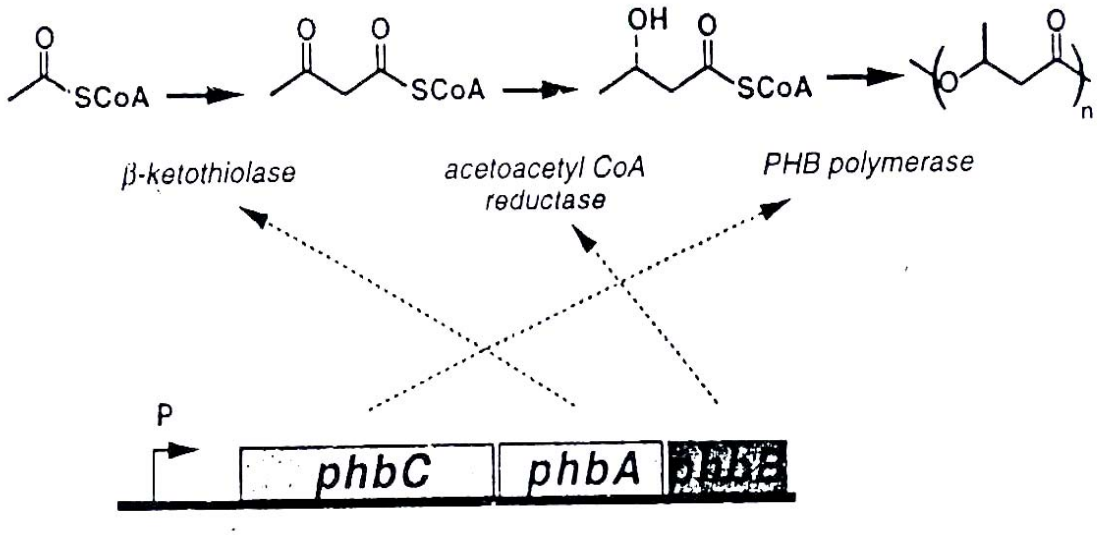


FIG 6: THE THREE STEP BIOSYNTHETIC PATHWAY FOR P(3HB) AND THE THREE ENZYMES ENCODED BY THE *phb*CAB OPERON (Madison and Huisman, 1999)

PhbA -β-ketoacyl-CoA thiolase, PhbB-Acetoacetyl CoA reductase, PhbC-PHB synthase, P- promoter

out nature from higher eukaryotes to prokaryotes. They are divided into two, based on their substrate specificity.

1. Class I thiolases.
2. Class II thiolases.

Class I thiolases have specificity for β-keto acetyl-CoA's ranging from C_4 - C_{16}. This class of enzymes are involved mainly in the degradation of fatty acids and is located in the cytoplasm of prokaryotes and in mitochondria and peroxisomes of mammalian and plant cells.

The second class of β-ketothiolases is considered biosynthetic and has a narrow range of chain length specificity from C_3-C_5. Throughout nature these biosynthetic thiolases are specialized for a variety of roles such as ketone body formation, steroid and isoprenoid biosynthesis and PHB biosynthesis (Madison and Huisman, 1999).

The most studied ketothiolase is that of *Ralstonia eutropha*. It contains two enzymes, enzyme A and enzyme B based on their substrate specificity. Enzyme A is a homotetramer of 44-KDa and converts acetoacetyl-CoA and 3-ketopentanoyl-CoA. Enzyme B is a homotetramer of 46-KDa subunits and cleaves acetoacetyl-CoA, 3-ketopentanoyl-CoA, 3-ketoheptonyl-CoA, 3- ketohexanoyl-CoA, 3-ketooctonyl-CoA, and 3-ketodeconoyl-CoA. Enzyme B is the primary source of 3HV monomer for P (3HB-HV) formation.

The enzymatic mechanism of phb A consists of two part in its reaction that result in the condensation of two acetyl-CoA molecules into acetoacetyl-CoA.

In the first part of reaction, an active site cysteine attacks an acetyl-S-CoA molecule to form an acetyl-s-enzyme intermediate. In the second part of reaction, a second cysteine deprotonates another acetyl-CoA resulting in an activated acetyl CoA intermediate that is able to attack the acetyl-S-enzyme intermediate and form acetoacetyl Co A.

Work on ketothiolase in *Zoogloea ramigera* has revealed that the ketothiolase is 1,173 nucleotides long and codes for a polypeptide of 391 amino acids. The work on inhibition of these thiolases by sulfydryl blocking agents revealed that cysteine was involved in the active site of thiolases. The cysteine involved in the acetyl-s-enzyme intermediate was identified as cys 89. The second cysteine in the active site was determined as cys 378. This deprotonates the second acetyl Co A molecule. So far all PHB thiolases contain these two active site cysteines. PHB thiolases use the same enzymatic mechanism to condense acetyl-CoA with either acetyl-CoA or Acyl-CoA.

A required step in the production hydroxyvalerate moiety of PHB-Co-HV is the condensation of acetyl coenzyme A (AcetylCo- A) and propionyl-CoA to form β-ketovaleryl CoA. This activity has been reported to be done by a bktB gene which encodes another β-ketothiolase that is capable of forming β-ketovaleryl-Co A. Thus establishing that there are multiple β-keto thiolases involved in the copolymer synthesis (Slater et al 1998).

Acetoacetyl Co A reductases

Acetoacetyl-CoA reductase is a (R) – 3 – hydroxyacyl-CoA dehyrogenase and catalyses the second step in the PHB synthetic pathway by converting acetoacetyl-CoA into 3-hydroxybutyrl-CoA. The aceto acetyl Co A reductase from *Z ramigera* is a homotetramer of 25 KDa subunits and is NADPH dependent reductase.

PHA synthases

PHA synthases are key enzymes of PHA biosynthesis. Enzymatic studies have shown that PHA synthases are localized in a soluble form in the cytoplasm when PHA is not being synthesized but gets sedimented together with granules when PHA synthesis starts. They are bound to the PHA granules and are localized at the surface of the granules. These enzymes catalyze the covalent linkage between the hydroxyl group of one and the carboxyl group of another hydroxyalkanoic acid. The substrates of PHA synthases are coenzyme A thioesters of hydroxyalkanoic acids. PHA synthases are classified into three types based on size structure and substrate specificity.

1. Type I PHA synthases
2. Type II PHA synthases
3. Type III PHA synthases

Type I PHA synthases are represented by *Ralstonia eutropha* enzyme and the subunit exhibits a molecular weight of 64.317 daltons and is encoded by $phaC_{Re}$. This type also occurs in the phototropic non sulfur bacteria and in many

heterotrophic bacteria except *Pseudomonas.* These prefer short chain length HA CoA monomers (C3-C5)

Type II pha loci is found only in *Pseudomonas* strain and has two pha synthase genes namely pha C_1 and pha C_2 and pha Z which is located in between. Pha Z codes for a depolymerase. Another gene pha D is also located in the type II pha loci. PHA synthase of *Pseudomonas oleovorans* encoded by $phaC1_{PO}$ has a molecular weight of 62.334 Da. The active forms of these have more than one subunit. These preferentially catalyses medium chain length HA CoAs.

Type III is represented by the enzyme of *Chromatium vinosum* and is encoded by pha EC_{CV}. It consists of two subunits, $phaC_{CV}$ and $phaE_{CV}$ with a molecular weight of 39,730 and 40,525, respectively. It catalyses the short chain length HACoAs. But the enzyme is composed of two types of subunits phaC and phaE as opposed to one type of subunit phaC in type I and type II synthases (Zhang et al 2001; Zhang et al 2001a). $phaC_{CV}$ is the catalytically active unit and the function of $phaE_{CV}$ is yet to be elucidated. Type III PHA synthases are also seen in phototrophic purple sulfur bacteria such as *Thiocystis violacea* and *Thiocapsa pfennigii* and in cyanobacteria such as *Synechosystis* and *Synechococcus*.

An alignment of the amino acid sequences of 36 PHA synthases (phaC gene products) revealed significant homologies between all PHA synthases.

a. PHA_{MCL} synthases from *Pseudomonas* are similar.

b. Pha C1 and Pha C2 proteins are different.

The alignment also revealed that there are 8 highly conserved amino acid residues that were identical. These 8 amino acids were Ser-260, Cys-319, Gly-322, Asp-351, Trp-425, Asp-480, Gly-507 and His-508.

The PHA synthase of *Bacillus* is different and novel. *Bacillus megaterium* pha C_{BM} is 362 amino acids in length (41.5K Da) and has the known salient features of pha C. It mostly closely resembles the PhaC of class III synthases in size and sequence having identity to them in the range of 32.7 to 29.9%. Pha C_{BM} is also distinctly different in having a putative lipase box having 15 non-variable amino acids. Thus *Bacillus megaterium* in particular and *Bacillus* genus in general forms a new class of PHA synthases (Mc Cool and Cannon, 2001).

Intermediates of the metabolism have not so far been identified as inhibitors or activators of PHA synthases. The only exception is the inhibition by Co enzyme A, whose physiological relevance is still not known.

Research about regulation of mcl PHA synthesis on a molecular basis shows a promoter region containing one or more promoter sequences upstream of the *pha* gene cluster. PHA production from simple carbon sources requires an intact Rpo N sigma factor, while PHA accumulation from fatty acids does not. It was also shown that pha F, one of the granule associated proteins which is homologus to prokaryotic histone H1 like proteins, is involved in transcriptional regulation of pha gene expression. Regulation of mcl PHA synthesis is therefore,

atleast partly based on enhanced PHA polymerase production by activation of transcription via specific environmental signals, such as lack of nitrogen or phosphate in the growth medium.

PHASINS: GRANULE ASSOCIATED PROTEINS

Proteins that are associated with the PHA granule are called as phasins. Phasins are low molecular weight proteins that are proposed to promote PHA synthesis in cells. They must accumulate to high levels in order to promote PHA synthesis. The Pha P gene is involved in its biosynthesis. Pha P accumulation requires PHA synthesis to take place. Phasin levels generally match PHA levels and their expression can be used as a marker for the production of PHB in individual cells. The Pha P is found to encode a 24KDa protein that binds to P (3HB) granule. This protein is always granule bound and no free pha P is seen in the cytoplasm. In vivo, this protein is likely to be involved in the maintenance of the optimal intracellular environment for PHB synthesis and utilization and as such provides guidance during the process of granule formation. First, soluble P (3HB) polymerase interacts with the 3-hydroxybutyryl-CoA in the cytoplasm, resulting in the priming of the enzyme by an unknown mechanism. Enzymes act on the ester bonds at the hydrophobic vesicle and water. PHA polymerase is a quite remarkable enzyme since it performs an esterification reaction under typically unfavorable conditions. During

an initial lag phase HB oligomers are slowly formed and extruded from the enzyme. The HB oligomers then form micelles as the oligomers increase in length and hydrophobicity. Consequently the micelle like particles provide a two-phase boundary with the polymerase located at the interphase. The enzyme then rapidly proceeds with 3-PHB synthesis extruding more PHB into the growing granule. Eventually the micelles coalesce into larger granules that can be visualized by microscopy. A layer consisting of specific proteins and phospholipids covers the granules. These surface layers cover the core of the granule that consists of PHA in the amorphous state. A granule of a diameter of 350 nm will contain approximately 40,000 PHB molecules.

Phasins form a layer at the surface of the PHA granules and they influence the size and number of PHA granules (Maehara et al, 2001). The over expression of the protein results in an increase in the number of PHA granules but a decrease in the size of the granules. The morphology of the granules is determined by the cooperative effect of Pha C gene and Phasins (Shin et al, 2002). Phasins stabilize the PHA granules, prevent them from coalescing and control the granule size. Phasins either bind to the PHA synthase or depolymerase or bind to the granules themselves and prevent binding of proteins not related to PHA metabolism (Korotkova et al, 2002).

Apart from phasins there are other regulators that effect PHA accumulation. Pha R has a negative regulation in PHA accumulation. In *Sinorhizobium meliloti* a homolog of Pha R, called as Ani A has been reported.

This protein functions in the partitioning of carbon flow in cells and affects not only PHA production but also production of extracellular polymeric substances and nitrogen fixation. Ani A is also seen in *Rhizobium etli* and acts as a global regulator of protein expression and carbon energy fluxes (Korotkova et al, 2002).

DEVELOPMENT OF EFFICIENT RECOVERY PROCESSESS FOR PHA FROM THE ORGANISMS

PHAs have been readily extracted by chloroform and other chlorinated hydrocarbons from bacterial cells. Development of an efficient recovery process is one of the important requirements to make PHA production economically feasible. Different methods of extraction of PHA have been adopted. They can be broadly classified as follows.

1. Extraction using sodium hypochlorite

2. Extraction using solvents

3. Extraction using solvent and hypochlorite dispersions

4. Enzymatic lysis of cells and solvent extraction

5. Use of surfactants and chelating agents

Extraction using hypochlorite is the usual method of extraction (Williamson and Wilkinson, 1958). But this method is highly destructive as it degrades the

polymer during extraction and lowers the polymer molecular weight (Ramsay et al, 1992). Extraction from solvents is being routinely done. PHA is refluxed with chloroform and the debris is removed by filtration and then polymer is precipitated with methanol or ethanol leaving low molecular weight lipids in solution and with hexane (Anderson and Dawes 1990). Longer side chain PHAs show less solubility than PHB and are soluble in acetone. Other solvents like ethylene carbonate and propylene carbonate have also been used. One of the processes for separating PHA from biomass comprises of treating the biomass with a PHA solvent and a marginal non solvent for PHA, removing any insoluble biomass, thereby leaving behind a solution of PHA and a marginal non-solvent for PHA. The PHA solvent is removed leaving precipitated PHA in the marginal solvent. (Noda, 1998). Another process is described where the biomass after separation is suspended in acetone or isopropanol and the separated and dried cell mass is dissolved in an unchlorinated inert organic solvent. The desired poly beta hydroxyalkanoic acid is obtained by evaporating the clear filtrate which is recovered after separating the solid mass in the form of a stable polymeric film (Ohleyer, 1995). A process describing separation, by adding a solvent which is immiscible with water and which has a boiling point of below 100^{o}C is also used. Here there is separating off of the aqueous phase and the organic phase is injected into hot water, causing the dissolved polyhydroxyalkanoate to precipitate (Blauhut et al, 1993).

To take advantage of both differential digestion by hypochlorite and solvent extraction, dispersions of hypochlorite and chloroform has been used in the recovery of PHB. Here the degradation of polymer is reduced because of shielding effect of chloroform. PHB of 97% purity was obtained by this method and an average molecular weight of 1,000,000 was obtained (Hahn et al, 1993). Further optimization of the method has revealed that PHB recovery was maximized upto 30% of hypochlorite concentrations (Hahn et al 1994).

Various acids (HCl, H_2SO_4), alkalies (NaOH, KOH, NH_4OH) and surfactants such as dioctylsulfosuccinate sodium salt, hexadecylmethyl ammonium bromide, sodiumdodecyl sulfate, polyethylene-p-tert-octylphenol (TritonX100) and polyoxyethylene (20) sorbitan monolaurate (Tween20) have been examined for their ability to digest non - P (3HB) cellular materials (Choi and Lee, 1999).

Various enzymes such as proteases, lipases and a mixture of enzymes have been used for PHA extraction. All these methods involve large amounts of solvents, chemical reagents and enzymes, which is an obstacle to reducing recovery cost on an industrial scale. Utilization of phage lysis genes to disrupt recombinant *E coli* that produces PHA has been reported. A novel self-disruptive strain of *Bacillus megaterium* that responds to substrate exhaustion has been reported (Hori et al, 2002).

BIODEGRADATION OF PHA

It is a general rule that microbial products can be degraded by microorganisms. Biodegradation is the ability to be utilized as a carbon source by microorganisms and converted safely into CO_2, biomass and water. Microbial attack starts where carbonyl group is present. Hence ester linkages are important in biodegradability. Polyesters are known to be non-enzymatically hydrolyzed by water, but enzymatic hydrolysis accelerates degradation (Kawai, 1995). Both extracellular and intracellular degradation of PHAs has been studied. However, majority of work is based on extracellular degradation of PHA because of applied interest with regard to environmental pollution. Degradation of PHB and its co polymer P (3HB-co-HV) has been studied both in natural as well as laboratory conditions. Under natural conditions degradation is studied in compost, soils and waste, although the rates varied depending on the environment and increased based on temperatures. Anaerobic methanogenic degradation of PHAs is also known (Budwill et al, 1992).

Various bacteria, fungi and actinomycetes are known to biodegrade PHA, although their composition and number varied depending on the environmental conditions. Quantitative analysis of microbial population adhering to commercial PHA samples such as Biopol was studied. Bacteria such as *Pseudomonas, and Bacillus strains,* fungi such as *Aspergillus, Penicillium*, *Cladosporium, Cephalosporium* and *Verticillium* colonised on PHA. Actinomycetes are also reported to degrade PHA. Biodegradation of PHA varied depending on several

factors such as temperatures, monomer composition, relative quantities, exposure, oxygen, blending with polysaccharides, polymer thickness, exposure surface, time, environment, film making technique, origin of polyester and crystallability, tacticity and diad content.

Microflora degrading PHAs also varied with temperature. P (3HB) and P (3HB-Co-3HV) degrading microflora consists of a wide range of different organisms at mesophilic temperatures whereas only a few species are involved in degradation at higher temperatures. At 15^{o}C the microflora consisted mainly of gram –ve bacteria and S*treptomyces*, whereas at 28^{o}C gram-positive bacteria and molds were frequently isolated. At 40^{o}C, molds and *Streptomyces* predominated. The restricted ability to degrade PHA_{lcl} polymers can be explained as due to the restricted ability to produce such polymers by different bacteria and it was found that those bacteria that can accumulate such polymers only degrade them.

Generally copolymers were found to be more biodegradable than homopolymers. This observed difference in susceptibility, of the depolymerase to the ester linkages by the presence of bulkier ethyl side chains along with the polymer backbone. Copolymers of P (3HB-Co-3HV) with 20% HV content degraded faster than that with 10% HV content.

PHB can be in two biophysical conformations. In vivo, the polymer is completely amorphous and is covered by phasins and lipids. After release from the cell the polymer becomes paracrystalline (Handrick et al, 2004). Crystallinity

of the polymer may be a primary factor in its degradability. PHAs of amorphous type are more degradable than those of crystalline type. Extracellular PHA products are usually highly crystalline (Hiraishi and Khan, 2003).

Biochemical pathway for PHA degradation

Biochemical studies have suggested a pathway for PHB catabolism in bacteria. Degradation is initiated with the action of a PHB depolymerase that releases the monomer 3-hydroxybutyrate. Both intracellular and extracellular PHB depolymerases have been documented. Accumulated PHB can be hydrolyzed by the accumulating strain itself during the periods of starvation (intracellular PHB hydrolysis by intracellular PHB depolymerases) or by other microorganisms after release of the polymer from the accumulating strain (extracellular PHB hydrolysis by extracellular PHB depolymerases). More than 20 extracellular PHA depolymerases (Pha Z) and several intracellular PHB depolymerases have been analyzed at the molecular level (Handrick et al, 2004).

3-hydroxybutyrate depolymerase (Bdh) enzymes from a number of bacteria have been purified and biochemically characterized. Enzymatic studies in various bacteria have suggested that Bdh plays a key role in the control of PHB degradation because its activity is regulated by some or all of the following compounds: NAD (P) H, pyruvate, oxaloacetate, 2-oxoglutarate, and acetyl-CoA. This enzyme has been observed only in bacteria that are able to accumulate PHB. The enzyme 3-hydroxybutyrate depolymerase (EC 1.1.1.30; Bdh) catalyses

the reversible oxidation of the released 3-hydroxybutyrate to acetoacetate, which is then activated to acetoacetyl-Coenzyme A by a CoA transferase. A ketothiolase cleaves the acetoacetyl –Co A to yield two molecules of acetyl –Co A which are metabolized via the tricarboxylic acid cycle and glyoxylate shunt.

Enzymology and genetic basis of PHA degradation

The PHA depolymerase protein and structural gene has been well studied. It was found that the molecular mass (40-50Kda) and effect of nonionic detergent diisopropylfluorophosphate, dithiothreitol and trypsin (abolition of PHB depolymerase activity but not the D-3- hydroxybutyric acid dimer hydrolase activity of all the enzymes) of all fine PHB depolymerases of bacteria isolated from various sources resemble those of the *Alcaligenes faecalis* enzyme.

Among all the PHA degrading bacteria known so far only *Pseudomonas lemoignei* was found to have five depolymerases, the rest are known to have only one depolymerase. The structural genes of most depolymerases have been cloned and sequenced. Biochemical analysis of the purified depolymerase proteins and analysis of the DNA deduced amino acid sequences revealed that PHA depolymerases apparently possess a catalytic triad consisting of serine, histidine and aspartate residues which have been demonstrated to form the active site in bacterial lipases. X-ray crystallographical studies have revealed a conserved folding pattern, which has been reffered to as the α-β-hydrolase fold and recognized as a consensus fold found in different hydrolases. The main

structural feature in these hydrolases is a central part consisting of predominantly parallel β- sheets with the active site located inside the protein. The central active-site serine residue in lipases is part of a consensus pentapeptide, Gly-X1-Ser-X2_Gly that is conserved in nearly all lipases. This central serine residue acts as a nucleophile, which is stabilised by a histidine and an aspartate (or glutamate) residue, all three residues forming the catalytic triad. Although no significant overall sequence homology to lipases or to other hydrolases was found, the lipase consensus sequence motif Gly-X1-Ser-X2_Gly was observed in all PHA depolymerases, the X1 residue was a leucine in PHA depolymerase instead of a histidine in bacterial lipases. The results have shown that PHA depolymerases may share a comparable mechanism to substrate hydrolysis. The hydrolysis of PHA_{SCL} by lipases has also been reported (Jaeger et al, 1995).

In mammals, the Bdh enzyme is involved in the ketone body metabolism during periods of starvation. The mammalian enzyme is located in the matrix face of the inner mitochondria membrane whereas the bacterial enzyme is cytoplasmic. In contrast to the bacterial enzyme, the mammalian enzyme has an absolute requirement for phosphotidylcholine for activity. The primary sequence of rat Bdh places it in the short chain alcohol dehydrogenase family (SCAD) superfamily. But there is no established molecular basis to convincingly compare between the mammalian and bacterial enzymes.

Rhizobium meliloti has a single Bdh. The *R meliloti BdhA* is a member of the short chain alcohol dehydrogenase family (SCAD) superfamily. Members of

this family are 250 –300 amino acids long and are either dimeric or tetrameric. Most of the conserved residues in the members of the SCAD superfamily are glycine residues, which are believed to be involved in coenzyme binding and contribute to the maintenance of the tertiary structure by allowing bend formation. In *R meliloti*, the only organism in which studies on the genetics of PHB degradation has been addressed, loci involved in PHB degradation appear to be scattered on the genome, with two loci on the chromosome and two on one of the megaplasmids, pRmeSU47b.

The *R meliloti* megaplasmid pRmeSU47b carries a number of catabolic genes, including those encoding enzymes for the utilization of carbon sources such as C_4-dicarboxylates, dulcitol, lactose, melibiose, HB and acetoacetate as well as genes encoding enzymes for phosphate utilization. PRmeSU47b also carries *glpK*, a gene required for utilization of glycerol as sole carbon source and *xdhA*, which is involved in the utilization of purines as a sole nitrogen source. The organization of these two genes, *BdhA* and *xdhA*, have roles to play in carbon and nitrogen starvation, respectively. This reflects a physiological relationship between these two starvation responses.

BdhA is negatively regulated by NAD(P)H, adenosine nucleotides, pyruvate, oxaloacetate and 2-ketoglutarate.A mutation in the *BdhA* has no effect on the ability of *R meliloti* to accumulate PHB (Aneja and Charles, 1999).

PRODUCTION OF PHA BY RECOMBINANTS AND TRANSGENIC PLANTS

Natural PHA producers usually produce PHA with a long generation time and require relatively demand stringent nutrient conditions. They also pose problems of down stream processing. Recombinants have an edge over natural producers (Lee and Choi, 2001) in that they have

1. Faster growth to high cell density
2. Accumulation of large amount of PHA
3. Ability to utilize cheaper carbon sources
4. Easy down stream processing
5. Lack of intracellular depolymerases
6. Molecular weight of PHA in the cells can be controlled.
7. Release of PHA granules by introduction and expression of bacteriophage lysis gene E
8. Digestion of *E coli* cells by NaOH is sufficient to release the granules. This lowers the production cost and helps in simpler downstream processing.

Several strategies have been adopted for developing recombinants.

1. Homologous and heterologous over expression of PHA biosynthetic enzymes in various organisms has been attempted.
2. PHA biosynthetic pathways introduced into non-PHA producers having more robust central metabolic pathways for more effective PHA production.

Expression of PHA genes heterologously increased PHA production but increase in the *pha* copy number did not affect PHA levels. This result explained the multilayered regulation of PHA biosynthesis. Increase in the copy number of PHA synthase altered the type of polymer and decreased its molecular weight. Recombinant *E coli* with *phb* genes from *R eutropha* is the first recombinant expressed in a heterologous host. Typical *E coli* strains such as XL1 Blue, JM109, HB101 accumulate PHB upto 75 to 85% of the cell weight. Although recombinants produce high amount of PHB these lack the ability to accumulate levels equivalent to natural producers. Growth of *E coli* cells is impaired by filamentation in a defined medium. Other challenges are to obtain stable and constant expression of *phb* genes during fermentation. PHB production from recombinants may be hindered by loss of plasmids from majority of bacterial population. Recombinant *E coli* lacks the phasin gene and produces heat shock proteins as a result of stress (Kabir and Shimizu, 2001). Most of the

recombinants prefer glucose, which is not economical, compared to natural producers which grow on sucrose and other cheaper sources such as molasses.

Plants serve as alternate systems for PHA production (Valentin et al, 1999). But the amount of PHA achieved is very less compared to bacterial fermentation. The capacity of plants for PHA production was first demonstrated by the transfer of genes for bacterial PHB synthesis to the model plant *Arabidopsis thaliana*. Pha B and Pha C genes were expressed in the cytosol. But only 1.4 mg / g dry weight in the leaf was observed. Moreover transgenic plants observed severe growth retardation. In another approach all the three enzymes of PHA synthesis pathway were targeted in plastids, using plastidic fatty acid synthesis. In plants, plastid has a high flux of carbon through acetyl-CoA because it is the site of fatty acid biosynthesis and storage. The PHA yields in the leaves increased to 140 mg / g without growth retardation. After *Arabidopsis thaliana* leaf chloroplasts, PHA synthesis pathways in crop plants like rapeseed, tobacco, cotton and corn have been tried. The transgenic tobacco plant expressed PHA biosynthetic genes and the current yield is 1.5 g/ kg dry weight of tobacco which is not very high for practical use (Arai et al, 2001). PHA production in roots of a carbohydrate storing crop plant such as *Beta vulgaris* has also been tried. Accumulation of more than 1% of PHA was reported in *Brassica napus* (oil seed rape) with maximum storage capacity upto 7.7% in the seeds (Menzel et al, 2003).

ECONOMICS

Several interesting properties of PHAs such as thermoplasticity, malleability, biocompatibility, and biodegradability have made it possible for a number of applications. PHAs can be synthesized from hydrocarbons, waste products, CO_2 and also toxic substances such as phenol. It can be tailored by modifying biosynthesis and also post biosynthetically. However currently there seems to be no real demand for this bioplastic or its blends. The main reason is its cost. PHB has been sold at US $ 15-30 per Kg. Detailed analysis has revealed that a lower limit of US $ 5 per Kg may be realistic. With recombinant *E coli* the prices are expected to come down to $ 2per Kg. Bacterial productions of Mcl PHA‘s are also expected to come to $ 5 per Kg. If *E coli* recombinants can be grown to cell densities of 150–200 g/l cell dry mass and with an accumulation of 70-90% W/W of PHA then the rate of PHA would be 2-3 g/liter/hour. Productivities of PHB production are in the range of about 2-5 g/l/ h, which is two to three, orders of magnitude lower than analogous chemical processes of polymer synthesis. Recently Metabolix Inc. has reported that it has succesfully scaled up production of PHA specifically in the packaging arena. In May 2003, the US department of commerce’s advanced technology program has awarded Metabolix $ 1.6 million allowing scientists to develop even more efficient conversion methods of renewable sugars into PHA plastics (Pierce, 2004). Dramatic improvements in the efficiency of microbial fermentations, leading to lower power costs for PHAs are expected.

ENVIRONMENTAL CONCERNS

However, two additional factors affect the long-term perspectives of petroplastics. First although the oil prices have increased much more slowly than the price index over the past few decades, and global oil reserves are chronically underestimated, the oil supplies will ultimately become limiting, probably sometime during the second half of 21st century. There is consensus that CO_2 emissions should be stabilized and this will lead to reduce petroleum usage. Thus the continued and increased production of petroplastics will require very good management of basic raw material, which is likely to become considerably more expensive towards the middle of 21st century. As this scenario unfolds, PHAs will be produced in sustainable processes based either directly by plants or indirectly by bacteria on photosynthetically produced precursors, at prices, which will in time become competitive with those of petrochemical plastics. As a result PHA will become viable successors to petroplastics not only in specialized applications but also in bulk applications.

APPLICATIONS

PHA being thermoplastic polyester has the potential to replace petrochemical plastics in a majority of applications. The extensive range of physical properties and broadened performance obtained by compounding and blending is exploited in such applications. PHAs are being manufactured as molded containers, backsheet of hygiene articles such as diapers, coating agents and packaging materials. It is exploited in bulk applications such as coatings, low strength packing and medium strength structural materials. Medium chain length PHAs have interesting applications in coatings, medical temporary implants (such as scaffolding for the regeneration of arteries and nerve axons), water based latex paints etc. A promising application of PHA is the use of PHA as a solid substrate for denitrification of water and wastewater (Hiraishi and Khan, 2003).

Utilizing PHA in day to day applications such as packaging and consumer awareness will increase investment in this area and this will subsequently help in developing the market for biodegradable plastics.

Objectives and Scope of Investigation

Plastic wastes are accumulating in the environment at an alarming rate. They are not biodegradable and hence are a matter of serious concern. Development of biodegradable plastics is the most sensible alternative in order to minimize this problem. Bioplastics are expected to reduce the pressure on landfills, minimize litter and above all help in conserving fossil fuel. Biodegradable polymers are a relatively new and emerging field. But they have already spread their influence into areas such as packaging, agriculture, medicine etc. Among the various biodegradable plastics that are emerging "Polyhydroxyalkanoates" have attracted the attention of industries and researchers because they have properties comparable to synthetic plastics and can be utilized in various specialized applications. They are biodegradable, biocompatible and are produced by microorganisms.

Polyhydroxyalkanoates (PHA) are biodegradable polyesters, synthesized by numerous bacteria as intracellular carbon and energy source. These are accumulated in the cytoplasm of cells as granules under conditions of nutrient imbalance. Accumulation usually occurs when carbon is excess and if atleast one other nutrient, which is essential for growth, is depleted. Polyhydroxyalkanoates serve as storage polymers in microorganisms and are utilized during starvation.

Although several bacteria are known to accumulate PHA intracellularly, industrial production of PHA has been developed using a few organisms

such as *Ralstonia eutropha* and recombinant *E coli*. These organisms have limitations (such as stability, expensive carbon source requirement) and hence the potential of other microorganisms in this regard needs to be explored. There is a lot of diversity among bacteria with regard to quantity and quality of PHA accumulated. Because of this diversity there is scope to discover better producers. Hence there is a need to explore indigenous bacterial cultures for PHA accumulating capability.

Occurrence of PHA in *Rhizobium* was reported as early as 1958 (Forsyth et al, 1958). But its formation in *Rhizobium sp*. is not commonly studied for production of bioplastics. PHA formation in *Rhizobia* is seen both during the bacteroid stage as well as free-living state (Madison and Huisman, 1999). *Rhizobial* PHA production has been dealt extensively only to study the interplay between cellular metabolism and polyester formation with regard to efficient nitrogen fixation. Extensive work has been done regarding the utilization of PHA as a source of energy in the bacteroid state. But little is known regarding the physiology of free living *Rhizobia* and PHA accumulated by them. Though *Rhizobia* are strict aerobes they are also capable of adapting to microaerophilic conditions in free living state wherein they accumulate PHA (Encarnacion, 1995). The members of the family *Rhizobiaceae* also differ in the mode of adaptations to stress and imbalanced nutrient conditions. They offer a variety of metabolic pathways directing carbon towards synthesis of biopolymers such as polysaccharides or polyhydroxyalkanoates. Polysaccharide synthesis is also a

common feature in *Rhizobia*. It is therefore interesting to study the intricacies of carbon metabolic traffic being directed either towards polyhydroxyalkanoate synthesis or polysaccharide biosynthesis with respect to *Rhizobia*.

This work aims at studying the capability of indigenous *Rhizobia* for production of biopolymer such as polyhydroxyalkanoate. This is an effort towards development of natural plastics from bacteria, which have potential to replace synthetic plastics and thereby in the long run eliminate the non-degradable plastics.

Objectives of the present work

1. Isolation and purification of *Rhizobia* from leguminous plants (such as root nodules). Screening of isolated cultures for the production of polyhydroxyalkanoates (PHA).
2. Identification of potent strain for the production of polyhydroxyalkanoates by morphological and biochemical methods. Optimization of cultural and nutritional parameters for biopolymer production.
3. Improvement of potent strain for biopolymer production by mutation using physical or chemical methods.
4. Isolation of specific biopolymer such as polyhydroxyalkanoate from the culture using physical, chemical or enzymatic methods.
5. Physicochemical properties of the isolated biopolymer (Melting point, solubility, molecular weight, composition etc).

Chapter 1

Isolation, Screening and Identification of Polyhydroxyalkanoate Producing Rhizobia

1.0 INTRODUCTION

The genus *Rhizobium* belongs to the family *Rhizobiaceae.* The soil bacteria that are able to colonize the roots of compatible leguminous plants and capable of inducing N_2 fixing nodules belong to the family *Rhizobiaceae*. This is a genus of aerobic, heterotrophic, non-sporeforming, bacteria able to invade the roots of leguminous plants and form nodules. The genera *Rhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium and Mesorhizobium* belong to this family. *Rhizobia* are capable of forming nitrogen-fixing symbiosis with more than 1130 species of leguminous plants and *Parasponia among* the *Ulmaceae*. Most *Rhizobia* are specific in their association with legumes (Stowers, 1985).

Metabolically various species of *Rhizobia* are split into two broad groups, fast growers and slow growers (Table 11). *Rhizobium* isolated from legumes of temperate origins such as *R trifolii, R leguminosarum, R phaseoli* and *R meliloti* are fast growers with a generation time of less than six hours. Whereas slow growers such as *R japonicum and R lupini* are isolated form legumes of tropical origin having generation time exceeding six hours. These bacteria exist in two different entities. The symbiotic N_2 - fixing bacteroid forms an intimate association with the host plant from which a steady supply of nutrients is derived. During nitrogen fixation the bacteroids exist as differentiated organelles that reduce N_2 to NH_3 into amino acids. They export NH_3 into the host cell cytosol where assimilation occurs via plant coded enzymes. The free-living form of *Rhizobia* competes for limiting nutrients with other soil inhabitants.

TABLE 11: ASSOCIATION OF A FEW IMPORTANT *RHIZOBIA* WITH LEGUMINOUS PLANTS

***Rhizobium* Species**	**Preferred hosts**
Fast growing Rhizobia	
R meliloti	*Medicago, Melilotus, Trigonella*
R leguminosarum	*Pisum, Viscia, Lathyrus, Lens.*
R trifolii	*Trifolium*
R phaseoli	*Phaseolus*
Slow growing Rhizobia (*Bradyrhizobium*)	
R japonicum	*Glycine*
R lupini	*Lupinus*
Cowpea miscellany	*Arachis, Vigna, Desmodium, Parasponia*

Source: Stowers, 1985

Our interest was concerned with the free-living forms of *Rhizobia*, their isolation and utilization for PHA production. The ability of the organism to survive in the adverse conditions depends on its capacity to develop or utilize different survival mechanisms. Rhizobia possess a variety of biosynthetic mechanisms to survive in different stages of their life. One of the them is polyhydroxyalkanoate production.

This chapter deals with the isolation, screening and identification of *Rhizobia* that produce PHA. Isolation and screening of different PHA producing *Rhizobia* is dealt with by three different methods.

1. **Traditional identification methods**
2. **Identification by polymerase chain reaction**
3. **Identification by Fourier transform infrared spectroscopy**

One culture that showed promising results was identified upto the species level.

1.1 MEDIA AND METHODS

STANDARD STRAINS

Various strains of *Rhizobia* were obtained from NCIM (National collection of industrial microorganisms, National Chemical Laboratory, Pune, India) and MTCC (Microbial type culture collection and gene bank, Institute of Microbial Technology, Chandigarh, India) for comparison purposes. The strains obtained were

Culture code	Organisms
MTCC 100	*Rhizobium meliloti*
MTCC 99	*Rhizobium leguminosarum*
MTCC 905	*Rhizobium trifoli*
MTCC 1954	*Ralstonia eutropha*
NCIM 2005	*Rhizobium leguminosarum*
NCIM 2002	*Rhizobium japonicum*
NCIM 2011	*Rhizobium sp* (A G Lochhead 347)
NCIM 2225	*Rhizobium sp* (Lima bean, NCIM isolate)
NCIM 2226	*Rhizobium sp* (Double bean, NCIM isolate)
NCIM 2227	*Rhizobium sp* (*Sesbania graniflora*, NCIM isolate)
NCIM 2231	*Rhizobium sp* (French bean nodule, NCIM isolate)
NCIM 2228	*Rhizobium sp.*

ISOLATION OF NEW STRAINS

Rhizobia are known to occur in soil, water and mostly in root nodules of *Leguminosae* family. These sources were used to isolate the bacteria. They were further screened for the production of PHA by staining and other procedures.

Samples

Red soils, soil from tea plantations, rhizosphere soil, water and root nodules of leguminous plants were used as sources. The roots of the following leguminous plants were collected locally for isolation of culture from nodules.

	Source of root nodules	**Designated number**
1.	Clustered bean (*Cyamopsis tetragonoloba*)	R 1
2.	Rain tree (*Pithecolobium saman*)	R 2
3.	Country bean (*Dolichos lablab*)	R 3
4.	Ground nut (*Arachis hypogea*)	R 5
5.	Cowpea (*Vigna sienensis*)	R 6
6.	Beans (*Phaseolus vulgaris*)	R 7
7.	Black gram (*Phaseolus radiatus*)	R 8
8.	Clitoria (*Clitoria ternatia*)	R 12
9.	Cassia (*Cassia oxidentalis*)	R 13
10.	Fenugreek (*Trigonella foenumgreacum*)	R 14
11.	Bengal gram (*Cicer arietinum*)	R 15

Method

Collected soil samples were serially diluted and plated on to Yeast mannitol agar (YMA). Root nodules of leguminous plants were also used to isolate bacteria. Well-grown nodules were carefully plucked out from the roots. Healthy nodules were selected and immersed in 0.1% $HgCl_2$ for 5 min for surface sterilization. The nodules were then rinsed several times in sterile water followed by 70% alcohol (3 min). They were again rinsed and macerated in known volume of sterile water. This was serially diluted and plated on to YMA. The colonies that grew well on YMA were isolated and screened as shown in fig 7.

SCREENING FOR PHA (Traditional method)

Gram's staining

The isolated cultures were characterized as Gram +ve or Gram -ve based on Gram's staining method (See page 106, Materials and methods)

Sudan black staining

Isolated cultures were characterized as PHA +ve or PHA –ve based on the sudan black staining of the cells taken from slant and liquid cultures (See page 107, Materials and methods). Here the PHA positive cells picked up the sudan black stain and appeared dark in colour when observed under the light microscope. Whereas negative cells were stained by the counter stain (safranin) only and they appeared pink.

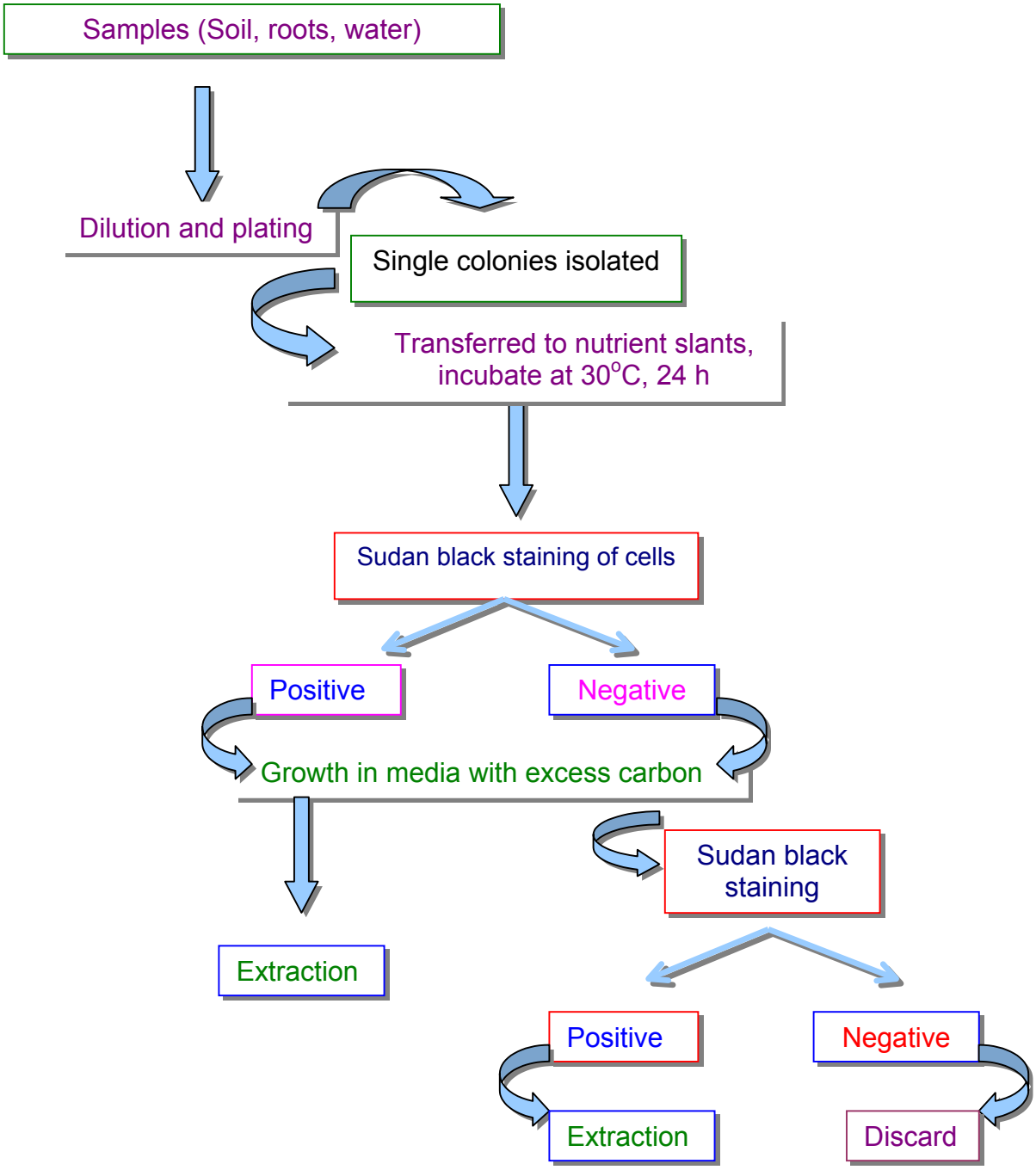


FIG 7: SCREENING MICROORGANISMS FOR POTENTIAL PHA PRODUCERS.

Nile blue staining

This is another efficient technique for identification of PHA producing bacteria (See page 107, Materials and methods). The cells that contain PHA fluoresce as bright orange clusters. Alternatively the culture was also grown in plates containing Nile blue stain. The colonies of PHA producing organisms appear orange red when observed under UV.

PHA estimation

PHA estimation was done by hypochlorite extraction (See page 103, Materials and methods).

IDENTIFICATION OF THE SELECTED CULTURE (R 14)

Yeast mannitol Agar (YMA)

The selected culture, R 14, was grown on YMA (See page 108, Materials and methods).

Congo red (Diphenyldiazo-bis-napthylamine)

10ml of sterile 1/400 aqueous solution of Congo red (25 mg/l final concentration) was added aseptically to each liter of melted YMA before tubing. R 14 was streaked on to these slants (Vincent et al, 1968). This was used for distinguishing between *Agrobacterium* and *Rhizobia*.

Bromothymol blue

R 14 was grown on YMA containing bromothymol blue. 5 ml of 0.5% alcoholic solution of bromothymol blue per litre was used with YMA. This was

used to distinguish fast (acid producing) and slow growing (non-acid producing) *Rhizobia* (Vincent et al, 1968).

Glucose Peptone Agar

Composition	**g/l**
Glucose	5
Peptone	10
Agar	15

To the sterile melted medium, bromocresol purple (10 ml of a 1% solution in ethanol) was added and sterilized at 115^{o}C for 10 min. This was useful for detecting the presence of many *Rhizobia*, which grow abundantly in 24-48 h at 30^{o}C commonly marked with a change in pH (Vincent et al, 1968).

Litmus Milk

Litmus milk was used for characterization of *Rhizobia.* It is prepared by adding a saturated alcoholic solution of litmus to fresh skimmed milk until a pale lavender colour is obtained. The prepared litmus milk was dispensed in 10-ml amounts in test tubes and autoclaved at 121^{o}C for 20min. Milk turns pink if there is acid formation. *Rhizobia* produce slow changes in litmus milk mainly towards slight alkalinity (Kersters and de Ley, 1971).

Catalase test

YMA agar slants were inoculated with the culture and incubated overnight at 30^{o}C. 1ml of 3% H_2O_2 was trickled down the slant. The slant was examined for

evolution of bubbles, the presence of which indicated a positive test for catalase (Kersters and de Ley, 1971).

Oxidase test

A filter paper strip was moistened in 1% solution of N,N,N^1,N^1,-tetramethyl-p-phenylenediamine-dihydrochloride.The growth of the test culture from a slant was placed on the paper. A platinum loop was used. Development of purple colour within 10 sec was due to the positive test for oxidase (Kersters and de Ley, 1971).

Antibiotic sensitivity

Antibiotic sensitivity of R 14 was tested. Antibiotic discs of ampicillin, caphalotoxime, cephalothin, cotrimoxazole, gentamicin, nalidicxic acid, nitrofurantoin, norfloxacin, erythromycin, penicillin, novobiocin, lincomycin, tetracycline were placed on YMA plates with R 14. Antibiotic resistance was measured by the inhibition zone formed around the discs and was measured in cm.

Growth of R 14 in different carbohydrate sources

Rhizobia show significant difference in growth when grown 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 substituted as carbon sources individually at 20 g/l (w/v) level in YMA medium.

Slants were prepared using this medium and R 14 was streaked on to it. Slants were incubated at 30^{o}C. Growth was observed after 72 h.

Growth of R 14 in amino acids

Different amino acids such as glycine, glutamic acid, cysteine, serine, tryptophan, leucine, histidine, proline, tyrosine, arginine, aspartic acid, alanine, lysine, threonine, valine, cystine, methionine, isoleucine and phenylalanine were used as nitrogen sources and growth of R14 was studied. These nitrogen sources (1.7 g/l) were incorporated into basal medium of YMA as nitrogen source. Slants were prepared using this medium and R 14 was streaked on to it. Slants were incubated at 30^{o}C. Growth was observed after 72 h.

Cell size

Cell size was measured using ocular micrometer (one scale =1/10 mm (100 μ) and stage micrometer (1 scale division =1/100 mm) in the microscope. The ocular micrometer was calibrated by using the stage micrometer. Under 10X lens each division corresponds to 11.2μ, with 25X, 4.6μ, with 40X, 2.8μ and with 100X, 1.1μ per division of ocular micrometer.

Scanning electron microscopy

To study the morphology, R 14 was observed under the scanning electron microscope. Fresh culture (12 h –18 h old) was centrifuged and washed twice in phosphate buffer 0.1M (pH 6.5). Centrifuged bacterial cells were fixed in 1-2% glutaraldehyde overnight. The cells were then separated by centrifugation at 8000 rpm for 5 min. In order to avoid initial osmotic damage, cells were

successively suspended in gradient ethanol (10-100%) followed by methanol. The sample was then dried in a desiccator. Dried cells were placed on aluminium stubbs and sputter coated with gold using argon as the ionizing plasma. Cells were scanned by using scanning electron microscope.

Nodulation test

The principle of this test is to grow host plants in sterile, nitrogen free medium with and without *Rhizobium* strain under test and to observe whether the nodules are formed in the presence of the bacteria.

Seeds of various leguminous plants such as clustered beans, fenugreek, cowpea, beans, double beans, country beans, pea, green gram, ground nut, black gram and soya bean were obtained locally for nodulation test.

Seeds were rinsed in 95% ethanol and immersed in 0.2% mercuric chloride solution for 3 min. Then they were washed with sterile water several times. The seeds were then placed separately on sterile wet paper (whatman no1). The sprouted seeds were placed on two different media, the composition of which is as given below:

Composition of Medium 1 (Jensen's medium)

Composition	**g/l**
$CaHPO_4$	1.0
K_2HPO_4	0.2
$MgSO_4.7H_2O$	0.2
NaCl	0.2
$FeCl_3$	0.1
Agar	8.0

Composition of Medium 2 (Fahraeu's Medium)

Composition	**g/l**
$CaCl_2.H_2O$	0.1
$MgSO_4.7H_2O$	0.12
KH_2PO_4	0.1
Na_2HPO_4 $12H_2O$	0.15
Agar	8.0

To obtain a clear and transparent substrate, gellan (1.5 g/l) was substituted for agar in the above media.

The seeds were also planted in sterile soil. Soil was sterilized by drying in oven at 120^{o}C for 4 h. Then it was cooled and bacterial biomass was inoculated to the soil before planting the surface sterilized seeds. Seeds were surface

sterilized with 70% alcohol. After a few weeks the plants were examined for root nodule formation.

IDENTIFICATION OF PHA PRODUCING ORGANISMS BY POLYMERASE CHAIN REACTION

Bacterial cultures were grown in 5 ml of sterile YMA for 24 h, at 30^{o}C and 250 rpm. The cells were harvested by centrifugation at 6000 rpm for 20 min. They were then washed thoroughly with distilled water and DNA was extracted according to Sambrook et al (1989).

DNA extraction procedure

The cell pellet was resuspended in 75 μl of 0.25-M sucrose and 50 mM Tris.HCl (pH 8.0) and vortexed. 15 μl of lysozyme (5 mg/ml) in TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) was added and the contents were mixed at 37^{o}C for 10 min. 50 μl of 20% SDS (w/v) in TE (warmed to 37^{o}C) was added and rocked gently, till complete lysis. To the lysed cells, 25 μl of ice cold 5 M sodium chloride was added, mixed thoroughly and kept on ice for 1 h. The cell debris was removed by centrifugation (11000 rpm) and supernatant was transferred to a fresh tube and extracted with chloroform – isoamyl alchohol mixture. 15 μ l of 3 M sodium acetate and 0.4 ml of ethanol were added and the mixture was kept for precipitation at –20^{o}C for about 10 min. DNA was pelleted by centrifugation and dissolved in 100 μl of TE buffer. Quality of DNA was checked by electrophoresis on 0.8% agarose gel.

Primer design

The nucleotide sequences of β-Ketothiolases and Acetoacetyl-CoA reductases were collected from different sequences available in the Gene Bank and was used for the design of Primers by Primer 3-primer design program. The sequences of the primers and the expected amplicon sizes are as follows:

Primer Name	Sequence	Corresponding region of gene	Accession number organism	Expected amplicon size (bp)
LF	**GTGACCGCGGGCAACGC**	3564-3581 (β-ketothiolase) 3008-3024 (β-ketothiolase	790549/*R meliloti* *4097185/A latus*	**966**
LR	**CTG(C)GCCGTTGAT (C)CGAC(G)GAGATGTT**	4553-4530 (Acetoacetyl Co-A reductase) 4024-4005 (Acetoacetyl Co-A reductase)	*790549/R meliloti* *4097185/A latus*	

The gene sequences for the primers were obtained from the gene bank and were subjected to multiple alignment, using primer 3-primer design program. A homology search of the database was performed using FASTA to investigate the specificity of the designed primers. LF (forward primer) were picked up from gene bank of *R meliloti*, accession number: 790549 phb A, β–ketothiolase (3564-3580), *Alcaligenes latus*, accession number: 4097185 phb A, 3-ketothiolase (3008-3024). LR (reverse primer) was picked up from gene bank of *R meliloti*, accession number: 790549 phb B, Acetoacetyl-CoA reductase (4553-4531),

Alcaligenes latus, accession number: 4097185, phb B acetoacetyl-CoA reductase (4024-4005).

Polymerase Chain Reaction

The optimized PCR reaction mixture contained 1x PCR amplification buffer [10 mM Tris- HCl (pH 9) 1.5 mM Mg Cl_2, 50 mM KCl and 0.01% gelatin], 200 μM each deoxynucleotide triphosphate (Bangalore genei, Bangalore, India), 1U *Taq* DNA polymerase (Bangalore, Genei), 2.5 μM each primer, in 25 ml PCR reaction mixture. The amplification was carried out using genomic DNA isolated earlier. The thermal cycle program was run on a Gene Amp PCR system. The cycle was 94^{o}C for 4 min, and 30 cycles of 94^{o}C 0.3 min, 60^{o}C for 0.45 min, 72^{o}C for 1.15 min followed by incubation at 72^{o}C for 10 min and a final incubation at 4^{o}C. PCR products were analyzed by gel electrophoresis in 1.2-% agarose gels. The amplified DNA fragments were visualized by UV illumination and the images were captured using a Hero LAB image analyzer (Wiesloch, Germany).

PHA estimation

All the samples tested by PCR were crosschecked by traditional extraction method (Materials and Methods, page 103).

IDENTIFICATION OF PHA PRODUCING BACTERIA BY FOURIER TRANSFORM INFRARED SPECTROSCOPY

Cells were collected by centrifugation (6000 rpm) from shake flask cultures and washed with distilled water. Then they were thoroughly dried by repeated washing in acetone and desiccated. Dried cells were suspended in chloroform. A drop of the suspension was placed on KBr windows and analyzed for PHA. Analysis was done by Fourier transform infrared spectroscopy (Model no. 2000, GC IR, Perkin Elmer, USA). The scanning conditions were a spectral range of 4000-400 cm^{-1}.

1.2 RESULTS AND DISCUSSION

Rhizobia were isolated from various natural samples such as soil and root nodules of leguminous plants. Based on sudan black staining nearly 20 colonies were isolated as positive PHA producers from a batch of 150 purified strains. They were further grown in the YMA medium along with standard strains and were examined for PHA production after hypochlorite extraction. PHA obtained was expressed in terms of % of dry biomass.

Occurrence of PHA in *Rhizobium* was reported as early as 1958 (Forsyth et al, 1958). Several species belonging to genera *Rhizobium, Bradyrhizobium* and *Azorhizobium* accumulate PHB in their free life cycle and in symbiosis. In contrast, in other species such as *Rhizobium meliloti*, the accumulation of PHB is observed only in the free living state or in the first steps of nodule development

but never in nitrogen fixing bacteroids (Cevallos et al, 1996). *Rhizobial* PHA production has only been dealt with regard to the interplay between cellular metabolism and polyester formation and factors affecting efficient nitrogen fixation. PHA formation in *Rhizobia* is seen mostly during free living state and is not beneficial for symbiosis (Madison and Huisman, 1999). Hence there is no thorough investigation of PHA production for industrial production purposes in *Rhizobium* (Tombolini et al, 1995). Synthesis and accumulation of PHB in different *Rhizobium* as reported by Manna et al (2000) vary between 1-38% of their dry biomass. The concentration of PHB accumulated by different *Rhizobia* has been reported by Tombolini and Nuti (1989) and according to them *Rhizobium meliloti* is known to accumulate PHB upto 55% of its cell dry weight and PHA in other strains vary from 35- 50%.

In the present study amount of PHA produced by the tested cultures varied from 5 - 65%. The standard strains produced PHA in the range of 11-47%. Amongst all the cultures that were tested only culture **R14** showed promising PHA yields of about 65% (Table 12). This was 10% higher than previously reported data and hence this culture was further investigated.

IDENTIFICATION OF R14

Identification of R14 was done according to Vincent et al (1968). R14 cells, which produced high amount of PHA, grew as whitish mucoid colonies on YMA (plate 1). They were gram negative, rods, motile, occurring singly or

TABLE12: PHA PRODUCTION IN A FEW ISOLATED AND STANDARD *RHIZOBIUM* CULTURES

Culture No.	Biomass (g%)	PHA (%w/w of biomass)
R 2	0.020	20
R 3	0.102	9.8
R 5	0.037	24.32
R 6	0.100	5.93
R 7	0.035	11.42
R 8	0.030	16.66
R 9	0.082	6.09
R 10	0.034	26.47
R 11	0.051	35.29
R 12	0.157	14.64
R 14	**0.143**	**65.73**
R 15	0.034	-
2226 (NCL)	0.216	30
2231 (NCL)	0.090	11
2227(NCL)	0.230	21
2228(NCL)	0.140	21
2011 (NCL)	0.220	18
2002 (NCL)	0.183	45
99 (MTCC)	0.700	41
100(MTTC)	0.610	47

PLATE 1: GROWTH OF *RHIZOBIUM MELILOTI* 14 ON YMA

in pairs. R 14 colonies appeared mucoid because of the production of exopolysaccharide. The cell size ranged from 1.98 μm to 2.9 μm in length and 1.1μ m in breadth. The cells were clearly observed under scanning electron microscopy (plate 2).

Young cells stained evenly (Gram's stain) but older cells appear banded because of the presence of PHA granules, when observed under phase contrast microscope. PHA granules are clearly stained by sudan black (plate 3). Presence of PHA granules were also confirmed by nile blue staining (plate 4). Nile blue A is a basic oxazine dye, which is water and ethanol soluble. The oxazone form of the dye (Nile pink) is formed by spontaneous oxidation of nile blue in aqueous solutions. The presence of PHA granules was confirmed by this staining method. PHA granules with bright orange fluorescence when observed under fluorescent microscope (460 nm exitation wavelength). Nile blue has a greater affinity to PHA granules than sudan black and is not easily washed off. Cell membranes and their lipid containing cell components do not absorb enough dye to be detected by fluorescence (Ostle and Holt, 1982).

Family *Rhizobiaceae* consists of species of *Rhizobium* and *Agrobacterium.* Here *Rhizobium* is most likely confused with *Agrobacterium* to which it is closely related. *Agrobacterium* can be distinguished from *Rhizobium* as it grows on peptone agar medium and absorbs congo red dye. Presence of PHB and ability to fix nitrogen in leguminous plants distinguishes *Rhizobium* from *Agrobacterium.*

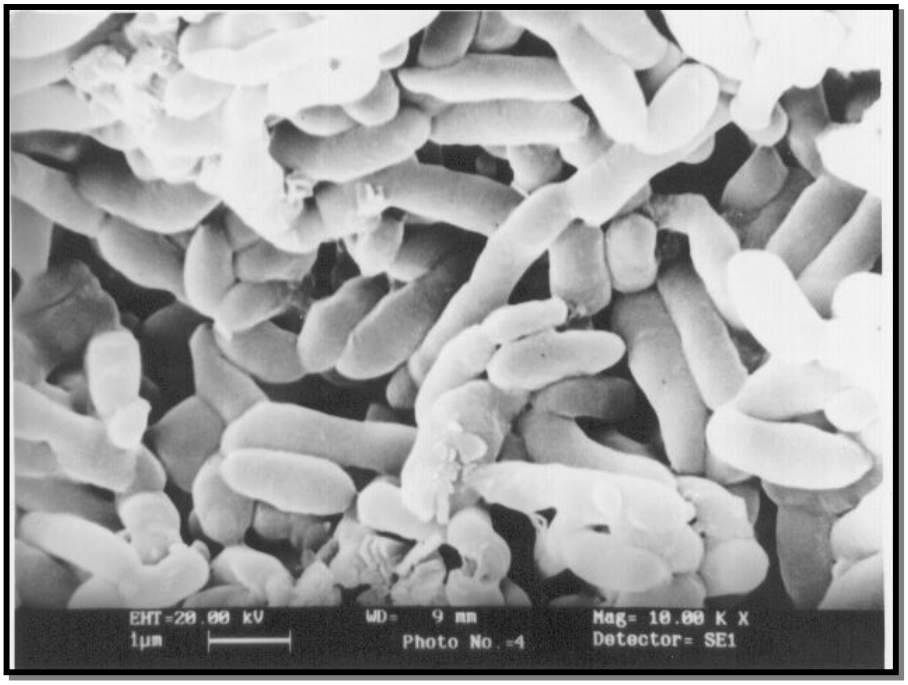


PLATE 2: SCANNING ELECTRON MICROGRAPH OF *RHIZOBIUM MELILOTI* 14

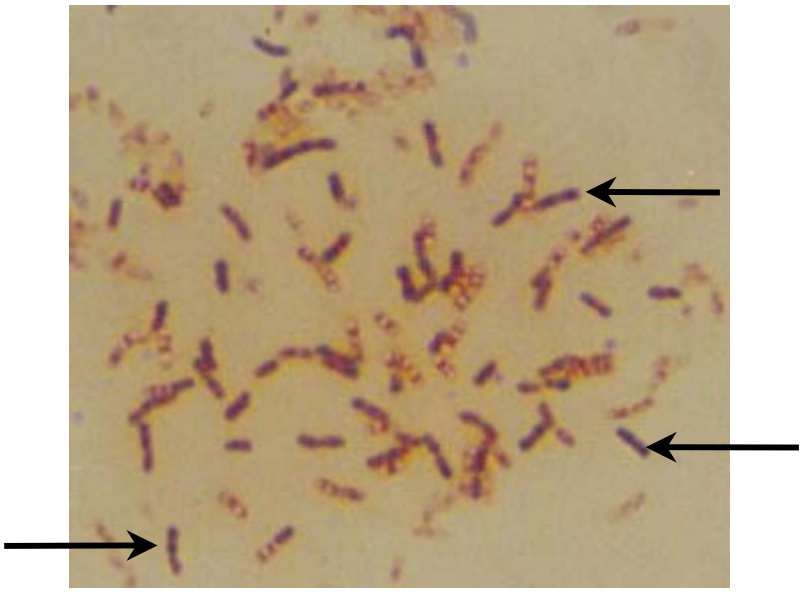


PLATE 3: SUDAN BLACK STAINING OF *RHIZOBIUM MELILOTI* 14. ARROWS INDICATE THE INTRACELLULAR PHA GRANULES, WHICH TAKE UP THE STAIN AND APPEAR DARK.

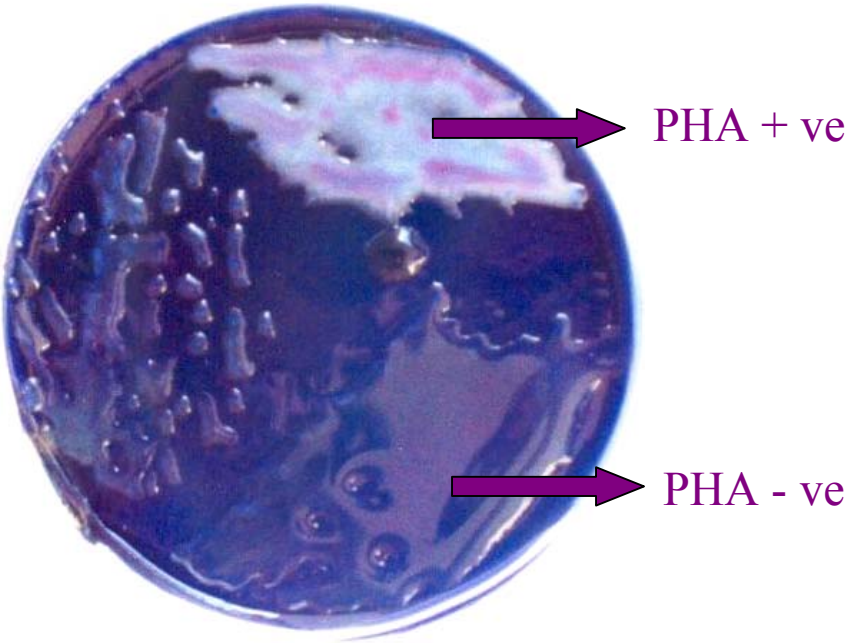


PLATE 4: GROWTH OF *RHIZOBIUM MELILOTI* 14 ON YMA CONTAINING NILE BLUE A.

R 14 showed no growth or pH change on peptone agar medium. This is because *Rhizobia* are weakly proteolytic. Whereas non-*Rhizobia* grow abundantly in this medium commonly marked with a change in pH (Vincent et al, 1968).

Growth in YMA supplemented with congo red dye detects non *Rhizobia* from *Rhizobia*. R 14 weakly absorbed congo red dye when grown on YMA supplemented with the dye. Non *Rhizobia* absorb the dye strongly (Plate 5).

The dye bromothymol blue helps in distinguishing fast growing *Rhizobia* from the slow growers. Fast growers have a mean generation time of 2-4 h and produce detectable colonies in 2-3 days and large colonies in 5 days at 25^{o}C on YMA. Where as slow growing *Rhizobia* have a generation time of 6-8 h and colonies are 1-2 mm in diameter after 10 days (Vincent et al, 1968). R 14 grew well in 48 h with 2-4 mm diameter colonies and produced enough acid to be detectable by bromothymol blue. R14 also showed tolerance to acidic condition (pH 4-6) as found in fast growers.

A comparison of R 14 with other *Rhizobia* is given in the table 13.

The change brought about by the organism in litmus milk is mainly due to their action on lactose and casein. Proteolytic activity of the organism leads to alkalinity. There was no clear serum zone formed in litmus milk by R 14 but milk turned bluish towards alkalinity. Catalase activity differentiates microorganisms from anearobes. R 14 was oxidase and catalase positive. The culture was least sensitive to erythromycin and cloxacillin, moderately sensitive to ampicillin,

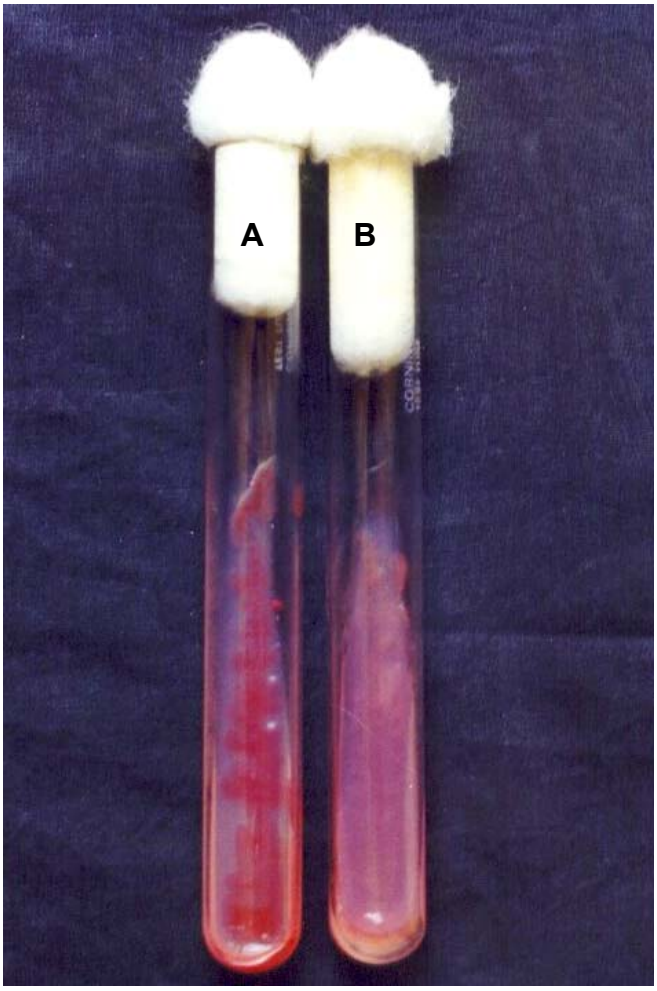


PLATE 5: ABSORPTION OF CONGO RED DYE BY *AGROBACTERIUM RADIOBACTER* (A) AND *RHIZOBIUM MELILOTI* 14 (B)

TABLE 13: COMPARISON OF R 14 WITH KNOWN CHARACTERISTICS OF STANDARD CULTURES

Characteristic feature	*Azorhizobium*	Bradyrhizobium	*Rhizobium*	*Sinorhizobium*	R14
Colony diameter on YMA with in 3 days at 28^{o}C	1-2mm	<1mm	2-4mm	2-4mm	2-4mm
Growth at 44^{o}C	+	-	-	?	+
Growth at pH 8	+	-	+	+	+
Acid production on YMA	-	-	+	+	+
Carbon utilization D-ribose,xylose, mannose, galactose, arabinose	-	+	+	+	+
Cellobiose	-	-	+	+	+
Maltose, lactose	-	-	+	?	+
Inositol, raffinose	-	-	+	?	+
Adipate	+	+	-	?	-
Dulcitol	?	-	+	-	+
Asparagine	?	-	+	-	ND*
Histidine	?	+	+	-	scanty
Growth in presence of 8% KNO_3	+	?-	?	?	No growth
Growth in presence of 1.5-4.5% NaCl	-	-	-	+	+
Oxidase and Catalase	+	?	?	+	+
Utilization of peptone	?	?	-	?	no
Hydrolysis of agar	?	?	-	?	no

* ND not determined

Details of all cultures except *R meliloti* 14 is from Bergeys manual of determinative bacteriology.

chloramphenicol, neomycin, gentamicin, polymyxin B and pencillin and very sensitive to streptomycin, kanamycin, tetracycline and novobiocin (Table 14). *Rhizobia* exhibit a great nutritional diversity with respect to carbon and nitrogen utilization. Fast growing *Rhizobia* are capable of growing on a wide range of carbon sources whereas slow growing *Rhizobia* are more limited in their ability to use diverse carbon sources. Fast growing *Rhizobia* utilize hexoses, pentoses, disaccharides, trisaccharides, and organic acids. Slow growing *Rhizobia* are unable to use disaccharides, trisaccharides and organic acids for growth. Slow growing *Rhizobia* are unable to metabolise sucrose whereas fast growing *Rhizobia* take up sucrose and other disaccharides (Stowers, 1985). Hence growth on carbon sources is used to classify *Rhizobia* and identify them. R 14 utilized hexoses, pentoses, disaccharides, trisaccharides and organic acids for growth even when provided as sole carbon sources. Among the carbon sources provided, R 14 did not grow on inulin (Table 15). Similarly utilization of amino acids also is a taxonomical character in *Rhizobia*. Amino acids are usually degraded into seven metabolic intermediates: pyruvate, α-ketoglutatrate, succinyl-Co A, fumarate, oxaloacetate, acetyl-CoA and acetoacetate. Thus they are either glucogenic or ketogenic. Overall R 14 was found to grow well on glucogenic amino acids and used these amino acids as sole nitogen sources (Table 16). Growth was good on glutamic acid, proline, arginine, aspartic acid and lysine. Poor growth was seen on ketogenic amino acids such as leucine,

TABLE 14: ANTIBIOTIC SENSITIVITY OF R 14

Antibiotic	Sensitivity	Inhibition zone (cm)
Ampicillin	+	0.1
Streptomycin	+++	0.8
Chloramphenicol	+	0.1
Neomycin	++	0.2
Erythromycin	-	-
Gentamicin	+	0.1
Kanamycin	+++	0.5
Polymyxin B	++	0.3
Pencillin	+	0.1
Novobiocin	+++	0.5
Cloxacillin	-	-
Tetracycline	+++	1.0

TABLE 15: GROWTH OF R 14 ON DIFFERENT CARBOHYDRATE SOURCES

Carbohydrate Source	Growth
Raffinose	+
Cellobiose	+++
Galactose	+
Rhamnose	++
Sucrose	+++
Sialin	+
Trehalose	+
Maltose	++
Dulcitol	+
Dextrose	++
Adonitol	+++
Fructose	++
Arabinose	++
Sorbitol	+
Xylose	++
Mannitol	+++
Mannose	++
Lactose	+
Mellibiose	+
Inulin	-

TABLE 16: GROWTH OF R 14 ON DIFFERENT AMINO ACIDS

Amino Acids	Growth
Glysine	+
Glutamic acid	++
Cysteine	+
Serine	scanty
Tryptophan	-
Leucine	scanty
Histidine	scanty
Proline	++
Tyrosine	scanty
Arginine	++
Aspartic acid	++
Alanine	scanty
Lysine	++
Threonine	scanty
Valine	-
Cystine	+
Methionine	scanty
Isoleucine	scanty
Phenylalanine	+

isoleucine, tryptophan, threonine and tyrosine. R 14 did not utilise histidine, serine, alanine, and methionine.

Recognition of a preferred host by the *Rhizobia* forms a final and important test to confirm it to species level or a group (Vincent et al, 1968). To confirm whether the bacterium isolated is species of *Rhizobium*, *s*elected bacterium was tested for its capacity to nodulate on various leguminous plants. The plants failed to grow properly in Jensen's medium and Fahraeu's Medium for lack of proper space in test tubes. Gellan did not support plant growth as the seeds were sunk in the medium and all the plants were desiccated before bacterial nodulation. But all the plants grew well (by 7 days) in sterile soil inoculated with R 14. Nodules were seen only on Fenugreek and *Alfalafa* roots after 3 weeks. (Plate 6). *Rhizobium meliloti* nodulates only *Medicago, Melilotus* and *Trigonella* (Zhan et al, 1990).

Based on the morphological, biochemical and nodulation tests R 14 was identified as a strain of *Rhizobium meliloti* (*Sinorhizobium meliloti*) and designated as ***Rhizobium meliloti* 14**.

Nodulation test of R 14 on *Trigonella foenumgraecum*

Nodulation test of R 14 on *Alfalfa*

PLATE 6: NODULATION OF *RHIZOBIUM MELILOTI* 14 ON *TRIGONELLA FOENUMGRAECUM* AND *ALFALFA*

IDENTIFICATION OF PHA PRODUCING ORGANISMS BY POLYMERASE CHAIN REACTION

Various phenotypic methods such as sudan black staining (Schlegel et al, 1970) , nile blue staining (Ostle and Holt, 1982), nile red staining (Gorenflo et al, 1999, Spiekermann et al, 1999) have been used to identify PHA producing organisms. These traditional methods are time consuming and labor intensive. Isolation and appropriate growth conditions prior to detection, and proper fermentation of the organism is necessary. Nutrient limiting conditions have to be provided to the bacterial cells to support PHA accumulation. Moreover these methods often don't clearly distinguish between PHA and lipid compounds (Sheu et al, 2000). Hence alternative methods have been developed for rapid detection of PHA producing bacteria. Genotypic methods involving Polymerase chain reaction technique is being used as a rapid detection method to identify PHA producing bacteria (Sheu et al; 2000; Solaiman et al, 2000; Shamala et al, 2003).

Enzymes such as β- ketothiolase, acetoacetyl-CoA reductase and PHA synthase are known to catalyze PHA synthesis (Fig 8). The synthesis route from acetyl-CoA to Poly (3HB) has atleast three steps and three enzymes are involved. β-Ketothiolases (Pha A) catalyze the first step in the PHA biosynthesis and is the key enzyme in PHB synthesis as well as metabolism (Oeding and Schlegel, 1973).

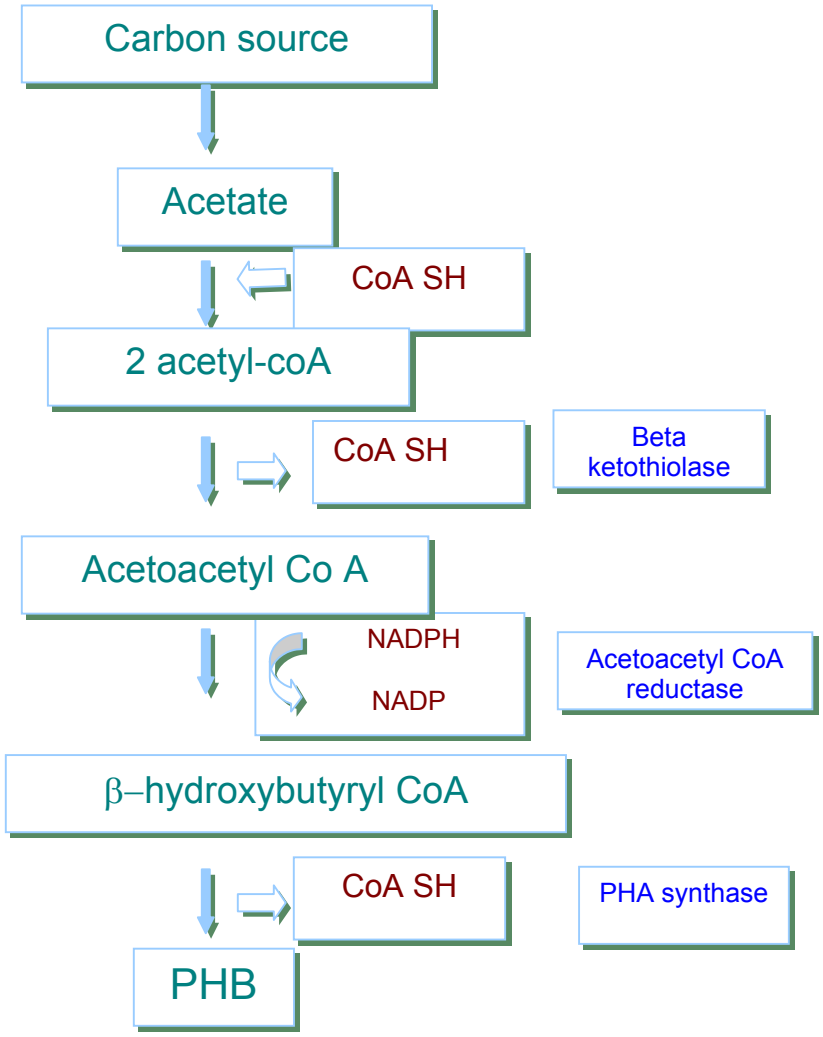


FIG 8: GENERAL BIOSYNTHETIC PATHWAY OF PHA IN BACTERIA

Acetyl-CoA, a central intermediate in the metabolism of all carbon compounds is either

a) Dissimilated to generate biologically useful energy.

b) Assimilated and used for growth and multiplication.

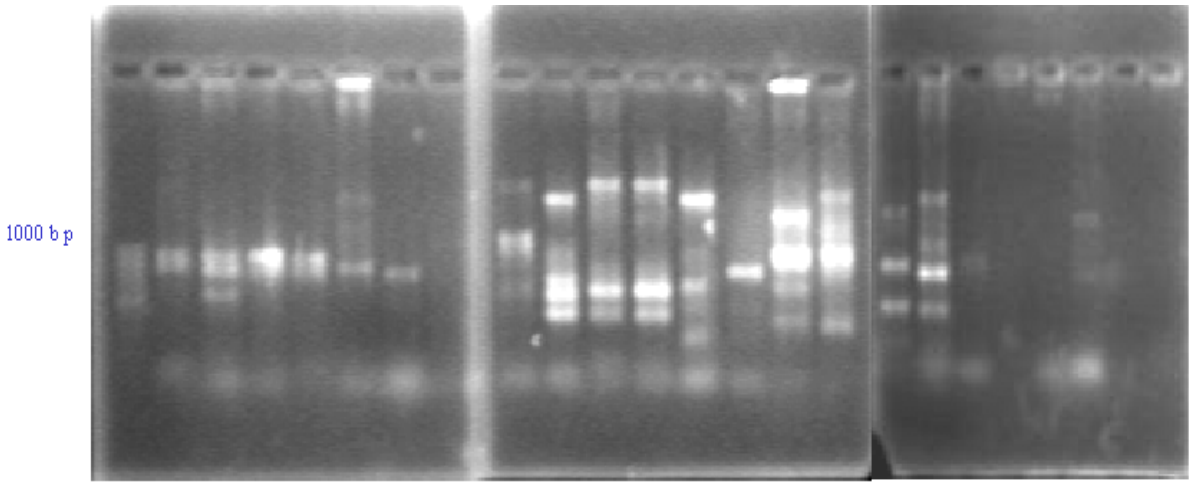
During abundance, the shortest and quickest way to store this carbon skeleton is to synthesize PHA via formation of acetoacetyl-CoA. During nutritional imbalance and high carbon levels the level of free CoA is reduced and activity of β - ketothiolases will be high. Thus β-ketothiolases are active in cells producing PHA. Though PHA synthase is the final and crucial enzyme in the PHA synthesis pathway (Anderson and Dawes, 1990; Madison and Huisman 1999), β-Ketothiolase and Acetoacetyl Co-A reductase are equally important enzymes in PHA biosynthesis (Tombolini et al, 1995). The presence of PHA synthase alone is not sufficient to allow synthesis of PHAs. PHA biosynthesis will not occur if genes encoding enzymes required for the synthesis of hydroxyacyl – coenzyme A thioesters are absent or if the pathways constituted by these enzymes are not functionally active. In this study primers were selectively designed based on gene of *Rhizobium meliloti* and *Alcaligenes latus* (both having Type I PHA sythases) whose β-ketothiolase sequences are known. PCR methodology for identification of PHA producers, so far has been exploited only with the PHA synthase gene (Sheu et al, 2000; Solaiman et al , 2000; Shamala et al, 2003).

Sensitivity of PCR for *Rhizobia*

β–Ketothiolase and acetoacetyl-CoA gene sequences of *Rhizobium meliloti* and *Alcaligenes latus* were aligned. Based on the conserved region sequences primers LF and LR were designed for PCR amplification. In prelimanary experiments it was observed that the primers amplified with the DNA of *Rhizobia* only and *Alcaligenes eutrophus, Pseudomonas* and *Bacillus* were not detected when PCR was done at 60^{o}C. The detection experiments with lower temperatures however showed amplification of non *Rhizobia* with multiple bands with both smaller and larger fragments than the expected size. β-ketoacyl- Co A thiolases are known to be ubiquitous in nature and there are reports of homologies between PHA specific β-ketothiolases (Oldenburg et al, 2000). The amplification of the genes of non *Rhizobia* may be due to the similarities. However under optimizted conditions at 60^{o}C, the primers amplified only *Rhizobial* PHA genes.

Isolation and screening of various environmental strains and standard strain s by PCR

A total of 22 strains both PHA positive and PHA negative strains were tested by PCR using the designed primers. *Rhizobia* isolated from root nodules of leguminous plants, cultures obtained from MTCC and NCIM culture collection centers were used. The results (Plate 7) show that there is a significant difference in the banding pattern of PCR amplicons between producers and non-producers of PHA. PHA estimation of these strains by the traditional methods



Marker 1000bp

PHA producers *1,2,3,4,5,10, 11,12, 14, 15.*

PHA non producers *6,7,9,13,16,17*

Producers with no amplification *22,19,8.*

Non producers and no amplification *18,20,21*

1: R 14	7: R 1	13: R 5
2: 100 (MTCC)	8: R 7	14: R 10
3: 99 (MTCC)	9: 2005 (NCIM)	15: R 2
4: 2011 (NCIM)	10: R 3	16: 905 (MTCC)
5: R 12	11: R 6	17:2002 (NCIM)
6: 2225 (NCIM)	12: 2227 (NCIM)	18: R 11

19: 2226 (NCIM), 20: 2228 (NCIM), 21:2231 (NCIM), 22: R 8

PLATE 7: PCR AMPLICONS OF PHA PRODUCERS AND NON PRODUCERS

showed that the strains R 14, 100 (*R meliloti*, MTCC), 99 (*R leguminosarum*), 2011, R 3, R 6, 2227, R 10, R2 and R 12 were the best producers. All the strains that amplified the expected PCR product were confirmed as PHA producers by hypochlorite extraction method. PHA % ranged between 18 to 57%. The PHA negative strains such as 905 (*R trifolii*), 2002 (*R japonicum*) showed non-specific amplification and strains 2228 and 2231 showed no amplification at all. Two standard *R leguminosarum* strains showed a clear difference in their amplification. Strain 99 (*R leguminosarum,* MTCC) which gave 41% PHA yield by extraction method amplified the expected PCR product. Whereas Strain 2005 (*R leguminosarum* NCIM) which was PHA negative showed nonspecific multiple amplification. The PHA positive strains invariably showed two bands; one band was of the predicted size (≈1000 bp) and the other of smaller size. This can be explained by the fact that there are multiple forms of β-ketothiolases (Slater et al 1998). It is also known that a *phb*A mutant of *A vinelandii* was able to accumulate 5% of PHB due to the presence of other ketothiolases (Segura et al, 2003). These strains were also analyzed by traditional extraction methods and the results were compared. The best producers showed the presence of two bands and this could be because of the presence of two types of the same enzymes in the bacterial system as reported in case of *Alcaligenes* (Steinbuchel and Hein, 2001). In many of the bacteria pha A, pha B and pha C form a single cluster in the genome. But in organisms such as *Zoogloea ramigera, Aeromonas caviae, Methylobacterium extorquens, Nocardia corollina, Rhodococcus ruber,*

Rhodospirillum rubrum, Rhodobacter spheroids and *Rhodobacter capsulatus* the genes do not co-localize. In *Rhizobium meliloti pha A and pha B* are not co-localized with PHA synthases (Fig 9). Hence it was expected that the primers designed for both β-ketothiolase and acetoacetyl reductase could detect the entire pha A B operon in *Rhizobium*. Alternatively it could also be hypothesized that the reason for the occurrence of two PCR products to single primer amplifications for ketotiolase and reductase enzymes. But the absence of any amplification by the PHA negative strains justifies the use of these primers for effective identification of PHA producing *Rhizobia*.

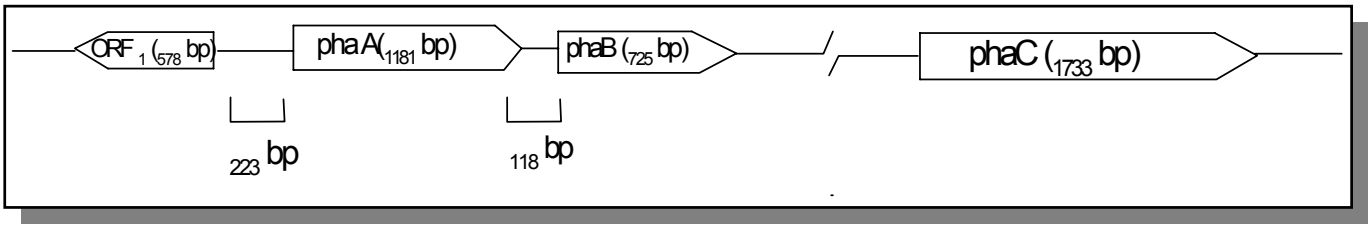


FIG 9: MOLECULAR ORGANIZATION OF PHA SYNTHESIZING GENE IN *RHIZOBIUM MELILOTI*

(Steinbuchel and Hein, 2001)

IDENTIFICATION BY INFRA RED SPECTROSCOPY FTIR

Fourier transform infrared spectroscopy has been used as a powerful tool for studying microorganisms and their cell components in their intact form. Almost any compound having covalent bonds, whether organic or inorganic will absorb frequencies of electromagnetic radiation in the infra red region of the spectrum. The radiation is vibrational and hence is not destructive. Bacterial cell components such as polypeptide, dipicolinic acid have been identified by FT-IR (Helm and Naumann, 1995). IR spectroscopy can thus provide a total simultaneous chemical analysis and the resulting spectra reflect the total biochemical composition of the cells such as proteins, polysaccharides, nucleic acids and PHAs. FTIR has also been used for quantification of PHAs (Kaniz et al, 2000).

FTIR is a useful method while screening a large number of bacterial samples to identify PHA producers. Other methods of screening are laborious, require extensive and complicated sample preparation and are time consuming. In FTIR, organisms can be probed by a single experiment, using simple uniform procedures that can be applied to all the samples (Lin et al, 1998). FTIR can identify polyhydroxybutyrate as well as its copolymers in a given sample (Hong et al, 1999, Misra et al, 2000).

About 10 locally isolated bacteria were screened by FTIR analysis. The prominent regions seen in many of the samples were bands at 1720 – 1730 cm^{-1}, 1280 cm^{-1}, 1375 cm^{-1}, 1455 cm^{-1} , 1465cm^{-1}, 1610cm^{-1} and 3424cm^{-1} (Table 17).

Polyhydroxyalkanoates are polyesters and C=O, C-O, -CH_2 -, -CH_3- and OH stretching groups are expected in the compound. These correspond to 1720-1740cm^{-1}, 1300-1000 cm^{-1}, 1465cm^{-1}, 1450-1375 cm^{-1}, and 3650-3600 cm^{-1} respectively in the IR spectra. IR peaks at 1725-1740cm^{-1} is typical of compounds containing carbonyl groups i.e esters. Non PHA producer such as R15 did not show any peak in this region. This is an important distinguishing factor as this peak will be the most prominent peak in bacteria that accumulate PHA. A peak at 1650 cm^{-1} corresponds to C=O of amides associated with proteins, which can be seen only in IR spectrum of samples where cells are directly analyzed. This peak will be absent in spectrum where extracted PHA is analyzed. Methylene groups (-CH_2 -) that have a characteristic absorption at 1450 cm^{-1} and absorption corresponding to methyl groups (-CH_3 -) at 1375 cm^{-1} were also seen. The intensity of these peaks is useful in identifying whether the samples also contain higher alkanoates other than PHB. The mode of extraction of PHA might affect the prominence of these peaks. Strong vibrations near 2928cm^{-1} were also seen. These bands could be characteristic of N-H or C-H whose contribution might be from both proteins and PHAs. A strong band here is a feature, characteristic of mcl PHA (medium chain length) subjected to confirmation. A band shift was also seen in carbonyl (C=O) vibrations towards higher wave number in intact cells in contrast to extracted samples. This is attributed to more complex environment surrounding the PHA in the cells (Hong et al, 1999). It was also noticed that a low concentration of PHA in the samples,

TABLE 17: CHARACTERISTIC FTIR REGION OF CELLS CONTAINING PHA

Name of locally isolated strains	Characteristic regions, FTIR (cm-1) wave number seen
R 2	1727,1280,1377,1455,1610,2917, 3424cm^{-1}.
R 3	1727,1280,1382,1450,1610, 2917 cm^{-1}.
R 6	1722,1270,1382, 1610, 2917, 3424 cm^{-1}.
R 8	1722,1270,1615,2917, 3375cm^{-1}.
R 10	1727,1275,1377,1446,1465, 1615,2919, 3375cm^{-1}.
R 11	1728,1270,1377,1620,2926, 3424cm^{-1}.
R 12	1727,1275,1615,2917,3424 cm^{-1}.
R 14	1727,1275,1377,1459,1620,2950, 3424cm^{-1}.
R 15 **(non producer)**	13 77, 1462, 1644,1537,2950, cm^{-1}.

which might be detected by gas chromatography, could go unnoticed in FTIR, as other esters present in the cells will disturb the characteristic bands. Thus FTIR also can distinguish between good and poor producers of PHA.

1.3 CONCLUSION

Rhizobia were isolated from various natural samples and the accumulation of PHA in the isolated cultures varied from 5-65%. Culture R 14 which showed promising PHA yields of 65% was identified as *Rhizobium meliloti*. The yield of PHA in R 14 was 10% higher than the reported yields in *Rhizobia.* Primers were designed to identify PHA producing *Rhizobia* from natural samples. PCR based identification specifically identified PHA producing *Rhizobia* and also the best producers among them. FTIR also proved to be useful in identification of PHA producers.

Chapter 2

Characterization of Cultural Conditions for Polyhydroxyalkanoate Production

2.0 INTRODUCTION

Physiological response such as accumulation of PHA in organisms may occur due to limitation or imbalance of a few nutrients. This phenomenon is also termed as overflow metabolism. Nutrients whose limitation results in PHA accumulation in the organisms are nitrogen, magnesium, oxygen, phosphate and potassium. However other nutrient limitations such as limitation of iron and sulfur also lead to PHA accumulation in microorganisms. Unbalanced supply of nutrients for growths or deficits of nitrogen or oxygen will reduce the complexity of metabolism and channel the flow of carbon skeletons into more unidirectional pathways such as PHA synthesis. (Babel et al, 2001; Squio et al, 2003). Thus PHA synthesis depends on levels and type of the substrate or nutrient provided. Thus it is imperative to understand the nutrient needs of the organism towards PHA synthesis.

This chapter examines the biochemical and physiological requirements of the selected culture, *Rhizobium meliloti* 14. Essential and limiting nutrient for growth and PHA production, aeration, pH and temperature requirements and also the growth kinetics of *R meliloti* 14 has been studied in detail in order to understand the requirements of the organism for efficient polymer production.

2.1 MEDIA AND METHODS

Media for growth of *Rhizobium meliloti* 14

Tryptone yeast extract medium, Rhizobium minimal medium (RMM) (Vincent et al, 1968) were first used for screening the organism *R meliloti* 14 for growth as well as PHA production, the composition of which are as follows:

(i) Tryptone Yeast extract medium (TY)

Components	**g/l**
Tryptone	5.0
Yeast Extract	3.0
$CaCl_2.6H_2O$	1.3
Agar	15.0

(ii) RMM medium

Components	**g/l**
K_2HPO_4	2.05
KH_2PO_4	1.45
$MgSO_4.7H_2O$	0.50
NaCl	0.15
$CaCO_3$	0.01
NH_4NO_3	0.5
Sucrose	2.0
	mg/l
$FeSO_4$	2.5
$MnSO_4$	0.5
$NaMoO_4$	0.5

continued…

	mg/l	
Biotin	0.01	Filter sterilized
Calcium pantothenate	0.01	
Thiamine	0.01	

(iii) Yeast Mannitol Agar (YMA) (see Materials and Methods page 108)

The above media (100ml) were sterilized (121^{o}C, 20 min) in 500 ml Erlenmeyer flasks. *Rhizobium meliloti* 14 was inoculated and grown at 30^{o}C and 250 rpm. Growth and PHA production was studied upto 72 h.

Growth kinetics of *Rhizobium meliloti* 14 with respect to PHA production

YMA medium (100 ml) was taken in 500-ml Erlenmeyer flasks and sterilized (121^{o}C, 20 min). The flasks were inoculated (in duplicate) with 10% (v/v) of 18 h old inoculum of *Rhizobium meliloti* 14. Thereafter the flasks were incubated at 30^{o}C, pH 7.0 and 250 rpm upto 120 h. Biomass, PHA, cfu/ml, polysaccharides were analyzed in duplicate flasks at 6, 12, 24,36, 48, 72, 96 and 120 h.

Effect of pH on growth of *Rhizobium meliloti* 14

YMA medium (100 ml) was taken in 500-ml Erlenmeyer flasks. pH of the media was adjusted to 3, 5.5, 7, 8, and 10 with 1 N NaOH and the flasks were

sterilized (121^{o}C, 20 min). *Rhizobium meliloti* 14 was inoculated and the culture was grown at 30^{o}C and 250 rpm. Biomass and PHA were analyzed after 72h.

Effect of temperature on growth of *Rhizobium meliloti* 14

Rhizobium meliloti 14 was inoculated into sterile YMA medium (100 ml). The flasks were incubated at 30, 37, 45^{o}C and at 250 rpm. Growth and PHA produced was monitored and recorded after 72 h.

Effect of agitation on growth of *Rhizobium meliloti* 14

YMA medium (100 ml) was sterilized in 500-ml Erlenmeyer flasks and flasks were inoculated with *Rhizobium meliloti* 14. The flasks were incubated at 30^{o}C and at 100, 200 and 300 rpm. Growth and PHA production was monitored and recorded after 72 h.

Cell size

Cell size was measured as described in Chapter 1, page 120. The cell size of *Rhizobium meliloti* 14 was seen when grown in different carbon and nitrogen sources.

Effect of various carbon sources on growth of *Rhizobium meliloti* 14

Mannitol, glucose, sucrose, arabinose, adonitol, fructose, cellobiose and mannose at (1%) were tested as carbon substrates for PHA production. These carbon sources were incorporated into basal medium (instead of mannitol in YMA). Each medium was then sterilized (121^{o}C, 20 min). *Rhizobium meliloti* 14 was inoculated into 100 ml of each of the flasks and was grown at 30^{o}C and 250

rpm. Biomass, PHA, polysaccharide and sugar utilized were analyzed after 72 h. Residual sugar was analyzed by using DNS reagent (reducing sugars).

Residual sugar analysis by DNS reagent

Reducing sugars were analyzed by DNS reagent. (See Materials and methods, page 104)

Effect of different nitrogen sources on PHA production

Different nitrogen sources such as peptone, tryptone, sodium nitrate, ammonium sulphate, ammonium phosphate, ammonium acetate, ammonium chloride, urea, sodium nitrite were tested as nitrogen substrates for PHA production. These were incorporated at nitrogen levels equivalent to that of total nitrogen present in 0.4 g/l of yeast extract. Total nitrogen content in yeast extract is 10.5%. These nitrogen sources were incorporated into basal medium (instead of yeast extract into YMA). The medium was then dispensed into 500-ml Erlenmeyer flasks and sterilized (121^{o}C, 20 min). The culture was grown at 30^{o}C and 250 rpm. Biomass, PHA and sugar utilized were analyzed after 72 h.

Effect of different concentration of phosphorus on growth of *Rhizobium meliloti* 14

Phosphorus at two different concentrations (0.3 and 1.2 (g/l) phosphorus levels) was added to the medium containing 10 g/l, 15 g/l and 20 g/l of glucose in six separate experiments. Na_2HPO_4 and K_2HPO_4 were used as source of phosphorus. The nitrogen levels in all the experiments were maintained at 4 g (8.5 g/l of urea) and magnesium sulphate at 0.2 g/l. Biomass, residual sugar, residual phosphate were analyzed as described earlier (Materials and Methods, page 103, 104 and 105) at 24, 48, 72 and 96 hours.

2.2 RESULTS AND DISCUSSION

PHA production in *Rhizobium meliloti* 14 was studied in reported media such as YMA, TY and RMM, in order to understand the nutrient requirement of the organism for PHA production. PHA accumulation inevitably requires a carbon source and *R meliloti* 14 accumulated PHA in media with either mannitol or sucrose as carbon source (Table 18). Mannitol and sucrose are the preferred carbon sources in *Rhizobium meliloti* and mannitol has been preferred for growth of *Rhizobium* (de Drets and Arias, 1970). The concentration of carbon source in the medium and appropriate limitation of any other nutrient is emphasized for PHA synthesis (Chen et al, 2001). *Rhizobium meliloti* 14 gave PHA yields upto 2 and 3.5 g/l with sucrose and mannitol, respectively. There was no accumulation of PHA in the medium containing only yeast extract and tryptone (Table 18).

TABLE 18: PHA PRODUCTION BY *RHIZOBIUM MELILOTI* 14 IN DIFFERENT MEDIA

Medium	Biomass (g/l)	PHA (g/l)	PHA (% w/w dry biomass)
Yeast mannitol agar	4.9	3.5	71.4
RMM medium	3.06	1.99	65.03
TY medium	1.5	nil	-

Carbon source is the basic necessity, although PHA synthesis is genotypically determined. Since PHA is not an essential constitutive component of bacteria, it is not formed under all circumstances (Babel et al, 2001). But as a rule PHA synthesis is initiated from the central intermediate of carbon metabolism, i.e acetyl-CoA. Hence a suitable carbon source such as mannitol or sucrose was taken up for experiments.

Growth kinetics and PHA accumulation in *Rhizobium meliloti* 14

Fig 10 gives details of growth pattern and PHA accumulation in *Rhizobium meliloti* 14 in YMA. The organism reaches stationary phase by 24 hours and PHA accumulation starts during the log phase at 6 h and increases with the onset of stationary phase. This is seen in *Rhizobium etli* where PHB is accumulated even in the exponential phase (Cevallos et al 1996). The log phase lasted upto 24 hrs and the biomass reached 3.1 g/l and PHA yield was 16% of (w/w) dry weight of biomass. Stationary phase was observed upto 72 h and biomass reached to 3.2 g/l. Accumulation of extracellular polysaccharide began at 36 h and reached to a peak at the end of stationary phase. It was also observed that both PHA and extracellular polysaccharide was produced simultaneously. This also suggests that the carbon source provided is being diverted to both the pathways and C/N ratio and other cultural parameters required are similar depending on the carbon source, in this case a sugar alcohol such as mannitol. Increase in the exopolysaccharide levels will increase the viscosity of the fermentation broth

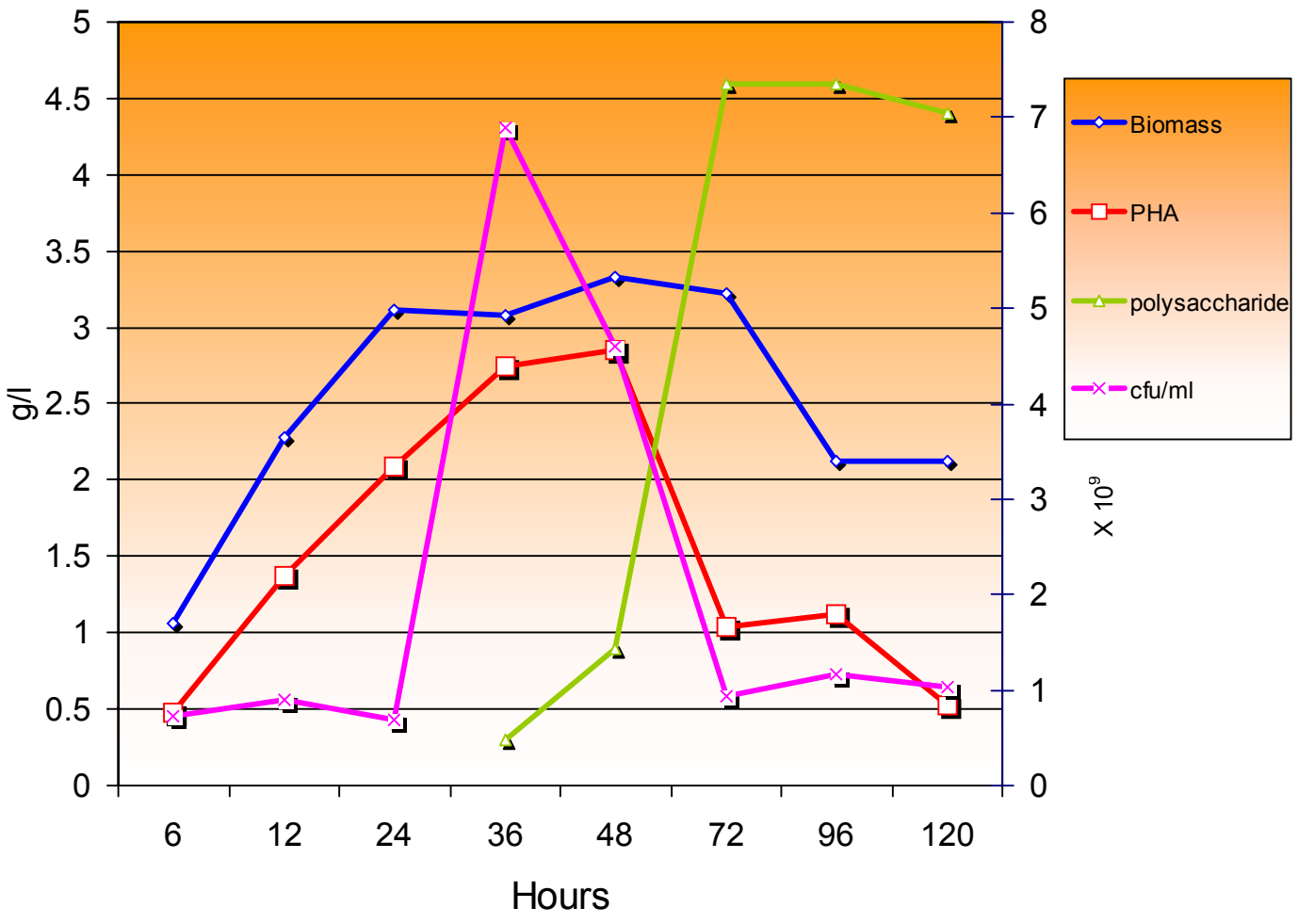


FIG 10: GROWTH KINETICS OF *RHIZOBIUM MELILOTI* 14

leading to decrease in oxygen transfer to the cell. Oxygen depletion in the medium may also help in the PHA production. But carbon source available to the organism is limited as most of it might be channeled towards exopolysaccharide production. Polysaccharide production continued even after growth had stopped. Synthesis of extracellular polysaccharide constitutes a waste of substrate when seeking to optimize PHB production (Segura et al, 2003). Hence, for improvement of PHA there should be less polysaccharide production. Growth kinetics of R 14 reveals that PHA production is growth associated. PHA production starts at the lag phase of cell growth and depolymerase seems to be active after 96 h. Reduction in culture time, increase in cell concentration and PHA content have to be taken into consideration in order to enhance productivity.

Optimization of pH, temperature, aeration, inoculum concentration of *Rhizobium meliloti* 14

R meliloti 14 grew well on YMA medium and at an optimum temperature of 37^{o}C (Fig 11). But PHA accumulation was highest at 30^{o}C. The optimum pH for growth and PHA was 7.0 in YMA (Fig 12). Moderate agitation of 200rpm was favorable for PHA production (Fig 13). There was poor growth and PHA production at higher rpm. These initial experiments seem to suggest that the organism produced PHA under oxygen limiting conditions. Oxygen limitation could be an important factor in PHA production. It has been postulated that in case of oxygen limitation, the nicotinamide nucleotides are not deoxidized.

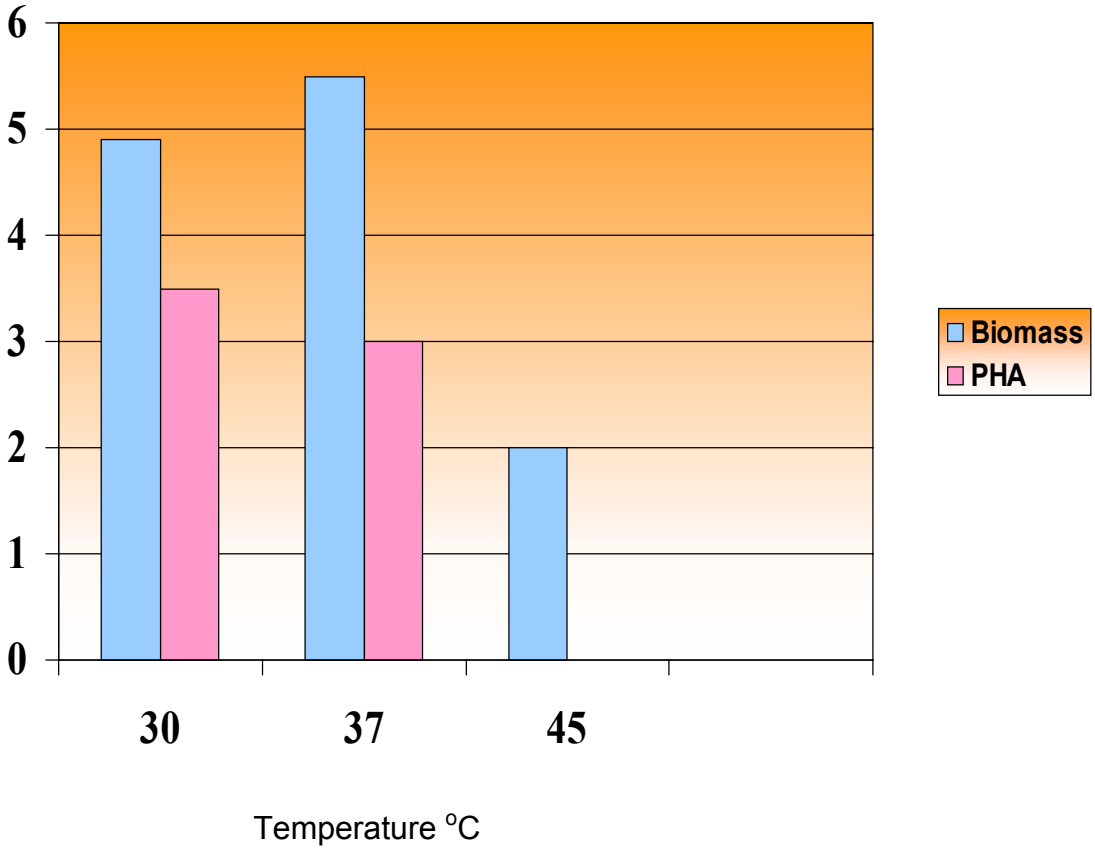


FIG11: EFFECT OF TEMPERATURE (°C) ON GROWTH AND PHA PRODUCTION IN *RHIZOBIUM MELILOTI* 14

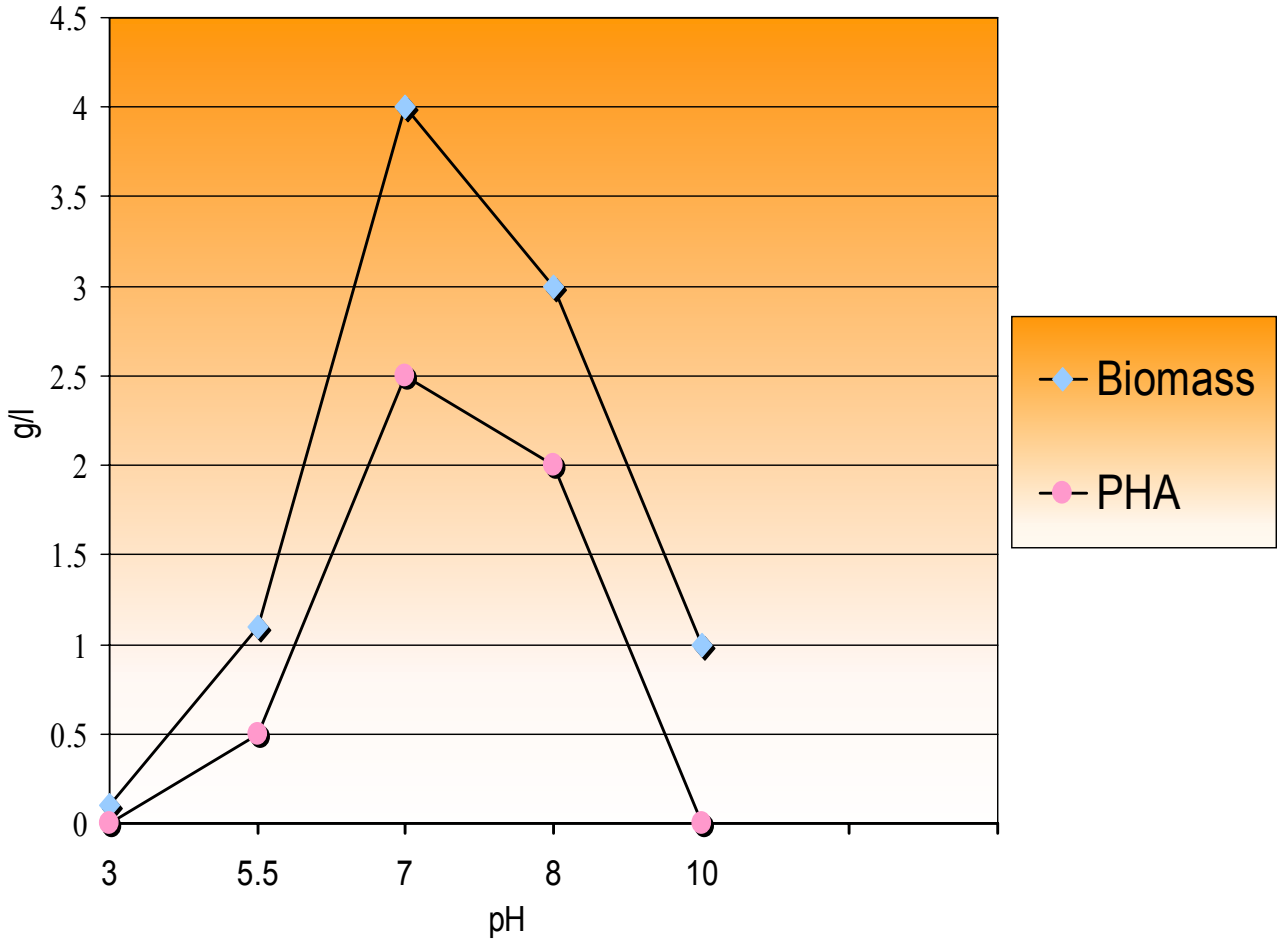


FIG 12: EFFECT OF pH ON GROWTH AND PHA PRODUCTION BY *RHIZOBIUM MELILOTI* 14

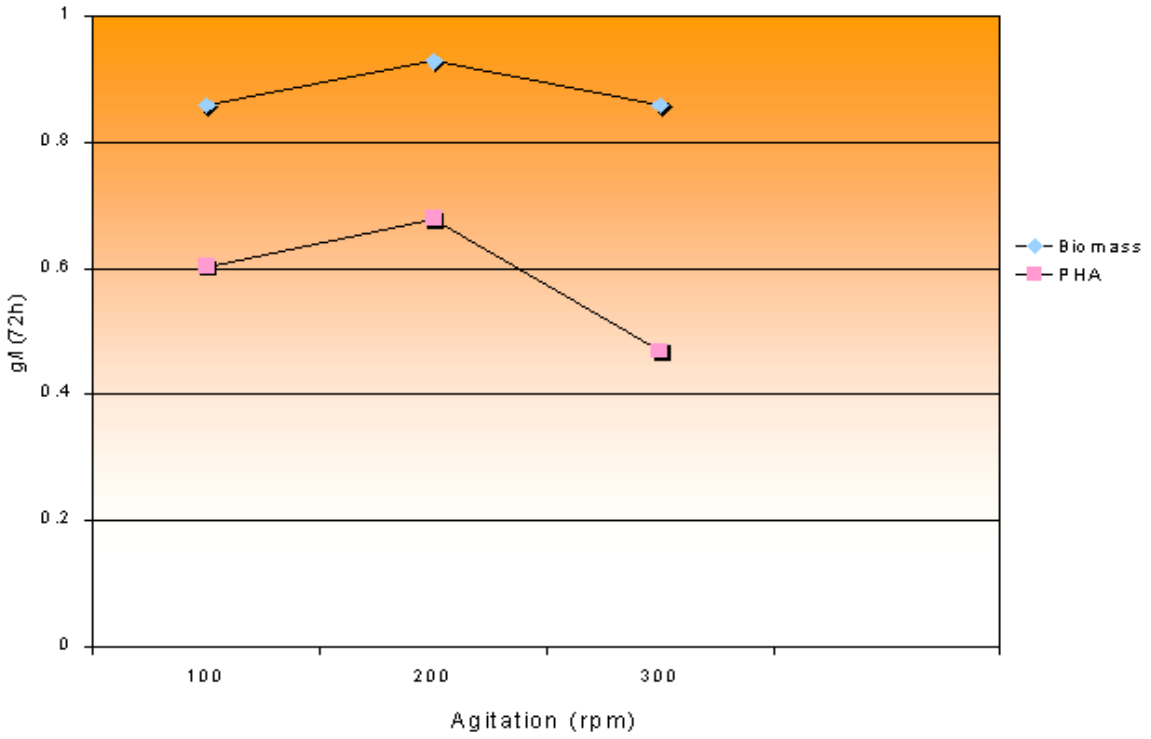


FIG 13: EFFECT OF AGITATION (rpm) ON GROWTH OF *RHIZOBIUM MELILOTI* 14

Thus effectiveness of the TCA cycle is reduced because NADH decreases the activity of citrate synthase and isocitrate dehydrogenase. Limitation of oxygen will also result in accumulation of acetyl-CoA and a low intracellular concentration of free Co ASH. The increase of the acetyl –Co A / CoA SH ratio partially relives the inhibition of β-ketothiolase, which favours formation of PHA. *Azotobacter vinelandii* and *Alcaligenes eutrophus* is known to accumulate PHA under oxygen limitation (Du et al, 2000).

Growth was better on complex media containing yeast extract and glutamate but simpler media also supported growth. Complex nitrogen source such as yeast extract was increased in the medium (0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4 and 5 g/l) with mannitol as a carbon source. Mannitol was maintained at constant levels (10 g/l). An increase in the nitrogen provided in the medium increased biomass but decreased PHA yield (Fig 14). Biomass levels increased upto 3 g/l at 5g/l of yeast extract but PHA levels decreased to 0.07g/l. A simultaneous increase in the sugar (mannitol) levels along with yeast extract increased PHA only at 0.6 g/l of yeast extract and 15 g/l of mannitol (Fig 15). Higher levels of nitrogen and carbon decreased the PHA levels.

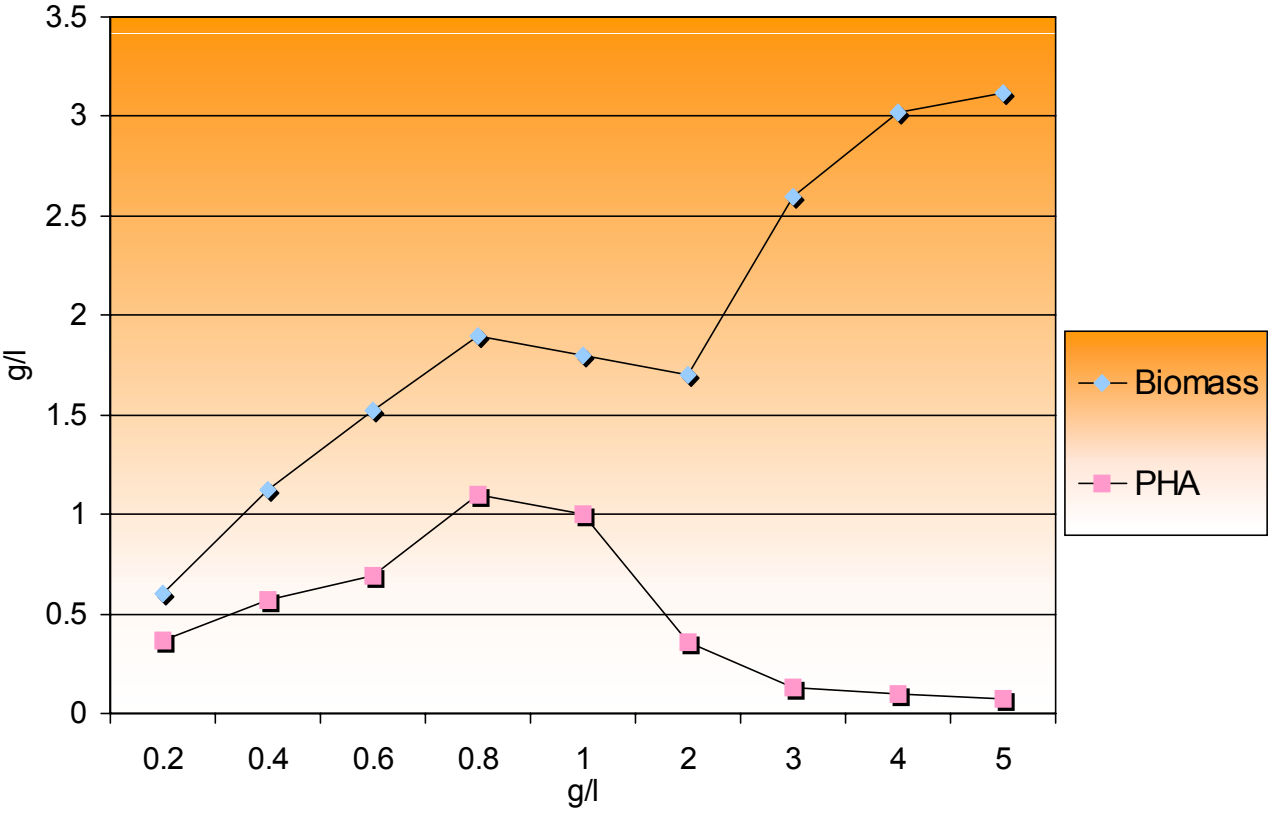


FIG 14: EFFECT OF CONCENTRATION OF YEAST EXTRACT ON PHA PRODUCTION

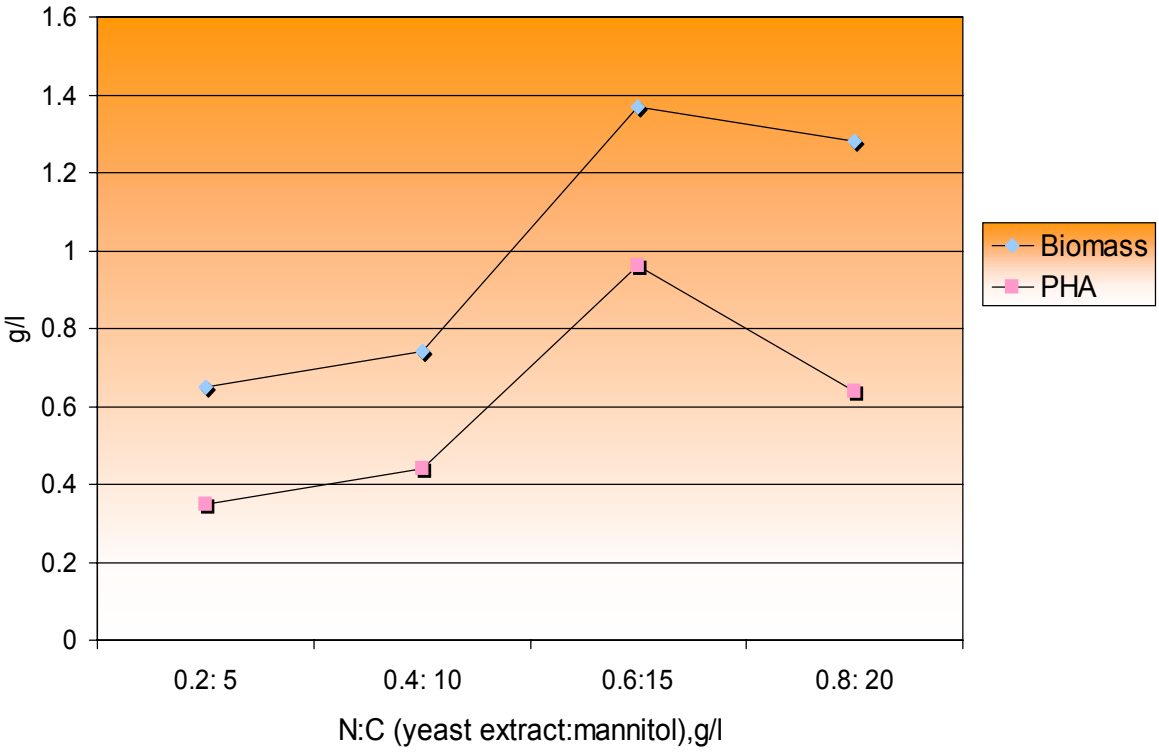


FIG 15: EFFECT OF INCREASING CONCENTRATIONS OF NITROGEN AND CARBON ON GROWTH AND PHA PRODUCTION IN *RHIZOBIUM MELILOTI* 14

Effect of various carbon sources on PHA production in *Rhizobium meliloti* 14

Various carbon sources such as fructose, glucose, arabinose, cellobiose, mannose, rhamnose, maltose, adonitol, sucrose, mannitol and glucose were used as sole carbon sources in the YMA medium. Mannitol was replaced by the corresponding carbon sources in the experiments. The sugars were added at 20 g/l level. The inoculum levels were maintained at 10% (v/v). High biomass levels were obtained in mannitol (4.9 g/l), adonitol (4.4 g/l), maltose (3.8 g/l), and mannose (3.5 g/l). PHA yield was seen only in mannitol (3.5 g/l), sucrose (2.14 g/l), fructose (1.61 g/l), adonitol (2.28 g/l), glucose (1.48 g/l) and cellobiose (1.371 g/l). It was observed that glucose was not favoured for growth or PHA production (Fig 16). Glucose uptake was only 22.5% of 2% sugar provided. EMP pathway is strain dependent in *Rhizobia*. The mechanism for lucose uptake has been observed in *Rhizobium meliloti*. *Rhizobia* do not posses phosphoenol pyruvate phosphotransferse system and most of the hexose metabolism proceeds through the Entner Doudoroff pathway. The presence of pentose phosphate pathway has also been observed in *Rhizobia* (Stowers, 1985). However fructose uptake was quite high, 61% of 2% sugar provided in the medium. Disaccharides such as sucrose, cellobiose and maltose were better suited for PHA production. The uptake of sucrose was however quite high and PHA production in sucrose containing medium was also high. Sucrose uptake is restricted to fast growers among *Rhizobia*. The enzyme invertase is inducible in

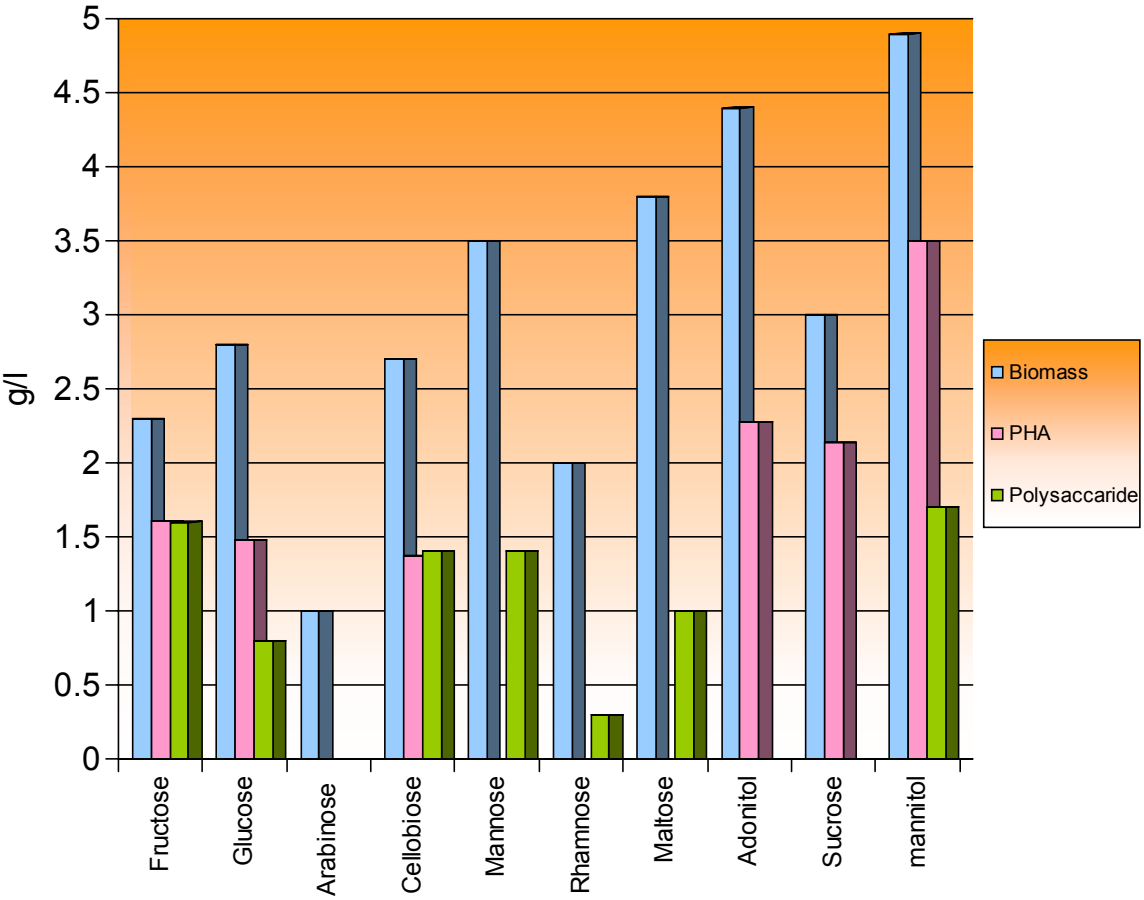


FIG 16: EFFECT OF DIFFERENT CARBON SOURCES ON PHA PRODUCTION IN *RHIZOBIUM MELILOTI* 14

nature and its activity is high only in sucrose fermentations. Uptake of mannose was 81% of 2% provided in the medium. Mannose is metabolized via mannose kinase, producing mannose –6-phosphate that in turn is converted to fructose -6-phosphate. Fructose -6- phosphate is further metabolized by ED pathway. Mannitol is metabolized via a mannitol dehydrogenase producing sucrose. When pentose such as arabinose was used as a sole carbon source, there was no PHA production. The biomass obtained was 1g/l and the broth turned slightly alkaline (pH 7.3). It was also observed that highly acidic pH, as in glucose (4.5), rhamnose (3.8), mannose (3.5), and highly alkaline pH as in adonitol (8.2), maltose (7.6) did not yield good PHA. But slightly acidic condition such as pH levels of 5.9 (sucrose) favoured PHA production. PHA production in fructose was higher compared to other monosaccharides though the pH was 7.5 after 72 h of fermentation. But sucrose was the best source apart from mannitol. Since sucrose is cheaper compared to mannitol, it was used as a carbon source in further studies. Other major elements required for growth and PHA production were magnesium and phosphorus.

Effect of various nitrogen sources on PHA production in *Rhizobium meliloti* 14

Nitrogen sources such as nitrates, ammonium salts and urea were preferred for growth and PHA production (Fig 17). Nitrogen sources such as peptone and tryptone did not favour PHA production in *R meliloti* 14. Peptone

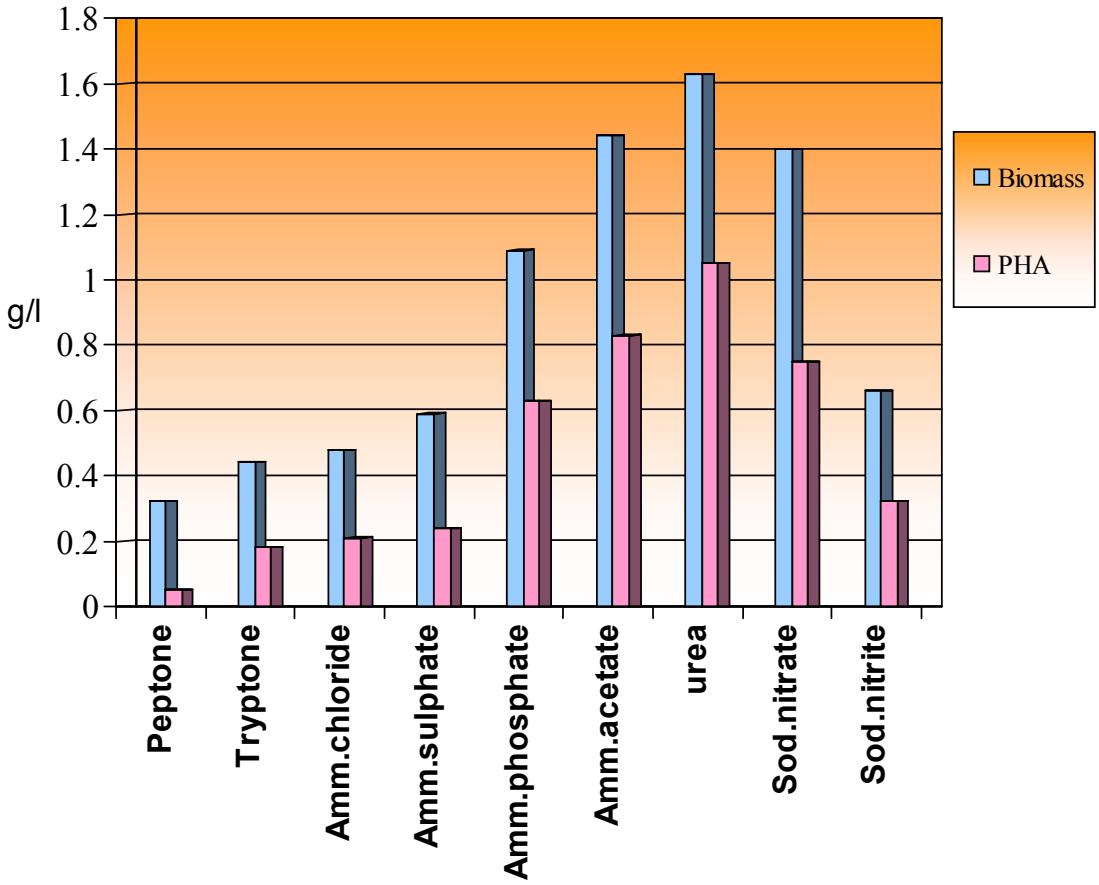


FIG17: EFFECT OF DIFFERENT NITROGEN SOURCES ON PHA PRODUCTION IN *RHIZOBIUM MELILOTI* 14

and tryptone which are water-soluble hydrolysates of protein and tryptic hydrolysate of casein contain a number of amino acids, peptides and proteoses. These organic nitrogen sources did not favour either growth or PHA production in *R meliloti* 14 although amino acids such as glutamate gave good growth. The bacterium did not require growth factors. Urea also could be used as an alternate source of nitrogen. *R meliloti* 14 grew well in urea with biomass 1.63 g/l and PHA yield of about 64.4% with mannitol as the carbon source. The yield of PHA in medium containing nitrates was (53.57%) better than that containing sodium nitrite (48.48%). The organism grew well in nitrates and biomass obtained was 1.4 g/l. PHA yield in ammonium salts such as ammonium phosphate, ammonium acetate, ammonium sulphate and ammonium chloride was 57.8%, 57.6%, 40.6% and 43.7% respectively in mannitol medium. Use of ammonium salts in the medium has an added advantage as it reduces the production of exopolysaccharides (Patriarca et al, 2002). This might indirectly favour the PHA synthesis.

Effect of different concentration of phosphorus on growth of *Rhizobium meliloti* 14

Effect of two levels of phosphorus on the growth of *Rhizobium meliloti* 14 was studied in six separate experiments (Fig 18). Increased supplementation of phosphorus (1.2 g P) did not increase the biomass levels. However it was seen

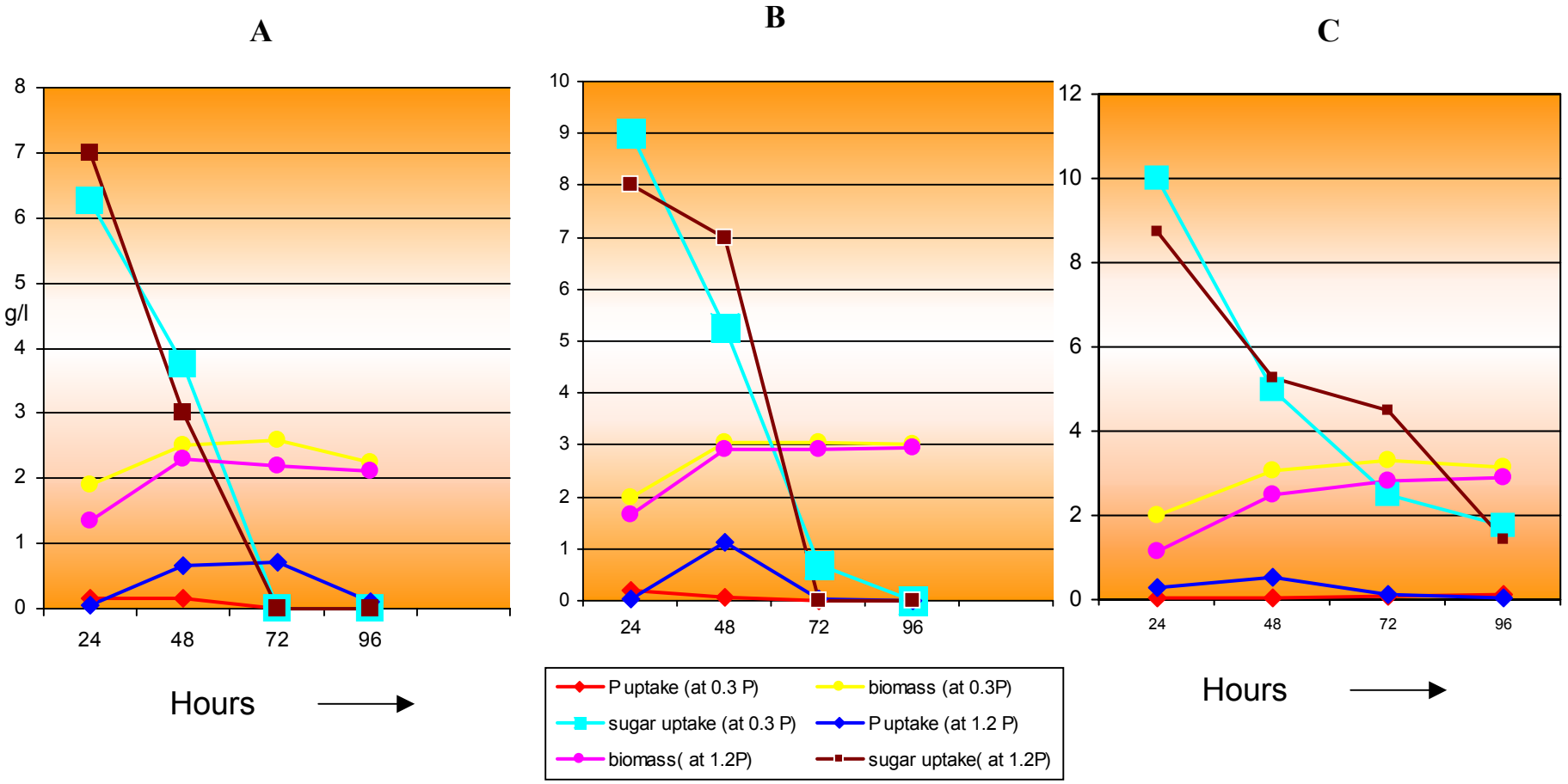


FIG18: PHOSPHORUS UPTAKE AT DIFFERENT LEVELS OF CARBON AND ITS EFFECT ON GROWTH OF *RHIZOBIUM MELILOTI* 14

A: 10 g/l sucrose, B: 15 g/l sucrose C: 20 g/l sucrose

that the uptake of phosphorus was faster in medium containing low C concentrationand slow in the medium substituted with high carbon levels. Glucose was used in the experiment, as it was known that *Rhizobium meliloti* 14 does not utilize glucose efficiently for PHA production. Hence effect of inputs of phosphorus was studied for high biomass in the absence of a suitable carbon source. The uptake of phosphorus was complete by 48h in medium supplemented with 0.3 g P in medium containing 10 g/l and 15 g/l of glucose. In medium with 20 g/l of glucose the uptake of phosphorus was 0.1g and residual P was 0.2 g. The results were similar in medium which was supplemented with high levels of phosphate (1.2 g P), however the up take levels were higher. The highest biomass level obtained was 3.3 g/l at 72 h with 0.3g of P with 20 g/l of glucose. There was no significant PHA production in the experiments. The levels of phosphorus provided may not favour the PHA production. PHA accumulation was observed after 72 h and the highest PHA yield was 17% after 96 h. The results suggest that *Rhizobium meliloti* 14 may require phosphate limitation for PHA production as PHA yields were observed after phosphate exhaustion in the medium. Phosphate feeding strategy with optimal nitrogen and carbon levels may be required for both biomass and PHA accumulation.

Effect of urea and sucrose as nitrogen and carbon source for optimizing media for PHA production

Urea and sucrose at different carbon and nitrogen levels were examined for efficient PHA synthesis (Fig 19) in *Rhizobium meliloti* 14. PHA yields increased from 0.4 g/l to 2.56 g/l when the level of sucrose and urea were raised from 10 g/l and 0.08 g/l to 30 g/l and 0.2 g/l respectively. A highest yield of PHA and highest PHA accumulation (70.7 %) was obtained with the C: N ratio of 16.8: 0.1. It was observed that for efficient production of biomass and PHA the concentration of urea and sucrose in the medium should be at least 0.1 g/l of urea and 20 g/l of sucrose. Increase in the quantity of both urea and sucrose had to be done maintaining the C: N ratio of 105 (8.4: 0.08). However the organism accumulated PHA even at lower concentration of urea and sucrose at same C: N ratio. The residual biomass (g/l) (Total biomass – PHA) doubled by doubling the quantity of carbon and nitrogen at same ratio. These experiments formed a basis and defined a range of carbon (sucrose) and nitrogen (urea) levels, to be used in further optimization studies.

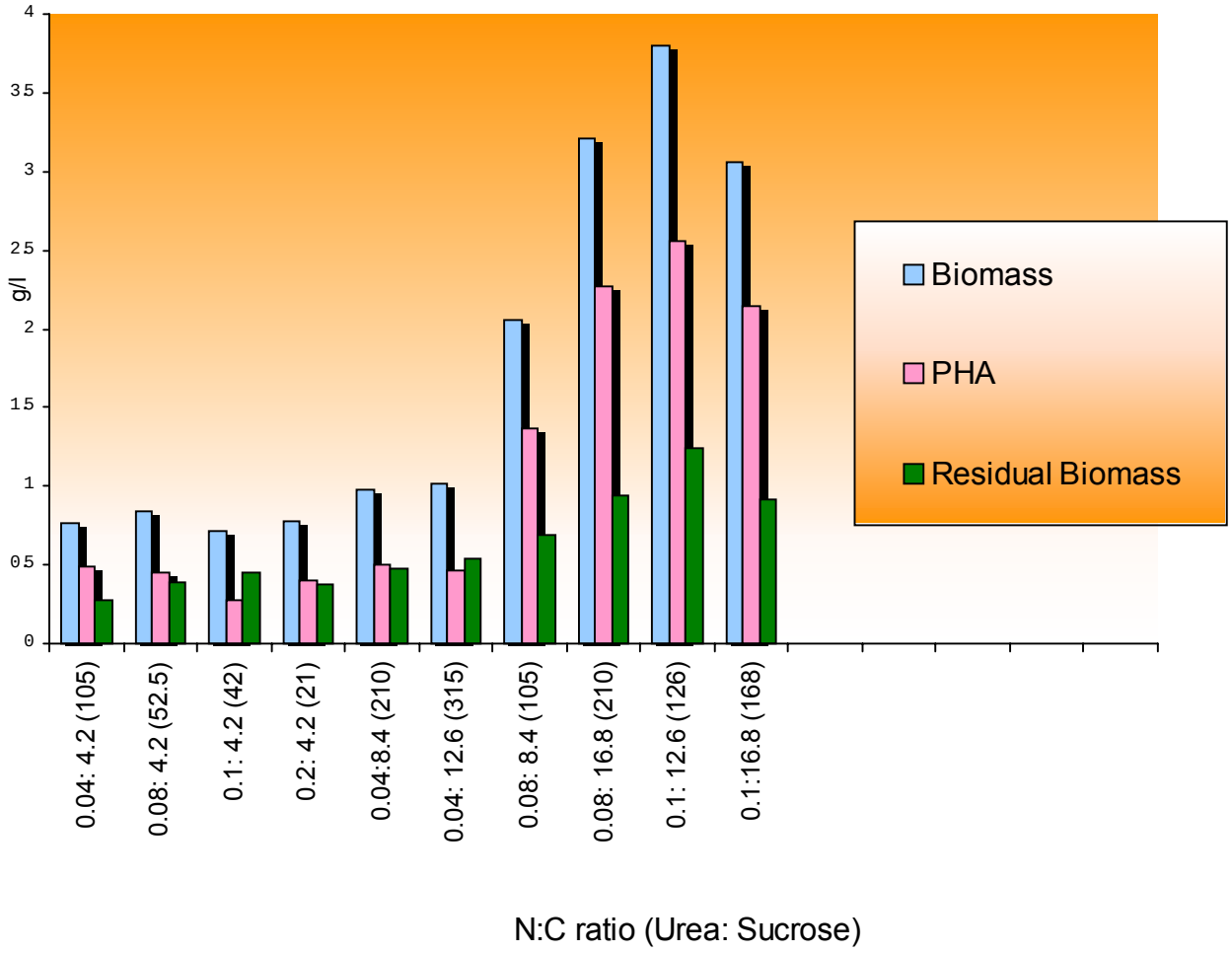


FIG 19: EFFECTS OF UREA AND SUCROSE AS NITROGEN AND CARBON SOURCE FOR OPTIMIZING MEDIA FOR PHA PRODUCTION IN *RHIZOBIUM MELILOTI* 14

2.3 CONCLUSION

In conclusion, *Rhizobium meliloti* 14 requires an optimum pH of 7 and temperature of 30^{o}C at 200 rpm for good growth as well as PHA production. Maximum PHA yields was obtained at stationary phase of the culture although the organism accumulated PHA during the logarithmic phase also. High aeration in the medium decreased the PHA levels suggesting the probability of oxygen limitation favouring PHA accumulation. The organism did not require any complex nitrogen sources such as yeast extract for PHA production. However, yields of biomass increased considerably with the addition of yeast extract in the medium. Other nitrogen sources such as urea, ammonium salts and nitrates favoured PHA production. The most favoured carbon sources for PHA production were mannitol and sucrose. The yields of PHA in sucrose were higher when urea was used as the nitrogen source. Acidity in the medium seemed to favour PHA production rather than alkalinity. Uptake of phosphorus was high at low carbon concentration and phosphorus depletion in the medium seemed to favour PHA synthesis. A carbon and nitrogen ratio of minimum 105 was necessary for high PHA yields when sucrose and urea were used. The highest percentage of PHA obtained was 70.7% with a yield of 2.27g/l.

Chapter 3

Strain Improvement for Enhancement of Polyhydroxyalkanoate Synthesis

3.0 INTRODUCTION

Rhizobium sps are known to accumulate upto 55% (PHB) in the cells (Tombolini and Nuti 1989). One possibility of obtaining better yielding organisms is by developing mutants. A mutant impaired in alginate production is reported to have increased accumulation of poly-β-hydroxybutyrate in *Azotobacter vinelandii* (Martinez et al, 1997). However reports regarding development of mutant strain for enhanced production of PHA are scanty (Takashahi et al, 1998). Hence efforts were made to develop mutants of *Rhizobium meliloti* 14 in order to obtain better yielding strains.

3.1 MATERIALS AND METHODS

Mutation

Mutagens such as Novobiocin and K_{14}-10- (4^1-N-Piperidinobutyl-2-chlorophenoxazine)(PBCP) were used for mutation. Mutagens were obtained from Dr. Arun Chandrashekar, Scientist, Department of Food microbiology, CFTRI, Mysore. Novobiocin was used at 0.02 μg/ ml to 0.1 μg/ ml in the medium (Yeast mannitol broth). The compound PBCP was dissolved in 25% dimethylsulfoxide (DMSO), filtered through 0.45 μ. Millipore filter and used at 1 to 10 μg /ml level in the culture broth. The cultures were grown at 30^0C for 24 h along with control. Experiment was carried out as shown in Fig 20.

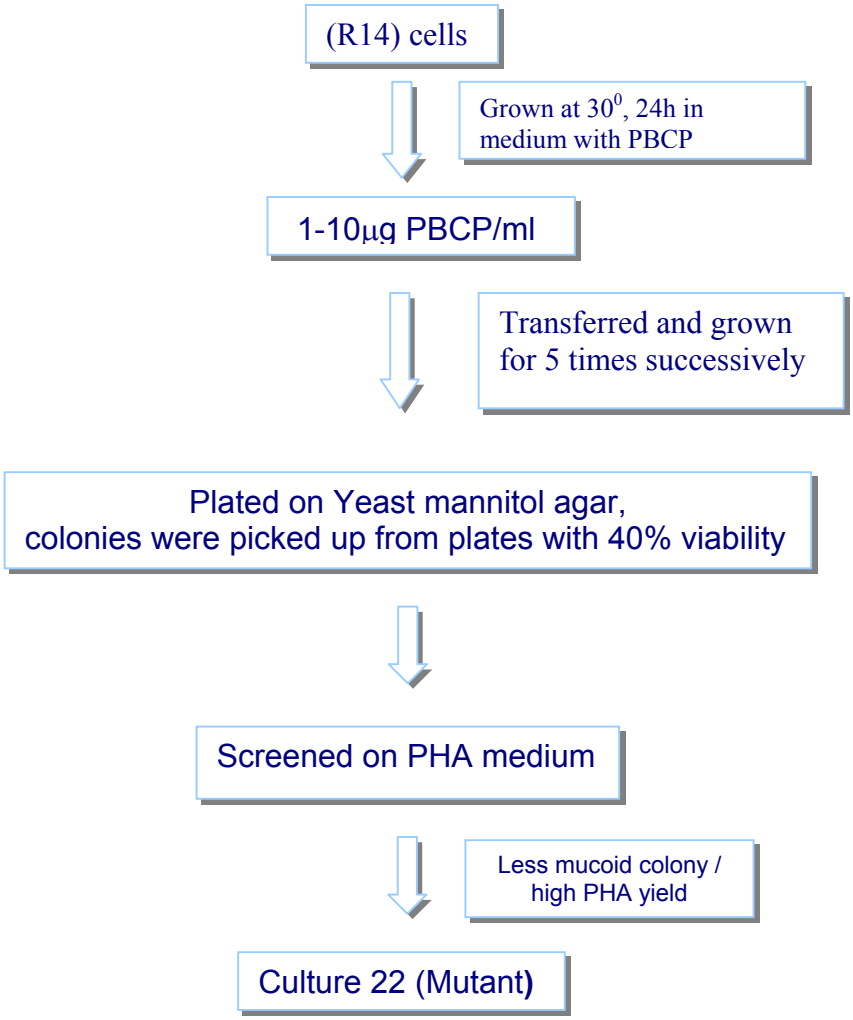


FIG 20: MUTATION PROCEDURE

These cultures were successively transferred five times into fresh medium containing the mutagen. Final samples were plated on Yeast mannitol Agar (YMA) and isolated colonies were picked up from the plates showing about 40% viability. The cultures were maintained on YMA slants.

Screening of Mutants

Strains obtained after mutation were further screened for low polysaccharide production. These were grown in PHA production medium (Materials and methods, page 109) and analyzed. PHA production was carried out in duplicate in 500-ml capacity Erlenmeyer flasks. Different C: N ratios were compared for PHA production. This consisted of $(NH_4)_2 SO_4$, 1.0 g / l with 10, 20 or 30 g/ l of sucrose or 25 g/ l with 1,2 or 3 g/ l of $(NH_4)_2 SO_4$. Cultivation was carried out on a shaker at 250 rpm at 30^0C for 72 h.

Analysis

Biomass, PHA and polysaccharide were estimated as already described (Materials and methods, page 103 and 105).

Plasmid profiles of R 14 and its mutant

Plasmids were isolated from *Rhizobium meliloti* 14 and the mutant and their profiles were compared. The isolated mutant was termed *as* ***Rhizobium meliloti*** **22.**

Plasmid isolation (Modified from Andersen and Mckay, 1983)

The test sample was inoculated into 5 ml LB broth and incubated for 12 h. 1 ml of the culture was taken in 1.5-ml eppendorf tube and centrifuged for 3 min. Supernatant was completely drained and to the pellet 200 μl of water and 0.5 M sucrose (1:1) were added and centrifuged at 11000 rpm for 5 min. The supernatant was discarded completely.

400 μl of 6.7% of sucrose, 50 mM tris and 1 mM EDTA (pH 8.0) were added to the pellet. Then the cell pellet was vortexed thoroughly and incubated at 37^{o}C for 15 –20 min. 100 μl lysozyme (Sigma) (10-mg/ ml in 25 mM Tris pH 8.0) was added and incubated at 37^{o}C with intermittent mixing for 30-45 min. Cell lysis was observed.

50 ml of 0.25 M EDTA and 50 mM Tris pH 8.0 was added to the lysed cells. 30 μl of freshly prepared SDS (20-mg/ ml) in 50 mM Tris, 20 mM EDTA pH 8.0 was added to the tube and mixed immediately. The tube was incubated at 37^{o}C for 10 min and the mixture was gently vortexed. 30 μl of 3 N NaOH was added to the lysate and gently mixed with intermittent inversion of the tube and swirling for 10 min. 50 μl of 2 M Tris pH 7.0 was added and mixing was continued for 3 min. Finally 72 μl of 5 M NaCl was added.

700 μl of phenol saturated with 3% NaCl was used for extraction of DNA. The mixture was thoroughly vortexed for 1min and then centrifuged at 11000 rpm for 10 min. The supernatant was carefully collected into another tube to which 700 μl of chloroform- isoamyl alcohol (24:1) was added, mixed and centrifuged

(11000 rpm for 10 min). The above steps were repeated and supernatant was collected in a fresh tube carefully.

To the supernatant 1ml of isopropanol was added and kept for precipitation at –20^{o}C overnight. The precipitated DNA was centrifuged at 11000 rpm for 10-15 min and supernatant was discarded. This was washed with 300 μl of 70 % ethanol and centrifuged for 3-5 min. The supernatant was completely discarded and the pellet was dried thoroughly by drying at 40^{o}C. The dried pellet which contained plasmid DNA was mixed with 20 μl of Tris EDTA buffer and stored at –20 ^{o}C till further use.

8-10 μl of the DNA was mixed with 2 μl of loading dye and loaded on to agarose gel (0.8%). Electrophoresis was performed in Tris acetate buffer containing 40 mM Tris, 20-mM acetic acid and 2 mM Na_2 EDTA (pH 8). Gel was run at 100 V for 6 h then stained with ethidium bromide (0.5 μg/ ml). Bands were seen by UV illumination and the images were captured using a Hero LAB image analyser (Wiesloch, Germany).

3.2 RESULTS AND DISCUSSION

PHA production by mutant (*Rhizobium meliloti* 22) strain

Novobiocin treated cells of *Rhizobium meliloti* 14 did not produce effective mutants. Antibiotic sensitivity assay revealed that *Rhizobium meliloti* 14 growth was severely affected by novobiocin. The chemical PBCP used in the experiment to treat *Rhizobium meliloti* culture is usually used as a plasmid-curing agent. More PHA was obtained with respect to mutated strain (67%) compared to parent strain (57%) (Fig 21). The results suggest that the enzymes involved in PHA synthesis may be situated chromosomally and may not be plasmid borne as a plasmid curing agent was used for the selection of the *Rhizobium meliloti* 22 (Lakshman and Shamala, 2003). Synthesis of PHA occurred throughout the active growth phase.

Effect of Carbon and nitrogen concentrations

Accumulation of PHA in batch culture by *R meliloti* was studied comparatively in parent *(Rhizobium meliloti* 14) and mutant (*Rhizobium meliloti* 22) strains using culture media with different C: N ratios. When *Rhizobium meliloti* 14 cultures were grown for 72h in the presence of 1, 2 and 3 g/l of $(NH_4)_2 SO_4$, (0.2, 0.4 and 0.6 g of N) with 25 g/l of sucrose (10.5 g of C) PHA obtained was 60, 37 and 33% of dry biomass weight, respectively (Table 19). Corresponding values for mutant strain was 67, 57 and 56%. The carbon conversion efficiency (per g of carbon utilized) in the parent was 0.5, 0.3, 0.3

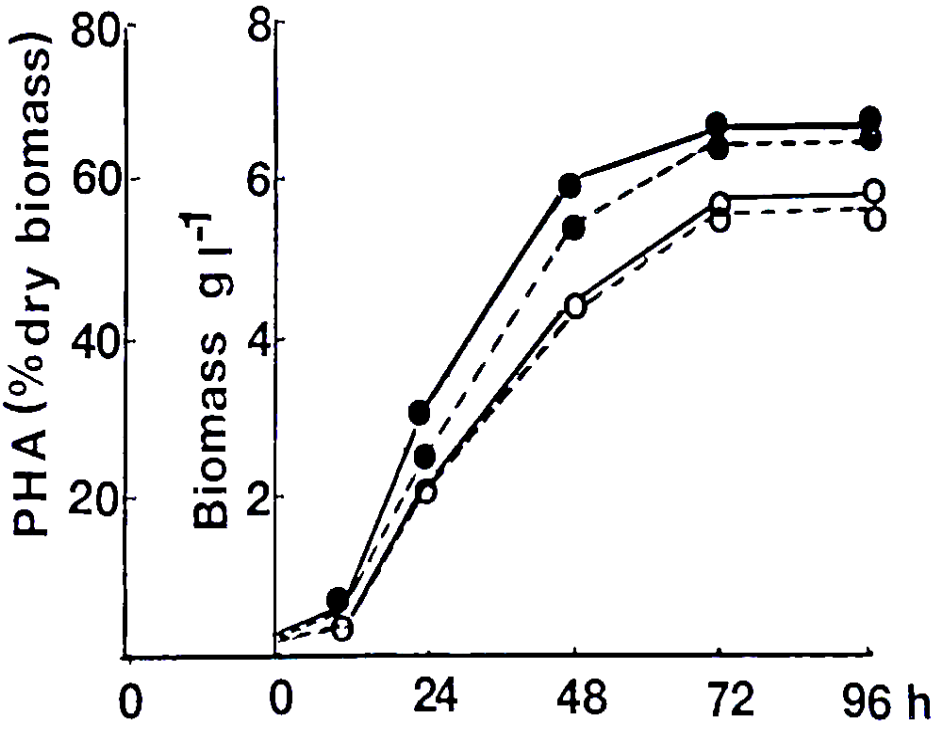


FIG 21: COMPARISON OF BIOMASS (-----) AND PHA PRODUCTION (——) BY *RHIZOBIUM MELILOTI* 14 PARENT (o) AND *RHIZOBIUM MELILOTI* 22 MUTANT (•) STRAINS.

TABLE 19: PHA PRODUCTION BY PARENT AND MUTATED STRAIN OF *RHIZOBIUM MELILOTI* GROWN ON DIFFERENT RATIOS OF CARBON AND NITROGEN.

Carbon (gl^{-1})	Nitrogen (gl^{-1})	Biomass (dry wt .gl^{-1})		Sugar utilized (%)		PHA (gl^{-1})		PHA(%) of Biomass, w / w)	
a	b	1	2	1	2	1	2	1	2
10.5	0.2	5.18	4.76	57.6	58.4	3.12	3.22	60.23	67.44
10.5	0.4	3.72	4.30	33.6	39.6	1.38	2.46	37.09	57.20
10.5	0.6	4.04	4.18	33.6	40.0	1.34	2.36	33.16	56.45
4.2	0.2	3.84	3.94	94.0	96.0	1.78	2.20	46.35	55.85
8.4	0.2	5.14	4.94	62.0	71.5	2.34	3.32	45.52	67.20
12.6	0.2	5.58	5.20	43.0	53.3	3.42	3.70	61.29	68.77

1= Parent strain (R 14), 2= Mutant strain (R22), a=Sucrose, b=$(NH_4)_2SO_4$

and percentage of carbon uptake was 57 %, 33% and 33% respectively. Whereas in the mutant the carbon conversion efficiency per g of carbon utilized was 0.52, 0.59, 0.56 and percentage yields were 58%, 39% and 40% respectively (Fig 21A). It was seen that the carbon uptake and conversion into PHA was higher in the mutant compared to the parent. The other ingredients in the medium were maintained at constant levels in all the experiments. PHAs are usually synthesized when bacteria are grown under excess carbon but with other nutrient such as N, P, S, O etc being limited. Polymer accumulation in *Rhizobium meliloti* 14 is affected by nitrogen concentration and lower nitrogen concentration in combination with excess carbon was favourable to obtain higher polymer yields. In *Rhizobium meliloti* 14 culture medium containing 0.4 and 0.6 g/l of nitrogen final pH dropped considerably (3.9) compared to others (5.7 to 7.0). Lower concentration (0.2g nitrogen g/l) in combination with excess of C (12.6 g/l) resulted in more PHA compared to culture medium containing higher nitrogen concentration. The PHA yields were 3.42 g/l in the parent and 3.7 g/l in the mutant strain. The carbon uptake of parent was 43% and in the mutant it was 53%. But the carbon conversion efficiency of the parent was higher in the parent (0.63 g/l) at high C: N ratio (Fig 21A). Whereas the mutant at high carbon and significantly low nitrogen levels (0.2 g/l of nitrogen) showed less conversion of carbon into PHA although the yields were quantitatively higher compared to the parent. Noticeable difference was observed in PHA yields between parent (45%) and *Rhizobium meliloti* 22 strain (67%), when grown with 8.4 g/l of C and 0.2 g/l N

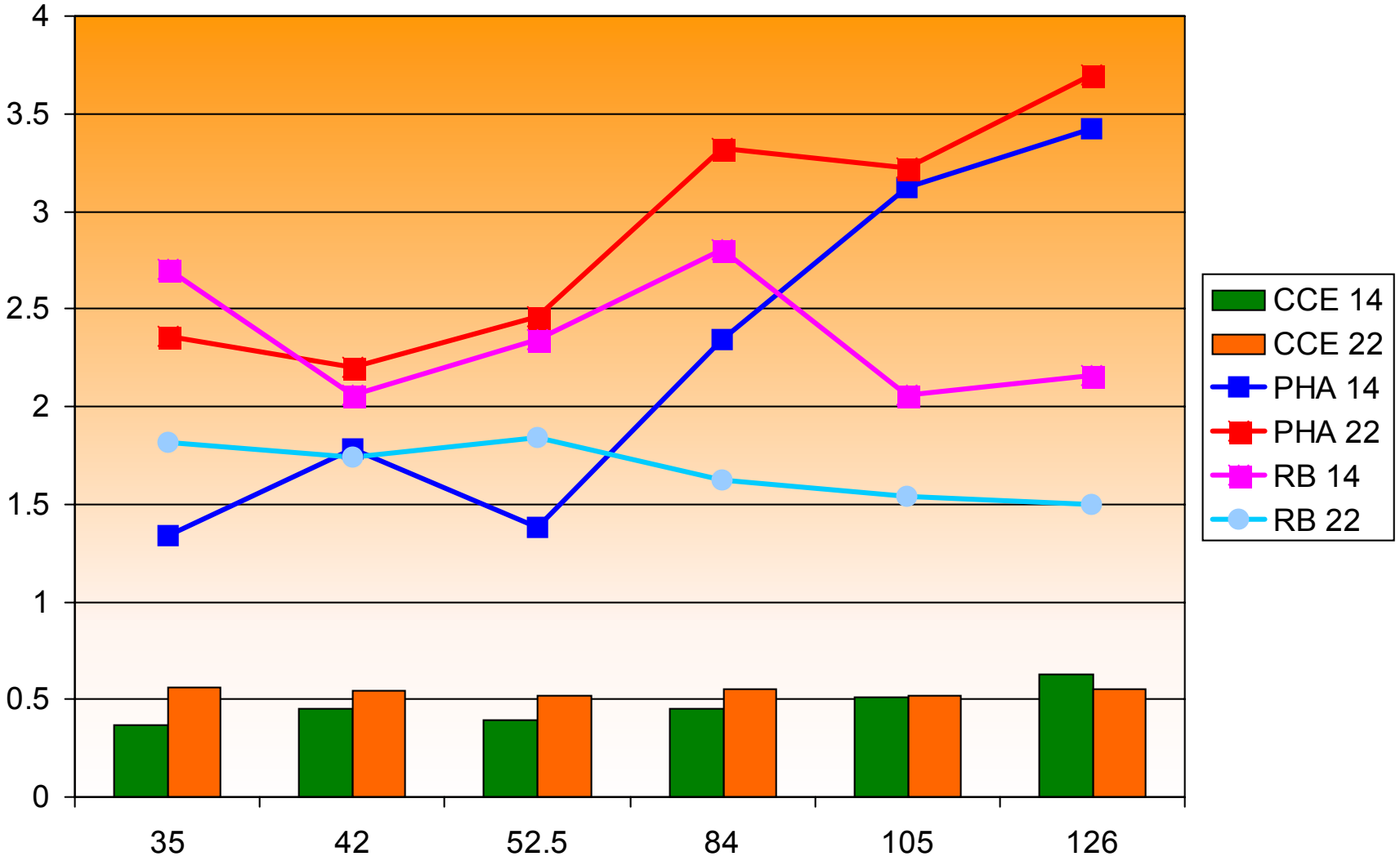


Fig 21 A: Carbon conversion efficiency [CCE] (PHA g / g carbon), Residual biomass (Total biomass-PHA) (RB g/l) and PHA (g/l) accumulation in *Rhizobium meliloti* 14 and *Rhizobium meliloti* 22 at increasing C/N ratio

source. Respective concentrations of exopolysaccharide synthesized by culture were 5.7 and 2.8 g/l. It has been shown in *Azotobacter vinelandii* that a 50% increase in PHB production obtained by constructing an alginate impaired mutant (Martinez et al, 1997). The *Rhizobium meliloti* 22 strain has the potential of converting the carbon source more efficiently for PHA production. These data suggest that in *Rhizobium meliloti* 22 strain there is enhanced metabolism of carbon towards the synthesis of PHA, which may result in higher concentration of PHA compared to the parent strain. According to Huisman et al (1992) a strain of *Pseudomonas oleovorans* lacking PHA depolymerase did not result in a net increase of PHA levels. Despite similar PHA levels the final cell density of the fermented broth involving mutant strain was higher than that of the parent strain. It has been proposed that this may be due to efficient conversion of hydrocarbons into cell mass. It is evident that the cell mass produced by the *Rhizobium meliloti* 22 (mutant) was more compared to *Rhizobium meliloti* 14 but in addition to this there was an increase in the conversion of carbon supplied to cellular PHA also.

Comparison of Plasmid profiles

Some members of the family *Rhizobiaceae* have very complex genomic architecture when compared with that of other well studied prokaryotes. One of the most interesting features of genomes of *Rhizobium meliloti* is the existence of large extra chromosomal replicons known as mega plasmids (Sobral et al, 1991).

Exopolysaccharide genes are known to be present on these plasmids (Finan et al, 1986). Engineering of metabolic pathways can optimize the formation of a product and increases the efficiency in the utilization of nutrients during fermentation. A block in the exopolysaccharide alginate synthesis pathway increased the volumetric PHB accumulation upto 10 fold due to more efficient flux of carbon source to the synthesis of the polyester and to better growth in *Azotobacter vinelandii* (Segura et al, 2003). Hence less mucoid colonies were picked up while screening so that a mutant which does not produce exopolysaccharide can be obtained. It was seen that *Rhizobium meliloti* 22 produced nearly 50% less polysaccharide when compared to *Rhizobium meliloti* 14 (2.8 g/l and 5.7 g/l). Mutation was expected in the plasmid DNA of *Rhizobium meliloti* 14. Plasmid profiles of *Rhizobium meliloti* 14 and *Rhizobium meliloti* 22 showed the lack of a high molecular weight plasmid in the mutant (Plate 8 & 9). In *Rhizobium* species plasmid DNA represent upto 50% of the total DNA. Apart from mega plasmids harbouring symbiosis genes there are additional large plasmids (Hartmann and Amarger, 1991). The loss of one of these plasmids may be responsible for low level of exopolysaccharide content in *Rhizobium meliloti* 22.

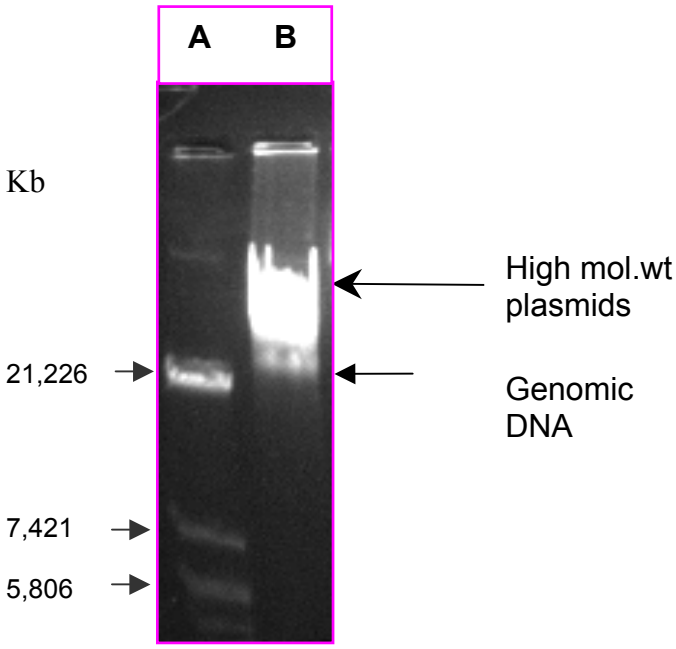


PLATE 8: AGAROSE (0.8%) GEL ELECTROPHORESIS OF PLASMID ISOLATED FROM *RHIZOBIUM MELILOTI* 14.

A: λ *Eco* RI marker

B: R 14 (probable high mol. wt plasmids found)

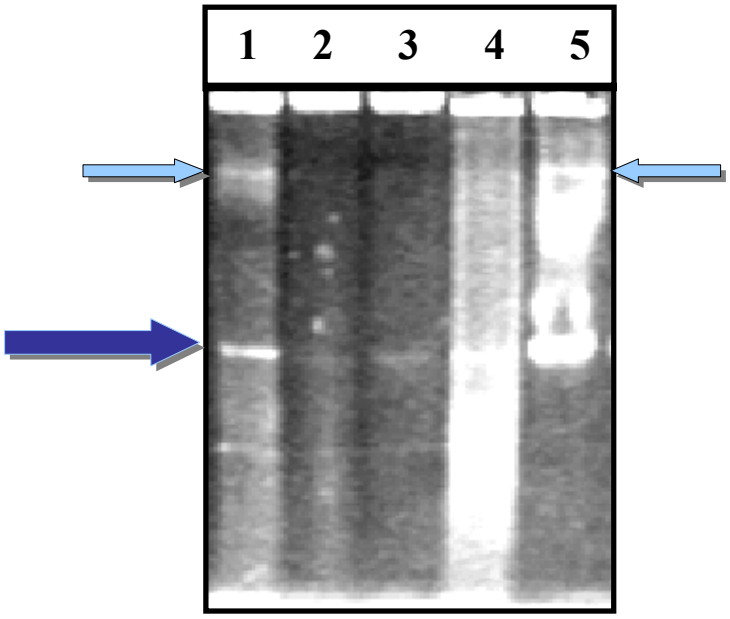


PLATE 9: AGAROSE (0.8%) GEL ELECTROPHORESIS OF PLASMID ISOLATED FROM *RHIZOBIUM* SPP.
1: R 14, 2: R 22 (MUTANT), 3& 4: *RHIZOBIUM* SPP, 5: STD *R MELILOTI* CULTURE.

Big arrow indicates the chromosomal DNA band common in all the lanes and small arrow indicates probable high molecular weight plasmids, seen in lane 1 and 5.

3.3 CONCLUSION

Mutation of *Rhizobium meliloti* 14 resulted in a strain that accumulated nearly 10% more of PHA. A highest yield of 3.7 g/l of PHA was obtained in the mutant. Carbon uptake and the efficiency to convert it to PHA were significantly higher in the mutant (*Rhizobium meliloti* 22). The exopolysaccharide produced by the mutant was 50% less compared to the parent. A comparison of the plasmid profiles of the parent and mutant showed the loss of a high molecular weight plasmid in the mutant. Hence it was concluded that this could be responsible for less polysaccharide production in the mutant and also the conversion of carbon into PHA.

Chapter 4

Optimization of Medium and Cultural Parameters for Polyhydroxyalkanoate Production

4.0 INTRODUCTION

Once a proper understanding is developed regarding the nutrient requirement of the organism towards PHA production it becomes necessary to develop an optimized medium. This is an elaborate process, which requires to be experimented in detail. In addition to this, optimization of PHA production by traditional method is time consuming. The intricacy of the methodology adopted also contributes to more difficulties when several strains of the same bacteria are to be assessed in terms of their polymer accumulation capacity. In order to overcome these problems, it appeared essential to adopt an alternative method for the rapid assessment of such fermentation system. In bacterial fermentation, response surface methodology (RSM) has been used mostly for optimization of process parameters. By adopting this technique, factors and interactions, which affect the desired response, are tested for their effectiveness for optimizing the fermentation process parameters in a limited number of experimentation.

In this chapter RSM has been effectively utilized for the optimization of bacterial PHA yields. The technique has been extended to assess the nutrient limitation conditions favorable for PHA accumulation in bacterial cells and to compare the nutritional performance of the mutant (*Rhizobium meliloti* 22) strain discussed in the previous chapter with that of parent (*Rhizobium meliloti* 14) simultaneously with limited experiment using central composite rotatory design (CCRD) experiments (Lakshman et al, 2003).

4.1 MEDIA AND METHODS

Inoculum

First set of experiments with urea as nitrogen source

Inoculum of parent (*Rhizobium meliloti* 14) and mutant (*Rhizobium meliloti* 22) were prepared by transferring growth of a 24 h old slant to 10 ml of culture medium containing (g/l): K_2HPO_4, 1.0; urea, 0.5; $MgSO_4$ $7H_2O$, 0.2; sucrose, 10; yeast extract, 0.5; pH 7.0. This seed inoculum was again transferred into 90 ml of sterile medium and used as inoculum after 24 h of growth. Inoculated medium was incubated at 30^oC and 250 rpm for 24 h.

Second set of experiments with $(NH_4)_2SO_4$ as nitrogen source

Inocula were prepared by transferring growth of a 24 h old slant of *Rhizobium meliloti* 14 and *Rhizobium meliloti* 22 to 10 ml of culture medium containing (g/l): glucose, 10; yeast extract, 1.5; Na_2HPO_4, 4.4; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4$ $7H_2O$, 0.2; pH 7.0.The cultures were grown for 24 h at 30^oC and 250 rpm. This seed inoculum was again transferred into 90 ml of sterile medium and used as inoculum after 24 h of growth. Inoculated medium was incubated at 30^oC and 250 rpm for 24 h.

Mutant

The mutant used was *Rhizobium meliloti* 22 as discussed in chapter 3.

PHA optimization

Different concentrations of sucrose, K_2HPO_4, urea, $(NH_4)_2SO_4$ and inoculum were considered for optimization of medium for PHA production. Initial

experiments with various nitrogen sources showed that percentage of PHA was higher in cells grown on urea and ammonium sulphate when compared with other nitrogen source (Chapter 2, page 172). $MgSO_4$ $7H_2O$ (0.2 g/l) was used as a common component in the media. The inoculum used for experiments contained about 1.65 x 10^4 viable cells /ml.

Experimental design

A central composite rotatable design (CCRD) with 4 variables at 5 levels for first set and 5 variables at 5 levels for the second set was used to study the response pattern and optimum combination of the variables used (Tables 20 and 21). CCRD was arranged to fit the regression model using multiple regression program (Tables 22 and 23).

The variables used for the first set were (g/l): Urea (0.05-0.65), K_2HPO_4 (0.1 -1.0), sucrose (5-55), and inoculum concentration (10-250 v/v).

The variables optimized for the second set were (g/l): Na_2HPO_4 $2H_2O$ (0.5-6); KH_2PO_4 (0.5-2); sucrose (5-30); $(NH_4)_2SO_4$ (0.5-2) and inoculum (10-200 v/v).

The CCRD combines the vertices of the hypercubes whose coordinates are given by a 2n factorial design to provide for the estimation of curvature of the model. In the first set seven replicates (treatments 25-31) and in the second set six replicates (treatments 27-33) were included for estimation of a pure error of sum of squares.

TABLE 20: VARIABLES AND THEIR LEVELS FOR CCRD (SET 1 WITH UREA)

Variables	Symbols		Levels				
	Coded	Uncoded	-2	-1	0	1	2
Sucrose (g/l)	X_1	x_1	5	17.5	30	42.5	55
Urea (g/l)	X_2	x_2	0.05	0.20	0.35	0.5	0.65
Inoculum (v/v)	X_3	x_3	1	7	13	19	25
K_2HPO_4 (g/l)	X_4	x_4	0.1	0.325	0.55	0.775	1.0

Where X1 = (x_1 - 30)/12.5; X_2 = (x_2 - 0.35)/0.15; X_3 = (x_3 - 13)/6 and X_4 = (x_4 - 0.55)/0.225]

TABLE 21: VARIABLES AND THEIR LEVELS FOR CCRD (SET 2 WITH $(NH_4)_2 SO_4$)

Variables	Symbols		Levels				
	Coded	Uncoded	-2	-1	0	1	2
Na_2HPO_4 (g/l)	X_1	x_1	0.5	1.875	3.25	4.625	6
KH_2PO_4 (g/l)	X_2	X_2	0.5	0.875	1.25	1.625	2
$(NH_4)_2SO_4$ (g/l)	X_3	X_3	0.5	0.875	1.25	1.625	2
Sucrose (g/l)	X_4	X_4	5	11.25	17.5	23.75	30
Inoculum(v/v)	X_5	x_5	10	57.5	105	152.5	200

Where X1 = (x_1 - 3.25) /1.375; X_2 = (x_2 - 1.25)/0.375; X_3 = (x_3 - 1.25)/ 0.375; X_4 = (x_4 -17.5)/6.25; X5=(x_5 – 105)/47.5]

TABLE 22:TREATMENT SCHEDULE FOR 4-FACTOR CCRD AND THE RESPONSE FOR SET 1

Sl. No.	A	B	C	D	Parent strain		Mutant strain	
					Biomass (g/l) (Y_1)	PHA (g/l) (Y_2)	Biomass (g/l) (Y_3)	PHA (g/l)(Y_4)
1	-1	-1	-1	-1	2.00	1.20	2.30	1.30
2	1	-1	-1	-1	2.65	1.50	3.10	1.50
3	-1	1	-1	-1	3.10	2.00	3.50	2.00
4	1	1	-1	-1	3.70	2.20	4.30	2.40
5	-1	-1	1	-1	1.80	0.80	2.40	2.10
6	1	-1	1	-1	3.06	0.98	3.60	2.20
7	-1	1	1	-1	3.10	1.50	3.60	2.00
8	1	1	1	-1	4.10	2.10	4.70	2.70
9	-1	-1	-1	1	2.10	1.10	1.80	1.20
10	1	-1	-1	1	2.60	1.50	2.80	1.40
11	-1	1	-1	1	2.90	1.70	3.50	2.70
12	1	1	-1	1	3.80	2.10	4.50	3.10
13	-1	-1	1	1	1.70	0.74	2.50	2.00
14	1	-1	1	1	2.40	1.10	4.00	2.50
15	-1	1	1	1	3.20	1.50	4.40	3.10
16	1	1	1	1	4.10	2.10	5.80	3.40
17	-2	0	0	0	1.70	1.02	2.90	1.50
18	2	0	0	0	3.40	1.80	4.90	2.20
19	0	-2	0	0	1.75	0.60	2.80	1.60
20	0	2	0	0	4.10	2.10	5.60	3.30
21	0	0	-2	0	3.50	2.70	0.30	0.10
22	0	0	2	0	3.50	1.90	3.01	2.20
23	0	0	0	-2	2.70	1.10	3.60	2.30
24	0	0	0	2	2.10	0.98	4.10	3.30
25	0	0	0	0	2.70	1.65	3.35	2.10
26	0	0	0	0	2.90	1.68	3.80	2.50
27	0	0	0	0	2.95	1.50	3.40	2.11
28	0	0	0	0	2.80	1.63	3.40	2.40
29	0	0	0	0	2.65	1.75	3.70	2.10
30	0	0	0	0	2.82	1.58	3.80	2.45
31	0	0	0	0	3.09	1.60	3.45	2.01

A= Sucrose conc. (g/l) (X_1); B= Urea conc. (g/l) (X_2); C= Inoculum conc. (ml/l) (X_3); D= K_2HPO_4 conc. (g/l) (X_4)

TABLE 23: TREATMENT SCHEDULE FOR 5-FACTOR CCRD AND THE RESPONSE FOR SET 2

Sl. No.	A	B	C	D	E	Parent strain		Mutant strain	
						Biomass (g/l) (Y_1)	PHA (g/l) (Y_2)	Biomass (g/l) (Y_3)	PHA (g/l) (Y_4)
1	-1	-1	-1	-1	1	5.1	4.3	3.9	2.8
2	-1	-1	-1	-1	-1	2.4	1.8	3	2.1
3	-1	1	-1	-1	-1	2.9	2.6	2.5	1.9
4	-1	1	-1	-1	1	4.7	3.7	4.2	3.6
5	-1	-1	1	-1	-1	2.6	2.1	2.9	1.9
6	-1	-1	1	-1	1	6.6	3.5	3.8	3.1
7	-1	1	1	-1	1	5.2	3.9	3.7	2.6
8	-1	1	1	-1	-1	2.4	1.9	2.7	1.6
9	-1	-1	-1	1	-1	4.1	2.9	4.3	3.8
10	-1	-1	-1	1	1	4.9	3.9	5.2	4.4
11	-1	1	-1	1	1	5.3	3.8	5.2	3.8
12	-1	1	-1	1	-1	4.6	3.4	3.6	2
13	-1	-1	1	1	1	4.2	2.9	4.2	2.5
14	-1	-1	1	1	-1	3.5	2.1	3.6	1.4
15	-1	1	1	1	-1	3.9	3	4	2.7
16	1	1	1	1	1	5.9	4.6	5.5	3.6
17	-2	0	0	0	0	2.9	2.3	2.9	2.4
18	2	0	0	0	0	4.5	2.9	4.4	2.8
19	0	-2	0	0	0	4.3	3.5	4.5	3.3
20	0	2	0	0	0	2.8	2	4.4	2.4
21	0	0	-2	0	0	3.4	2.4	3.5	3.1
22	0	0	2	0	0	4.1	1.5	4	3.4
23	0	0	0	-2	0	2.8	1.7	2.6	1.8
24	0	0	0	2	0	7.1	6.1	4.5	3.5
25	0	0	0	0	-2	3.4	2.6	1.9	1.2
26	0	0	0	0	2	5.7	5.1	5.3	4.6
27	0	0	0	0	0	4.3	3.7	4	3.2
28	0	0	0	0	0	4.3	3.5	4.1	3.4
29	0	0	0	0	0	3.9	3.2	4.5	3.5
30	0	0	0	0	0	4.1	3.5	3.8	3.2
31	0	0	0	0	0	4.6	3.3	5	3
32	0	0	0	0	0	4.6	3.4	4.1	3.1
33	0	0	0	0	0	3.3	2.6	3.9	2.5

A = Na_2HPO_4 (g/l) (X_1); B=KH_2PO_4 (g/l) (X_2); C=$(NH_4)_2SO_4$ (g/l) (X_3); D= Sucrose conc. (g/l) (X_4); E=Inoculum Conc. (ml/l) (X_5)

Statistical analyses

A second order polynomial equation was employed to fit the experimental data presented in table 22. Proposed model for the responses Y_1, Y_2, Y_3 and Y_4 was $Y_1=a_0+a_1x_1+a_2x_2+a_3x_3+a_4x_4+a_{12}x_1x_2+a_{13}x_1x_3+a_{14}x_1x_4+a_{23}x_2x_3+a_{24}x_2x_4+a_{34}x_3x_4$ where Y_i (i=1-4) is the predicted response for biomass and PHA yield for the parent as well as the mutant strain respectively, a_o is the value of the fitted response point of design, a_i, a_{ii}, a_{ij} the linear, quadratic and cross product terms respectively. A non-linear mathematical optimization procedure of the Quattro Pro software (Quattro Pro, Version 4.0, Borland International Inc. USA) was used for the optimization of the fitted polynomials for biomass and PHA yields. Responses obtained were compared with the predicted models. The fitted polynomial equation was expressed as surface plots through which it was possible to visualize the relationship between the response and the experimental levels of each factor used in the experiments.

PHA production in optimized media

Fermenter studies

Fermenter trials were done in a 2L fermenter (NBS Bio IIO, BioFlo Modular Fermenter) to study the growth and PHA production in *Rhizobium meliloti* 14. Initial dissolved O_2 concentration was 100%, temperature and pH were maintained at 30^oC and 7 respectively. PHA production medium was used for the experiments.

Fermenter trials were done in a 10 L fermenter (Laboratory Fermenter LF 10 SIDA, Sweden) to study the growth and PHA production in *R meliloti* 14 based on the optimized nutrient conditions (for high biomass) obtained by RSM. The medium contained sucrose 55 g/l, urea 0.65 g/l, K_2HPO_4 0.39 g/l and inoculum 25 ml /l. Initial pH was 7.0 and rpm was set to 250.

Estimation of biomass and PHA

The cell biomass and PHA were estimated as described earlier (Materials and methods, page 103)

4.2 RESULTS AND DISCUSSION

Set 1

Assessment of nutrient conditions for PHA production

PHAs are accumulated as intracellular granules when surplus carbon is available but growth is restricted by some factor such as availability of essential nutrient. *Rhizobium meliloti* strain has been reported to produce up to 55% of polymer with respect to dry cell biomass (Tombolini and Nuti, 1989). During RSM studies based on treatment schedule and responses (Table 22) it was possible to compare parent and mutant strain and assess nutrient requirement and limitations for PHA production. Phosphate appeared to be limiting nutrient for PHA accumulation in the parent strain of *Rhizobium meliloti* 14. A decrease in the initial concentration of phosphate in the medium (treatment 1 and 9) from

0.78 g/l to 0.33 g/l resulted in 8% increase of PHA concentration in the cell indicating the effect of limitation of this nutrient for accumulation of polymer. As indicated in treatment No. 24, the mutant strain required moderate levels of sucrose (30 g/l), along with excess of phosphate (1 g/l) for high PHA content of cell biomass (80%) and yield (3.3 g/l). It is also evident from treatment 17 and 18 of table 22 that a surplus amount of carbon (sucrose) is required for PHA synthesis but it is essential to define its concentration otherwise excess carbon is diverted towards biomass build up. Unbalanced supply of the nutrient reduces the complexity of the metabolism that occurs in the bacterial cell and the flow of carbon is channeled into unidirectional path such as PHA synthesis (Babel et al, 2001). The flux of acetyl-CoA through TCA cycle may be reduced and hence excess but defined amount of carbon may lead to enhanced activation of the glycolytic pathway causing an increase in acetyl-COA / CoASH ratio that would lead to rapid PHA synthesis. Efficiency of biomass and PHA production was relatively more in mutant strain. Compared to the parent strain, PHA concentration in the mutant cells was not affected by variation in urea (N) concentration (0.05/ 0.65 g/l). Nitrogen is a component of protein, enzymes and nucleic acids and increased utilization would benefit overall functions of the cell. The data indicate that the efficient utilization of N towards PHA synthesis may be due to modification of carbon metabolism in the mutant. It is known that in *Rhizobium meliloti*, some of the factors responsible for carbohydrate metabolism such as polysaccharide synthesis are plasmid borne. A plasmid-curing agent

namely 4 -*N*-piperidinobutyl-2-chlorophenoxazine was used in the present study for the isolation of the mutant (Chapter 3), which might have given rise to the modification of the carbohydrate metabolism of the cells. PHA yield was also enhanced by high inoculum concentration (250 ml/l) using the mutant strain. It has been reported that PHA synthesis is also enhanced by insufficient oxygen supply to the cells (Tombolini and Nuti, 1989). Higher yields in the mutant strain compared to the parent strain in the presence of higher inoculum may be due to decreased polysaccharide production (Lakshman and Shamala, 2003) which would result in overcoming the insufficiency of oxygen supply to the cells at initial stages of fermentation and also due to better utilization of required nutrients.

The responses measured in the experiments were PHA and biomass yields. The effect of urea, inoculum and K_2HPO_4 on responses of biomass production and PHA yield are tabulated (Table 24) by the coefficient of 2^{nd} order polynomials. Interaction of the factors however had pronounced effect on PHA optimization indicating the importance of consideration of these factors for enhancement of PHA yields. The insignificant terms were omitted using t-test and the responses under different combinations were analyzed using analysis of variance (ANOVA) appropriate to the experimental design. It is evident from the data presented that first and second (quadratic and cross product) order terms were found to be significant and lack of fit was not significant. The high value of coefficient of determination (R^2) also suggest that the model is a good fit. The response surfaces based on the coefficients are shown in Fig. 22 and 23,

TABLE 24: ESTIMATED COEFFICIENT FOR THE FITTED SECOND ORDER POLYNOMIAL REPRESENTING THE RELATIONSHIP BETWEEN THE RESPONSE AND PROCESS VARIABLES FOR PHA PRODUCTION BY *RHIZOBIUM MELILOTI* 14 FOR SET 1

	Parent strain		Mutant strain	
	Biomass (g/l)	PHA (g/l)	Biomass (g/l)	PHA (g/l)
a_0	2.84 [a]	1.63 [a]	3.56 [a]	2.24 [a]
a_1	0.41 [a]	0.19 [a]	0.53 [a]	0.18 [a]
a_2	0.60 [a]	0.39 [a]	0.73 [a]	0.44 [a]
a_3	0.03 [ns]	- 0.17 [a]	0.44 [a]	0.36 [a]
a_4	- 0.08 [c]	- 0.03 [c]	0.12 [c]	0.22 [a]
a_{11}	- 0.07 [c]	- 0.06 [a]	0.11 [c]	- 0.07 [ns]
a_{22}	0.03 [ns]	- 0.07 [a]	0.19 [a]	0.08 [c]
a_{33}	0.17 [a]	0.17 [a]	- 0.45 [a]	- 0.25 [a]
a_{44}	0.10 [b]	- 0.15 [a]	0.10 [c]	0.17 [a]
a_{12}	0.02 [ns]	0.04 [c]	- 0.01 [ns]	0.05 [ns]
a_{13}	0.08 [c]	0.03 [ns]	0.10 [ns]	0.03 [ns]
a_{14}	0.03 [ns]	0.03 [ns]	0.06 [ns]	0.00 [ns]
a_{23}	0.09 [c]	0.06 [b]	0.01 [ns]	- 0.15 [b]
a_{24}	0.04 [ns]	- 0.02 [ns]	0.15 [c]	0.20 [b]
a_{34}	- 0.04 [ns]	0.04 [c]	0.19 [b]	0.05 [ns]

[a] Significant at 0.1%, [b] Significant at 1.0%, [c] Significant at 5.0%
[ns] Not significant even at 5% level

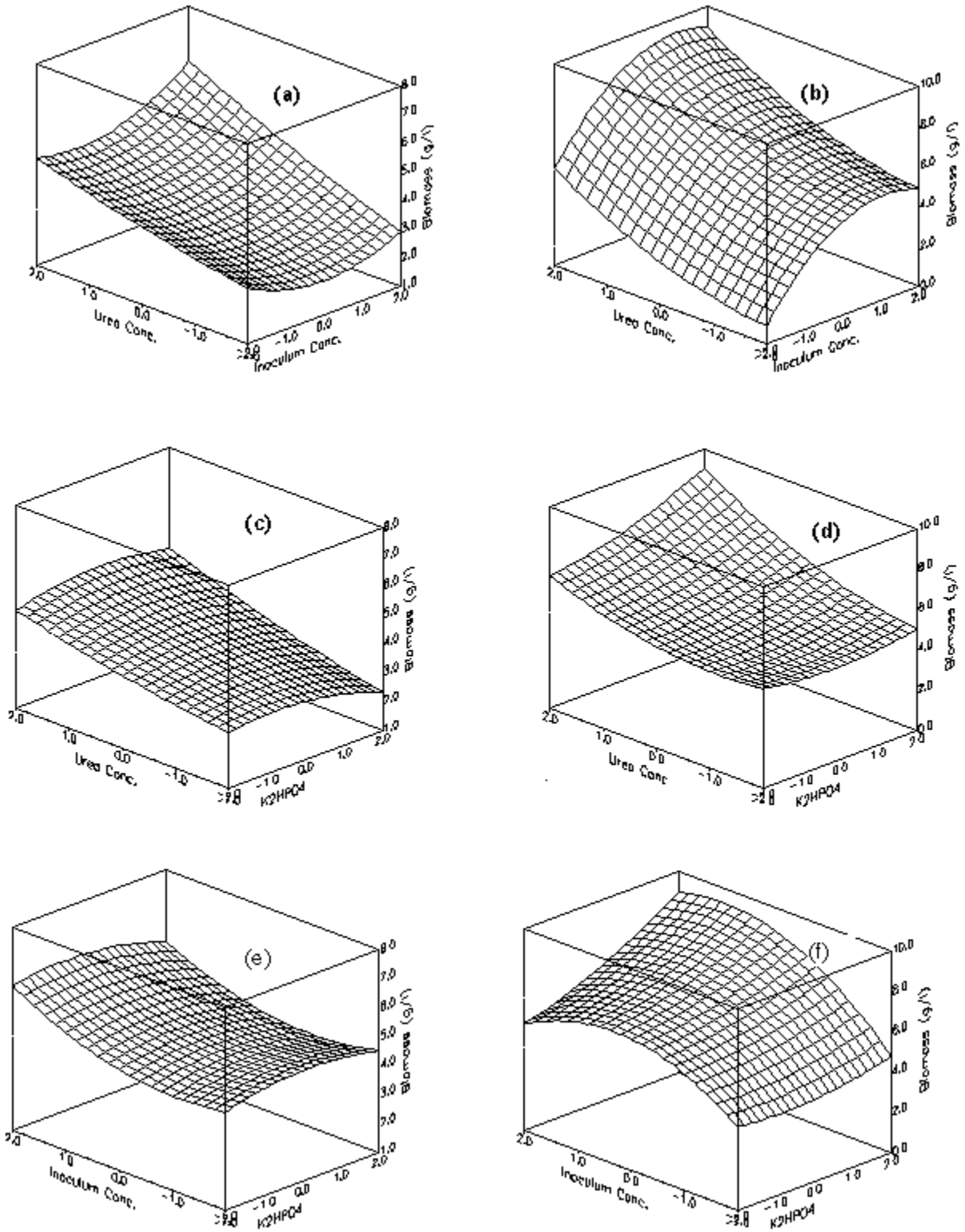


Fig. 22: Response surfaces showing the effect of inoculum and urea concentration (a, b); Urea and K_2HPO_4 concentration (c, d); inoculum concentration and K_2HPO_4 concentration (e, f) on biomass in parent (a, c and e) and mutant (b, d and f) strains of *R. meliloti*.

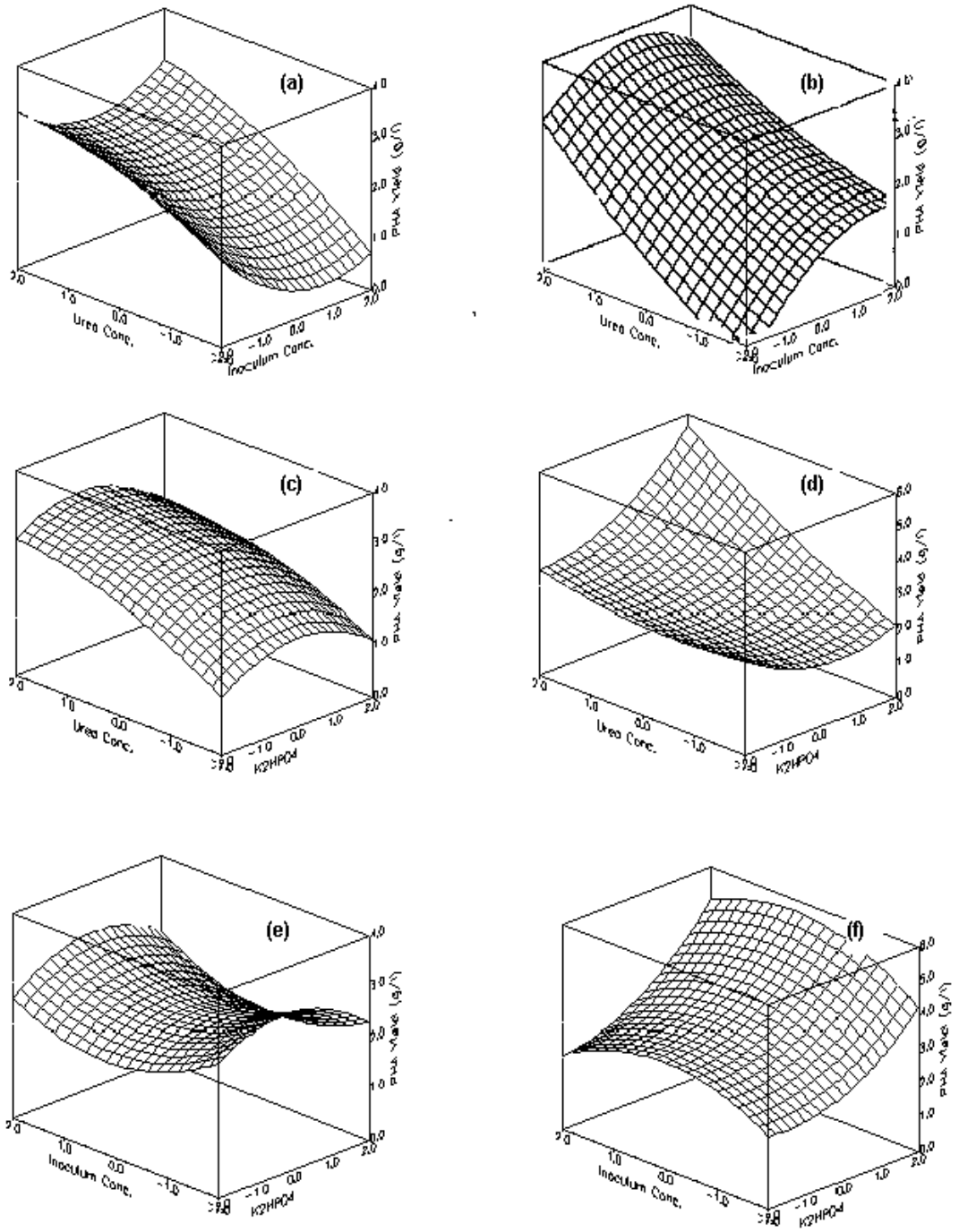


Fig. 23: Response surfaces showing the effect of inoculum and urea concentration (a, b); Urea and K_2HPO_4 concentration (c, d); inoculum concentration and K_2HPO_4 concentration (e, f) on PHA yield in parent (a, c and e) and mutant (b, d and f) strains of *R. meliloti.*

keeping two variables at optimum level and varying the other two within the experimental range. The optimum conditions for biomass and PHA yields are represented in table 25.

The response surface graphs for biomass and PHA yields appeared to show similar pattern but significant differences were seen between the parent and the mutant strain of *R. meliloti* (Fig. 22-23).

Effect of inoculum and urea concentration on biomass production and PHA yield

At the optimum level of K_2HPO_4 (0.41 g/l) and sucrose (55 g/l), highest concentration of urea (0.65 g/l) was found to be favorable for maximum biomass and PHA yield by the parent strain. Under these conditions the maximum biomass (6.23 g/l) and maximum PHA yield (3.09 g/l) was obtained at maximum (250 ml/l) and minimum inoculum levels, respectively (Fig 22 a and 23 a). In case of the mutant strain, under similar conditions, maximum biomass (9.29 g/l) and maximum PHA yield (5.66 g/l) was obtained at 200 ml/l and 150.58 ml/l inoculum level respectively (fig. 22 b and 23 b). Mutant strain showed enhanced biomass production and PHA synthesis as compared to parent strain.

Effect of urea and K_2HPO_4 concentration on biomass and PHA yields

At the optimum level of inoculum maximum quantities of biomass and PHA yield for parent strain was found at highest concentration of urea (0.65 g/l) and lower concentration of K_2HPO_4 (0.48 g/l) (Fig. 22 c and Fig. 23 c). The interaction of these factors for biomass and PHA yields were significantly

TABLE 25: FEASIBLE OPTIMUM CONDITIONS AND EXPERIMENTAL VALUE OF RESPONSE AT OPTIMUM CONDITION FOR PHA PRODUCTION BY *RHIZOBIUM MELILOTI* 14

Optimum conditions	Parent strain (*Rhizobium meliloti* 14)	Mutant strain (*Rhizobium meliloti* 22)
Sucrose (g/l) (X_1)	51.58	55.00
Urea (g/l) (X_2)	0.65	0.65
Inoculum (ml/l) (X_3)	10.00	150.58
K_2HPO_4 (g/l) (X_4)	0.48	1.00
Responses		
Biomass (g/l) (Y_1)	6.23	9.29
PHA (g/l) (Y_2)	3.09	5.66

different for mutant strain because excess of K_2HPO_4 (1.0 g/l) and 0.65 g/l of urea were required for maximum biomass and PHA production (Fig. 22 d and Fig. 23 d).

Effect of inoculum and K_2HPO_4 concentration on biomass and PHA yields

At the optimum level of urea, maximum concentration of biomass and PHA yield for parent strain were obtained at higher level of inoculum and of K_2HPO_4 (Fig. 22 e and Fig. 23 e). The interaction was significantly different for biomass yield of mutant strain, which required high concentration of K_2HPO_4. and inoculum (Fig. 22 f) The interaction was not significantly different for PHA yield by mutant strain as the yield increased with increased concentration of K_2HPO_4 (Fig. 23 f).

Exploration of the response surfaces for *Rhizobium meliloti* indicated a complex interaction between the variables such as sucrose, urea, inoculum and K_2HPO_4.. Concentrations (g/l) of sucrose 51.58, K_2HPO_4 0.48, urea 0.65, and inoculum 10 (ml/l) were the conditions optimized by RSM for maximum PHA yield (3 g/l) by the parent strain. Corresponding optimized concentrations of 55, 1.0, 0.65 and 150.58 resulted in higher yields of the polymer (5.66 g/l) by the mutant strain. A high inoculum level required for optimized PHA production and increased PHA yield indicated the suitability of the mutant for high cell density fermentation. From RSM studies it was possible to simultaneously find out that the limitation of phosphate (0.33 g/l initially) in the presence of excess sucrose results in maximum production of PHA (2.2 g/l) in the parent strain, whereas the

mutant strain required excess of phosphate (1 g/l) for higher PHA turnover (3.3 g/l).

There was a significant difference in the carbon uptake levels between the parent and the mutant. The carbon conversion efficiency (CCE) into PHA (g) /g of carbon utilized was significantly higher in the mutant compared to the parent strain (Fig 24 and 24a). In treatments 1, 2, 3 and 4 the CCE of the parent was 0.4, 0.2, 0.5 and 0.3 , whereas in the mutant CCE was 0.6, 0.6, 0.6, 0.5 PHA (g)/g of carbon utilized respectively. Sugar uptake levels at corresponding treatments were 41.7 %, 31.4%, 48.8% and 32.75 in the parent and 28%, 13.4%, 42.8% and 24.7% in the mutant respectively. Similarly CCE of the mutant was higher than parent as seen in treatment 11 (0.6), 12 (0.55), 13 (0.51), 14 (0.5) and 15 (0.54). It was evident that the CCE in the mutant was higher compared to parent. Treatments 17, 20 and 23 (Fig 24a) show higher carbon conversion rates in *Rhizobium meliloti* 14. But the growth of mutant was not good at these levels.

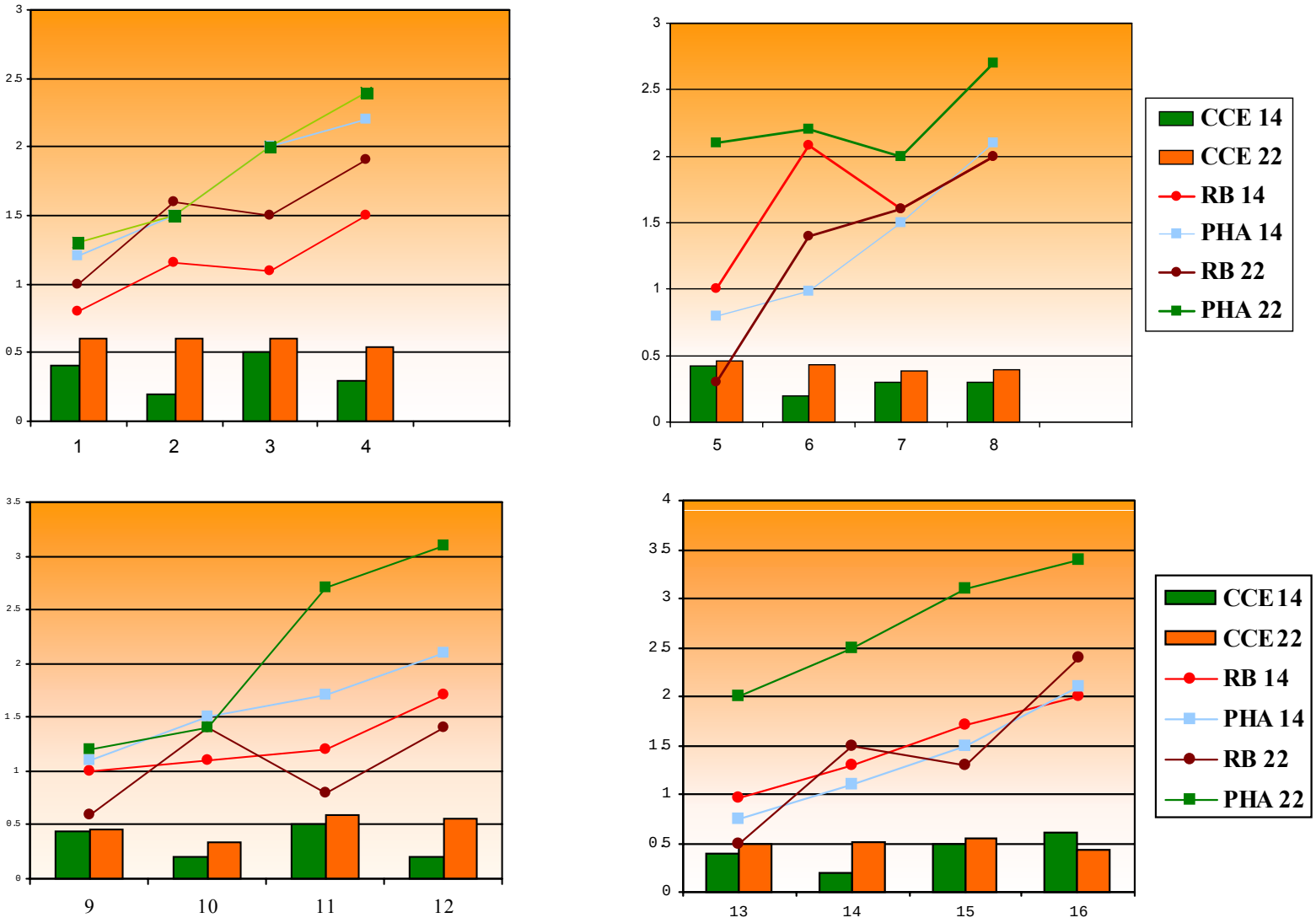


Fig 24: Carbon conversion efficiency [CCE] (PHA g / g carbon), Residual biomass (RB g/l) and PHA (g/l) accumulation in *Rhizobium meliloti*14 and *Rhizobium meliloti* 22 at different treatment levels according to table 22 …Continued as 24A

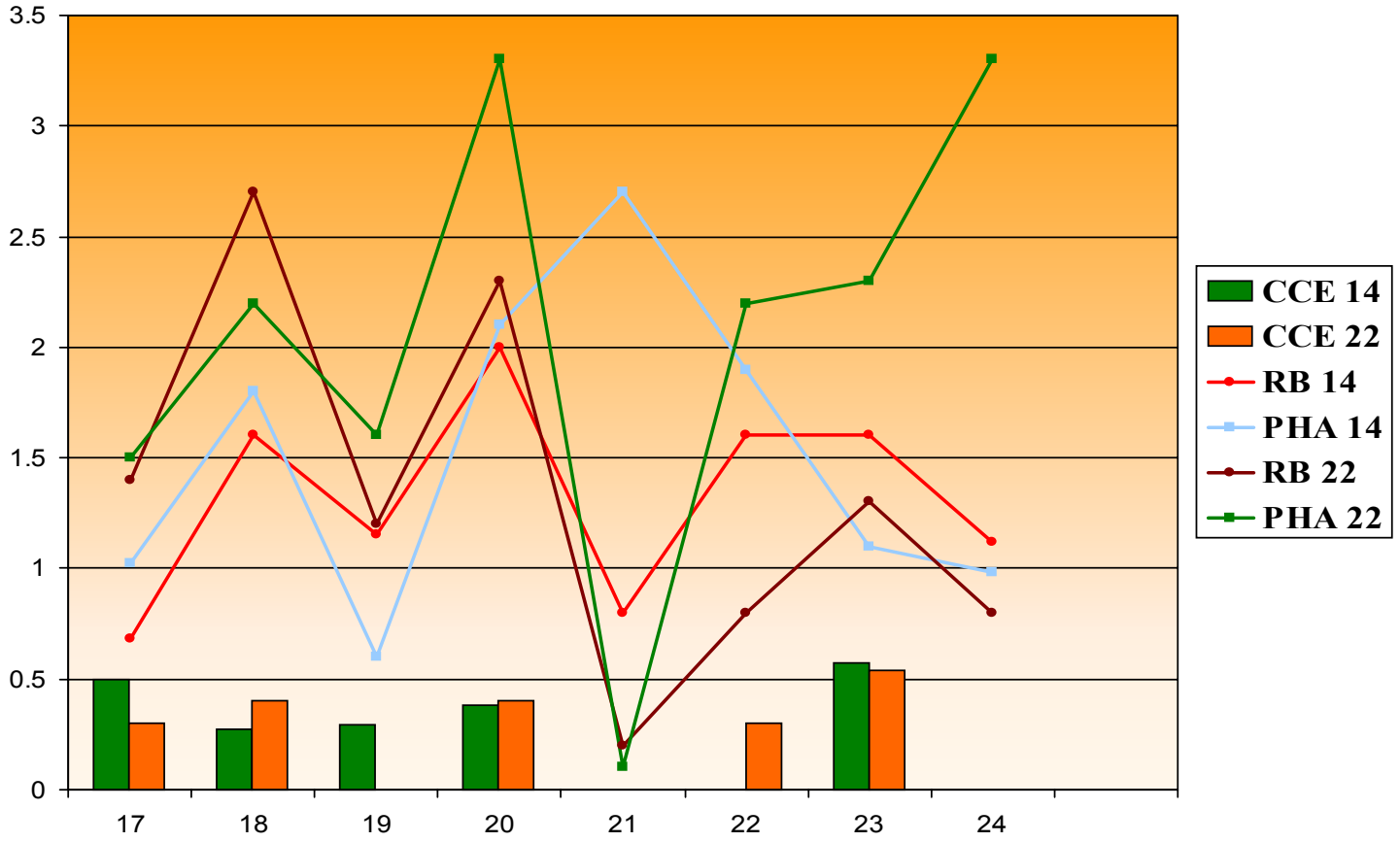


Fig 24 A: Carbon conversion efficiency [CCE] (PHA g / g carbon), Residual biomass (RB g/l) and PHA (g/l) accumulation in *Rhizobium meliloti* 14 and *Rhizobium meliloti* 22 at different treatment levels according to table 22

SET 2

Assessment of nutrient conditions for PHA production

In the second set ammonium sulphate was used as nitrogen source in the medium. High PHA accumulation was seen when surplus carbon was available. But as in set 1 the *R meliloti* 14 gave nearly 7.1 g/l of biomass and PHA yield was 6.1g/l (85%). The mutant accumulated 4.5 g/l biomass and 3.5 g/l of PHA. As seen in the set 1 phosphate was a limiting nutrient for PHA production in *R meliloti* 14 strain. As indicated in treatment-1 (Table 23), where in limitation of both KH_2PO_4 and Na_2HPO_4 gave high content of biomass and PHA levels, 5.1 g/l & 4.3 g/l respectively. Increase in phosphate levels of KH_2PO_4 increased biomass of *R meliloti* 22 to 4.2 g/l and PHA yield to 3.6 g/l (treatment 4). Phosphate limitation for PHA was observed for the *R meliloti* 14. Surplus amount of carbon in the form of sucrose 30 g/l was required for both PHA synthesis and high biomass production in *R meliloti* 14 (treatment 24). Where as *R meliloti* 22 did not grow or accumulate PHA at similar levels. Exopolysaccharide producing microorganisms have better intake of carbon as they direct considerable amount of assimilated carbon towards production of polysaccharide as in *R meliloti* 14. Where as in *R meliloti* 22, PHB production could be limited (intracellularly) because of the constrain of cell volume. This justifies the low levels of biomass but high percentage of PHA accumulation as seen in *Rhizobium meliloti* 22 compared to *Rhizobium meliloti* 14. At high levels of $(NH_4)_2SO_4$, (2 g/l) there was good growth of both *R meliloti* 14 and *R meliloti* 22 with 4.1g/l and 4g/l biomass

respectively. But PHA yields were 1.5 g/l (36.5%) and 3.4 g/l (85%). The *R meliloti* 14 strain also requires nitrogen at limiting levels to produce PHA. N_2 limitation for PHA production is well known. Where as the *R meliloti* 22 accumulated high PHA (treatment 22) at $(NH_4)_2SO_4$ 2 g/l. As explained in Set 1, *R meliloti* 22 was able to channalize nitrogen towards PHA synthesis. The global nitrogen regulatory (ntr) systems have been described and are active in *Rhizobia.* The ntr system senses the ratio of intracellular concentration of glutamine to α-ketoglutarate and through a cascade of regulatory proteins, regulates the activity of nitrogen regulation. Assimilation of NH_4 is known to take place through glutamine synthetase/ glutamate synthase pathway and at higher NH_4 concentration through glutamate dehydrogenase (GDH) (Patriarca et al, 2002). Thus enters the TCA cycle and enhances the biomass. NH_4^+ is also produced inside the *Rhizobia* from nitrate and aminoacids. Excess of carbon in these conditions may lead to PHA synthesis. NH_4 is known to reduce the ability to produce exopolysaccharide (Patriarca et al, 2002). In case of *Rhizobium meliloti* 14 ammonia is directed towards excess of biomass and a small amount of exopolysaccharide where as in the *Rhizobium meliloti* 22 does not seem to assimilate ammonia for growth.

High inoculum levels (200 ml/l) favoured the PHA production in both *Rhizobium meliloti* 14 and in *Rhizobium meliloti* 22 (treatment 26) with $(NH_4)_2SO_4$ as nitrogen source. Biomass and PHA yields were 5.7 g/l and 5.1 g/l (89%) in *Rhizobium meliloti* 14 and 5.3 g/l and 4.6 g/l (86%) in *Rhizobium meliloti* 22

respectively. Increase in either KH_2PO_4 or $(NH_4)_2SO_4$ levels along with high inoculum increased accumulation of PHA to 10% in *Rhizobium meliloti* 22 (treatment 1,4,6), where as it decreased in *Rhizobium meliloti* 14 (treatment 1, 4, 6) (Fig 25). This clearly states that *Rhizobium meliloti* 14 accumulates PHA on phosphate, potassium and nitrogen limitation, but *Rhizobium meliloti* 22 does not respond similarly to these limitations. Sub optimal levels of phosphate are described for PHA synthesis (Borah et al, 2002). But *Rhizobium meliloti* 22 show requirement for both phosphorous and nitrogen unlike parent. Decrease in KH_2PO_4 levels did not affect *Rhizobium meliloti* 22 but increase to 2 g/l affected PHA production (treatment 20). *Rhizobium meliloti* 22, when compared to parent produced nearly 18% more at low nitrogen levels and nearly 49% more PHA yield at high nitrogen concentration i.e., 2 g/l of $(NH_4)_2SO_4$ (Table 23, treatment 21 and 22). Low inoculum levels decreased the yields in all treatments (2,3,5,8,12,14,15). However increase in sucrose levels increased the PHA yield irrespective of low nitrogen and phosphate levels (treatment 9). *Rhizobium meliloti* 22 requires high inoculum and high sucrose for PHA accumulation. It requires either high KH_2PO_4, $(NH_4)_2SO_4$ and inoculum level or high carbon level for PHA production. *Rhizobium meliloti* 22 may channalize excess carbon into PHA without phosphate or nitrogen limitations where as the *Rhizobium meliloti* 14 cannot.

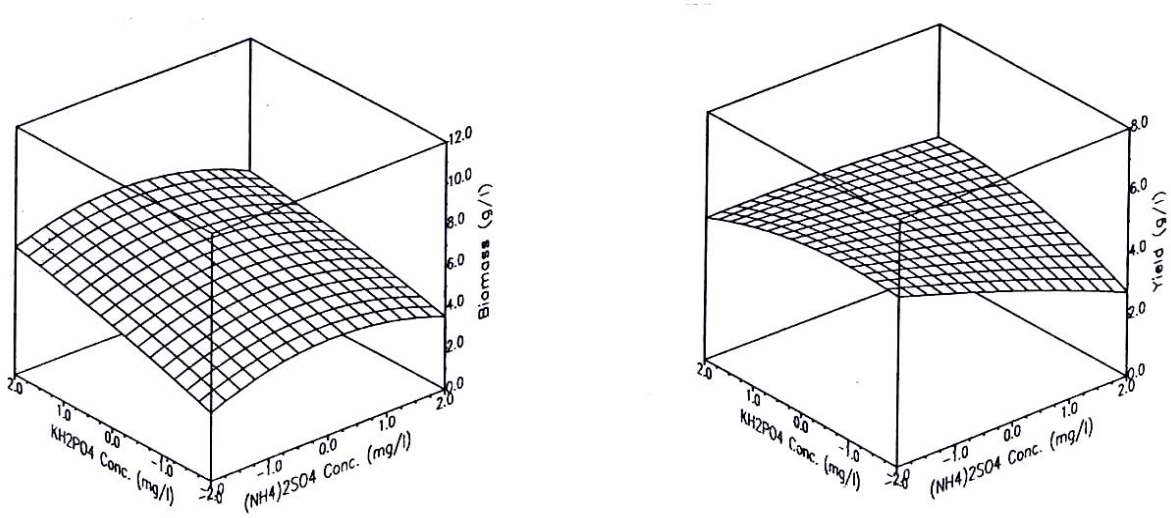


FIG 25: RESPONSE SURFACES SHOWING EFFECT OF KH_2PO_4 CONCENTRATION AND $(NH_4)_2SO_4$ CONCENTRATION ON BIOMASS AND PHA YIELDS IN *RHIZOBIUM MELILOTI* 22

Effect of KH_2PO_4 and Na_2HPO_4 levels on PHA and biomass levels

Rhizobium meliloti 14 showed low biomass or growth levels (2.9 g/l) with Na_2HPO_4 limitations but biomass increased to 4.5 g/l with increase in the Na_2HPO_4 (treatment 17 and 18, table 23). Whereas there was high biomass (4.3 g/l) in the presence of low KH_2PO_4 (0.5 g/l). An increase in KH_2PO_4 (2 g/l) decreases the biomass in *Rhizobium meliloti* 14 (treatment 20). Low level of Na_2HPO_4 severely affected the biomass in the mutant but not PHA accumulation. Growth was not altered by limitations of KH_2PO_4 (treatment 20) (Fig 26). Sodium and potassium are metals in biological systems and are involved strictly in maintenance of osmotic pressure, as components of enzyme, electron carriers in redox reaction, transport and generation of membrane potential. To maintain a concentration gradient the intracellular potassium concentration should be always low. An increase in potassium inside the cell when there is low sodium and high potassium in the medium might lead to adversaries in metabolism. The metabolic activity of cells in potassium limited condition is maintained at relatively high level compared to nitrogen limitation in which case the metabolic activity is severely suppressed. Although nitrogen limitation enhanced PHB production complete nitrogen deficiency considerably damaged microbial activity including PHB synthesis (Kim et al, 1996). *Rhizobium meliloti* 14 showed requirement of high level of Na_2HPO_4 and low level of KH_2PO_4, whereas *Rhizobium meliloti* 22 requires high levels of Na_2HPO_4.

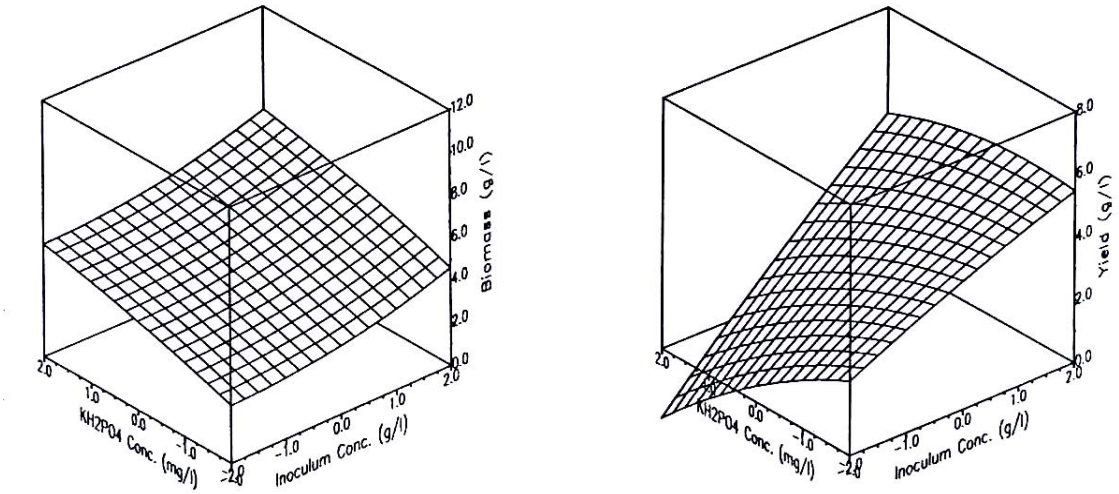


FIG 26: RESPONSE SURFACE SHOWING EFFECT OF KH_2PO_4 CONCENTRATION AND INOCULUM CONCENTRATION ON BIOMASS AND PHA YIELDS IN *RHIZOBIUM MELILOTI* 22

Effect of KH_2PO_4 concentration and $(NH_4)_2SO_4$ concentration on biomass levels and PHA yields

At low levels of inoculum (57.5 ml /l), with increase in KH_2PO_4 level and decrease in $(NH_4)_2SO_4$ (Fig 25), there was low growth in *Rhizobium meliloti* 14 (2.9 g/l) as well as *Rhizobium meliloti* 22 (2.5 g/l) (treatment 3 and 4). An increase in inoculum (152.5 ml/l) at similar concentration increased the PHA accumulation in *Rhizobium meliloti* 22 significantly. Decrease in KH_2PO_4 (0.875 g/l) levels and increase in $(NH_4)_2SO_4$ (1.625 g/l) levels with low inoculum (treatment 5 and 6) resulted in less growth and biomass. But with increase in high inoculum levels with similar condition there was a drastic difference between *Rhizobium meliloti* 14 and *Rhizobium meliloti* 22. Biomass obtained was 6.6 g/l and yield 3.5 g/l (53%) for *Rhizobium meliloti* 14 and biomass 3.8 g/l and 3.1 g/l (81%) for *Rhizobium meliloti* 22.

Ammonia supplementation is said to be beneficial for PHB accumulation (Bitar and Underhill, 1990) But comparably higher biomass of 4.7 g/l *Rhizobium meliloti* 14 and 3.6 g/l *Rhizobium meliloti* 22 as in treatment 4 was obtained at low ammonia levels. Ammonia cross through membrane by specific transport systems that are dependant on high affinity energy dependant transport system (Patriarca et al, 2002). Therefore the parent seems to utilize its phosphate and nitrogen sources to produce high-energy molecule and divert towards biomass production. There seems to be loss of some alteration in *Rhizobium meliloti* 22 that leads to poor uptake of ammonia (Fig 27).

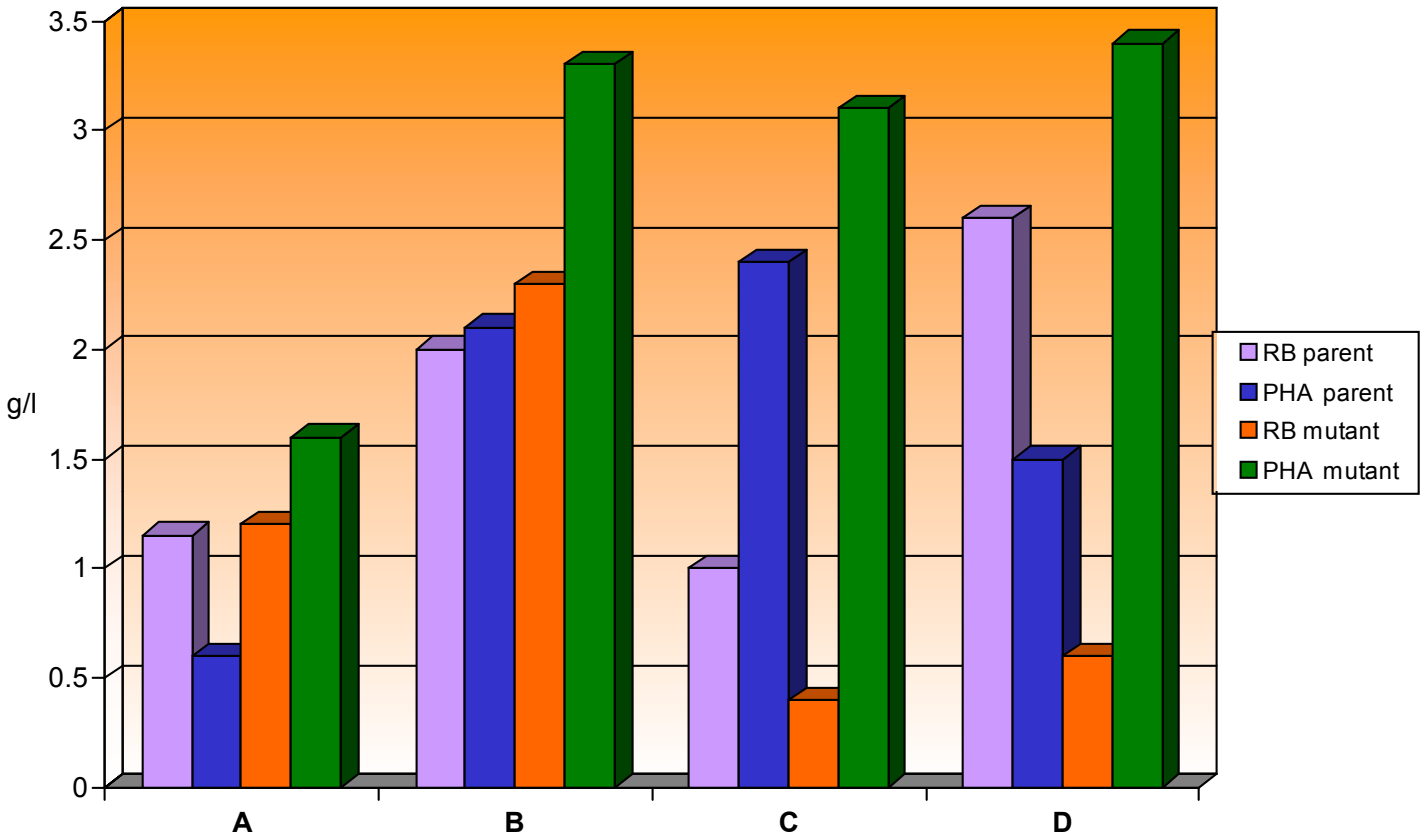


Fig 27: Comparison of nitrogen (Urea A, B and Ammonium sulphate C,D) assimilation on growth and PHA production in *Rhizobium meliloti* 14 (parent) and *Rhizobium meliloti* 22 (mutant)
A. Treatment 19, table 22, B. Treatment 20, table 22,
C. Treatment 21, table 23, D. Treatment 22, table 23
RB - Residual Biomass (Total biomass – PHA)

This was observed because of low biomass levels under high nitrogen concentration, but there was high PHA accumulation. Secondly during carbon limitation (low levels of carbon) as in treatment 6, the carbon uptake and metabolism must be from aminoacids and they will excrete ammonia intracellularly. This ammonia if not properly assimilated will lead to low bacterial growth. (Patriarca et al, 2002).

Effect of KH_2PO_4 concentration and Sucrose concentration on biomass levels and PHA yields

At Sucrose 23.75 g/l and KH_2PO_4 0.875 g/l, PHA accumulation in *Rhizobium meliloti* 22 was higher irrespective of low inoculum levels (treatment 9&10). Biomass and PHA yield were 4.1 g/l and 2.9 g/l in *Rhizobium meliloti* 14 and 4.3 g/l and 3.8 g/l in *Rhizobium meliloti* 22. At similar condition increase in $(NH_4)_2SO_4$ (treatment 13 and 14) there was drastic decrease to 50% low in PHA accumulation, 1.4 g/l in *Rhizobium meliloti* 22. High KH_2PO_4 (1.625 g/l) and sucrose 23.75 g/l under low inoculum levels was favoured by *Rhizobium melilotii* 22 but drastically decreased the PHA content (treatment 12, table 23). Increase in $(NH_4)_2SO_4$ at this situation increased biomass but not yield.

Fermenter trials

91% of PHA was accumulated when *Rhizobium meliloti* 14 was grown in a 2L fermenter, in medium containing ammonium sulphate as the nitrogen source (Table 26). But the yields were 1.62 g/l of PHA. The initial dissolved oxygen was set to 100 %. Results showed that the levels of PHA accumulation in the culture decreased with the increase in aeration. Eventhough the dissolved oxygen concentration lowered to 50%, the PHA levels did not increase. And by the end of 72 h the PHA content was 30%. The data indicates that in a fermenter under controlled conditions there is faster growth rate and at 24 h high PHA accumulate. There was 100% uptake of the sugar by 72 h and the biomass levels increased to 3.6 g/l. Continuos monitoring of pH, temperature and DO levels in fermenter favours the bacterium, as there is no nutrient stress on the organism. Further optimization at fermenter levels needs to confirm this.

Fermenter trials at 8L capacity were conducted to study the growth and PHA production of R 14 (Table 27, Fig 28, and Plate 10). There was a gradual decrease in the pH from 14 h to 72 h from 7.0 to 5.4. *Rhizobium meliloti* 14 accumulated upto 4 g/l of PHA after 72 h. Final biomass was 6.8g/l. The percentage of PHA obtained was however 58.8 %, which was less, than compared to batch shake flask cultures. Fig 28 shows a gradual increase in the biomass levels upto 48 h. There was no change in the residual sugar after 36-h indicating that there was no uptake of sugar after the culture reached stationary phase. The biomass levels went up to the expected levels (predicted values 6.29

TABLE 26: GROWTH AND PHA PRODUCTION OF *RHIZOBIUM MELILOTI* 14 IN 2 L FERMENTER

Hours	Biomass g/l	PHA g/l	% PHA based on dry wt of biomass	DO_2 %	Residual sugar g/l	Sugar uptake %
24	1.7	1.62	91	100	18	11
48	3.2	1.82	57	50	7	65
72	3.6	1.2	30	50	0	100

TABLE 27: GROWTH AND PHA PRODUCTION OF *RHIZOBIUM MELILOTI* 14 IN 10L FERMENTER

Samples (h)	Biomass (g/l)	PHA (g/l)	pH	Residual sugar (g/l)	Polysaccharide (g/l)
14	1.3	0.4	7.9	11	0.1
24	2.2	0.75	7.3	10	-
36	3.85	2.15	6.5	9.5	0.7
48	4.9	2.45	6.1	9.0	0.7
60	5.9	3.1	5.8	9.0	1.0
72	6.8	4	5.4	9.0	1.6

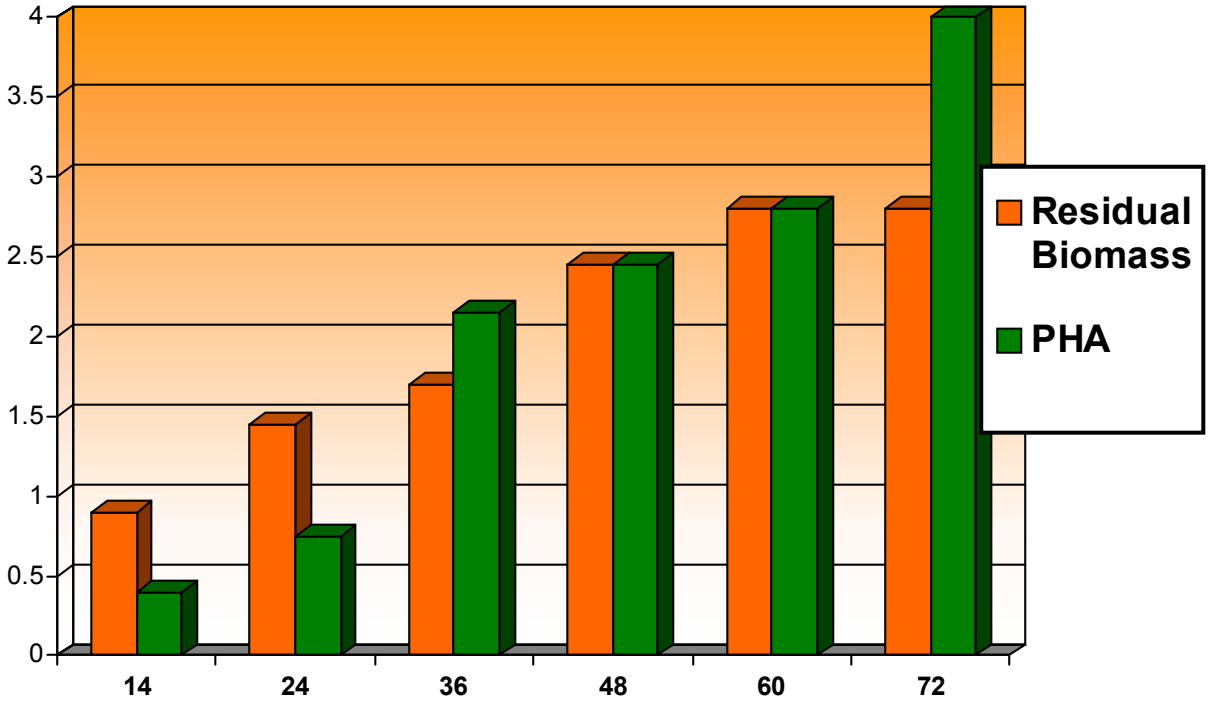


FIG 28: GROWTH AND PHA PRODUCTION OF *RHIZOBIUM MELILOTI* 14 IN 10 L FERMENTER

PLATE 10: GROWTH OF *RHIZOBIUM MELILOTI* 14 IN A 10 L FERMENTER.

and experimental 6.8 g/l) at 72 h. The PHA yield was slightly higher than predicted (Predicted value 2.95 g/l, experimental 4 g/l). An increase in the polysaccharide (1.6 g/l) content was also seen at 72 h.

4.3 CONCLUSION

A thorough comparison of biomass and PHA yields in the parent and the mutant were carried out based on Response surface methodology. Efficiency of produciton of biomass and PHA was higher in mutant compared to the parent Maximum yields of PHA obtained by the parent strain was 3.0 g/l in the medium containing urea as the nitrogen source. Highest yields obtained by the mutant strain was 5.66 g/l.

Phosphate and nitrogen appeared to be the limiting nutrient favouring PHA production depending on the culture conditions. Although the initial trials suggested the role of oxygen limitation, the effect of oxygen has not been thoroughly studied here. The mutant did not require phosphate limitation for PHA synthesis. The medium optimized for PHA production for *Rhizobium meliloti* 14 by RSM was sucrose (g/l) 51.58, urea (g/l) 0.65, inoculum (ml/l) 10 and K_2HPO_4 (g/l) 0.48. The quantitative yield of PHA and carbon conversion efficiency of the mutant was consistently higher compared to parent when grown in medium containing urea as the nitrogen source. The PHA yields in both the strains exceeded 80% and reached 85 to 89 % depending on the cultural conditions.

The highest PHA yield ranged between 5.5 g/l to 6.5 g/l in shake flask experiments. Biomass obtained ranged between 6.0 g/l to 9.0 g/l.

Highest yield of 91% of PHA based on dry weight of biomass was obtained in one of the fermenter trials. But the quantitative yields were 1.62 g/l, which was, less than the yields obtained in shake flask experiments. It was concluded that further optimization of conditions at scale up levels is necessary. However the data obtained in the shake flask experiments forms the basis as they provide information regarding the various limiting nutrient conditions favouring the PHA accumulation.

Chapter 5

Characterization of Polyhydroxyalkanoates

5.0 INTRODUCTION

Characterization of polyhydroxyalkanoates is done by various methods such as the infrared spectroscopy, gas chromatography, mass spectroscopy and nuclear magnetic resonance studies. All the above techniques are important to conclude about the molecular structure of any compound.

5.1 METHODS

Purification of PHA

PHA for characterization was obtained by solvent extraction in a soxhlet apparatus. Cells were collected by centrifugation form a 72-h old culture. They were washed thoroughly with distilled water and dried with acetone. Acetone dried cells were refluxed in chloroform for 6 h in the soxhlet apparatus. PHA in the cells dissolved in chloroform and a highly viscous solution was obtained after 6 h of refluxing. Insoluble matter that was found floating in chloroform was removed by filtering using glass wool followed by centrifugation. PHA was recovered from chloroform solution by precipitation with hexane. Five volumes of hexane were used against one volume of chloroform solution for precipitation of the polymer. PHA precipitated as a white cottony mass. PHA was collected by decanting the hexane chloroform mixture and then air-dried.

Crotonate assay by UV Spectroscopy

The polymer obtained by extraction method described above was used for crotonic assay. About 10 mg of the extracted polymer was taken in a test tube and was dissolved in 5 ml of concentrated sulfuric acid. This was digested in a water bath at 100^{o}C for 10 min to hydrolyze the product to crotonic acid. The absorbency at 235 nm of the solution was measured in an UV spectrophotometer (Shimadzu, UV 160, Japan) against sulfuric acid blank (Law and Slepecky, 1961).

PHA was quantified based on this assay by comparing with the purity of the standard PHB. A standard graph was drawn with standard PHB obtained from Sigma. Standard PHB was taken in 0.5, 1, 1.5, 2.0 and 10 mg and was treated as mentioned above. The absorbance was measured at 235 nm. Concentration of test sample was calculated from standard graph and purity was estimated based on concentration of PHA in known weight of sample used.

Sample preparation and Fourier Transform-Infrared spectroscopy

Extracted PHA was dissolved in chloroform. A drop of the chloroform solution was placed on the KBr windows and analyzed. Analysis was done by Fourier transform infrared spectroscopy (model no.2000, GC IR, Perkin Elmer, USA). The scanning conditions were a spectra range of 4000-400 cm^{-1}.

Gas Chromatography

Sample preparation

GC analysis of PHA was carried out by the method described by Brandl et al (1988). 10 mg of extracted PHA was taken in a glass test tube. 1ml of chloroform, 0.85 ml of methanol and 0.15 ml of sulfuric acid were added. All the chemicals used were of AR grade. The tube was sealed and kept for hydrolysis in an oil bath at 100^{o}C for 160 min. After hydrolysis contents were allowed to cool and were mixed with 5 ml of water. After phase separation the bottom chloroform phase was taken and used for GC analysis. PHB and PHB-CO-PHV (5 mol%) from Sigma were also prepared similarly as standards.

The methyl esters obtained were analyzed in a Fisons gas chromatograph (GC Fisons, 8000, CE instruments, Italy) with a DB 1(Durabond) capillary column (DB series, Shimadzu, Japan; Mfd by JW Scientific USA) and flame ionization detector. Nitrogen 1ml /min was used as a carrier gas. The temperatures of the injector and detector were 220^{o}Cand 230^{o}C, respectively. Temperature program used was 55^{o}C for 7 min; temp ramp of 4^{o}C per min upto 100^{o}C; 10^{o}C rise upto 200^{o}C followed by 10 min hold.

Gas chromatography mass spectroscopy

Sample preparation

Samples were esterified as mentioned above and were analyzed in a GC Mass Spectrophotometer (GC-17A Gas chromatograph and QP-5000 MS,

Shimadzu). Column used was DB 1 (Durabond) capillary column (DB series, Shimadzu, Japan; Mfd by JW Scientific USA) and flame ionization detector. Nitrogen (1 ml/ min) was used as a carrier gas. The temperatures of the injector and detector were 220^{o}C and 230^{o}C respectively. Temperature program used was 45^{o}C for 7min; temp ramp of 4^{o}C per min upto 100^{o}C; 10^{o}C rise upto 200^{o}C followed by 10 min hold. P (HB-co-HV) with 5% valerate (sigma) was used for comparison.

NMR spectroscopy

Nuclear magnetic resonance (NMR) analysis of PHA samples was done at sophisticated instruments facility, Indian institute of Science, Bangalore, India. ^{1}H and ^{13}C NMR spectra were recorded using purified samples. PHA was extracted by chloroform as mentioned under purification of PHA. 5 mg of sample were dissolved in deuterated chloroform ($CDCl_3$) and the solution was analyzed at 400 MHz on an AMX 400 (Bruker) spectrophotometer. The spectrum was recorded at 25^{o}C with a pulse repetition of 3 s. The enhancement of proton resonance in ^{1}H NMR was determined from a comparison with the spectrum of standard P (3HB-co-HV) obtained from sigma. Copolymer concentration was expressed as mol%. This was calculated from ratios of peak areas due to HV methyl resonance and the sum of HB and HV methyl resonances in ^{1}H NMR. The carbon resonance obtained in the ^{13}C NMR was compared with the peaks from a standard PHV resonance graph.

Differential scanning calorimetry

Differential scanning calorimetric (DSC) experiments was performed using a Perkin Elmer DSC apparatus. Analysis was done at Raman research Institute, Bangalore, India. Approximately 4-5 mg of PHA samples were subjected to the following protocol: (1) heat at 50 ^{o}C and hold for 1min, (2) heat to 183^{o}C at 5 ^{o}C /min and (3) Cool from 183^{o}C to 50 ^{o}C at 5 ^{o}C /min.

Viscometry

The molecular weight of PHA was estimated based on viscosity measurement to give a viscosity average molecular wt. (M_v). Predetermined amounts of the sample at a concentration of 0.1, 0.2, 0.3, 0.4 and 0.5% were analyzed for their reduced viscosity. The viscosity of PHA-chloroform solution was determined at 20^{0}C using Ostwald's viscometer. Time for the flow of the solvent and solution was recorded in triplicates. Density of solvent and solution were determined using a specific gravity bottle. Intrinsic viscosity was determined by plotting a graph with reduced viscosity versus concentration on y and x-axes respectively (Lundgren et al, 1965; Quagliano et al, 2001).

$$\text{Viscosity } \eta = \frac{\text{Density of solution}}{\text{Density of solvent}} \times \frac{\text{T1 solution}}{\text{T2 solvent}}$$

T1 and T2 are time in seconds.

Specific viscosity (η Sp) = η -1

M_V was calculated according to Mark – Houwink – Sakurada equation

$$\eta = K^1 (M_V)^a$$

$K^1 = 7.7X10^{-5}$dl/g and a = 0.82 for PHA

Film casting

Films were prepared by the solvent casting method (Savenkova et al, 2000a). A 2% solution of PHA in chloroform was prepared. Uniform, flat and scratchless glass plates were selected for casting. These plates were placed on a flat and levelled surface to get a film of uniform thickness. A spirit leveller was used to level the plates. The chloroform solution (80 - 100 ml) was poured on to plates (30 X 20 cm) and was left in a place without air turbulence for a minimum of 4h at room temperature. Care was taken not to disturb the plates during drying. After drying the films were peeled out of the plates.

Tensile strength

Tensile strength of PHA film was carried out according to ASTMD 882 using universal testing machine (Model Lx 5, LYOD ISNT). The test was performed in triplicates at room temperature. PHA strips of 1cm breadth and 10 cm length were cut from the films. Thickness of the film was measured using micrometer, model 549E (Testing machines inc., New York, USA) and was expressed in terms of gauge. The test specimens were placed in the grips of the test machine. The grip of the machine was tightened evenly so as to hold the filmstrip firmly during testing. The speed was set at the rate of 50 mm/min. As the test strip elongates the resistance of the strip increases and was detected by a load cell. The elongation of the strip was calculated as % elongation.

Tensile strength = Maximum load in N / cross section area in mm^2

% Elongation =extension (mm) / length of the sample in mm x 100

Water vapour Transmission rate (WVTR)

Water vapour transmission rate of PHA film was measured as per ASTM E 96-95 and carried out according to the desiccant method. Anhydrous calcium chloride was enclosed in aluminium dish, closed and the assembly was placed in a humidity chamber. The chamber was maintained at 38^{o}C and 90% RH. The initial weight was noted. Then the dish was covered with the test film (12-cm diameter) and sealed using paraffin wax. This assembly was placed in the humidity chamber maintained at 38^{o}C and 90% RH. The aluminium dish was

weighed at regular intervals of 60 min till a constant weight gain was achieved. Each time the assembly was cooled to room temperature before recording weight. Experiments were conducted in triplicates. The water vapour transmission rate was calculated using the following formula

WVTR = pick of moisture in grams per h / area of the specimen

Unit: g / m^2 / 24 h at 38^oC and 90% RH

Oxygen transmission rate (OTR)

Oxygen transmission rate of PHA film was measured as per ASTM D-1434-66. Experiments were conducted in triplicates. For oxygen transmission rate a permeability cell consisting of two stainless steel discs were used. The discs formed a cylindrical cavity when superimposed. The test film (9-cm diameter) was clamped between the two discs using 6 equally spaced bolts after placing 3 filter circles on the upper disc as support. A rubber gasket on it ensured a pressure tight fit. The cell was connected with a short plug of mercury contained in a capillary glass tube in a vertical position with an opening in the centre of the upper disc. Gas inlets and vent lines were provided on both sides of the cell. Oxygen was supplied at a constant rate, over atmospheric pressure to the bottom of the cell and the permeated gas was allowed to expand on the opposite side against atmospheric pressure. Pressure of oxygen was maintained at 20 psi. The upward displacement of mercury due to permeation of the gas through the film gives the rate of oxygen transmission. An electromechanical

vibration was used to avoid friction to the movement of mercury in the tube. The displacement was measured as a function of time and displacement of mercury versus time was plotted and the slope of straight line was obtained. OTR was calculated as follows

OTR = (VX6.566X10^{10}) / A X P in cc/ 24 h/ m^2/ atm^-

A = area of the test film, cm^2

P = test gas pressure differential, cm Hg

V = volume of the gas transmitted through the material (slope X a)

Slope = rate of rise in the capillary plug, cm/s. a = C/S area of capillary, cm^2.

5.2 RESULTS AND DISCUSSION

Characterization of PHA by UV spectroscopy

PHA can be converted quantitatively to crotonic acid by heating in concentrated sulfuric acid and the UV absorption maximum of crotonic acid is shifted to 235 nm when concentrated sulfuric acid is the solvent (Law and Slepecky, 1961) Carbonyl compounds absorb light below the ultra violet range and hence are difficult to detect by spectrophotometry. PHA gives rise to crotonic acid on dehydration or heating. Dehydration is done using concentrated sulfuric acid. The principle of UV absorption lies in the fact that the ultraviolet absorption maximum of α, β unsaturated acids undergoes a strong bathochromic shift or a shift to the lower frequency in sulfuric acid and can be recorded in the

UV range. The absorption maximum shifts to 235nm (Williamson and Wilkinson, 1958; Slepecky and Law, 1960). Here PHA can be detected even at 5 μg level. Fig 29 shows the UV graphs obtained. The purity of PHA obtained was observed by correlating the absorption of the compound and corresponding purity levels (μg) of standard PHB (Sigma). The purity of extracted sample was 96%. The results indicated the presence and levels of crotonic acid and confirmed the presence of polyhydroxybutyrate in the sample.

Characterization of PHA by Fourier transform infra red spectroscopy

Fourier transform infra red spectroscopy (FTIR) is a routine chemical technique used to study the molecular structure. It can be both qualitative as well as quantitative. Basically FTIR is used for determining the presence or absence of specific functional groups in a reaction mixture. Any compound with covalent bonds will absorb frequencies of electromagnetic radiation in the infrared region. In FTIR, as the temperature of a substance is raised it begins to emit radiant energy. The amount of emitted radiation forms a curve as a functional wavelength or frequency depending on the temperature of the substance and its emissivity. Organic substances exhibit characteristic group frequencies in infrared region. The absorption spectrum of a given mixture is generally additive i.e., the sum of the individual spectra of the components. The intensity of the absorption bands is related to the concentration of the substance

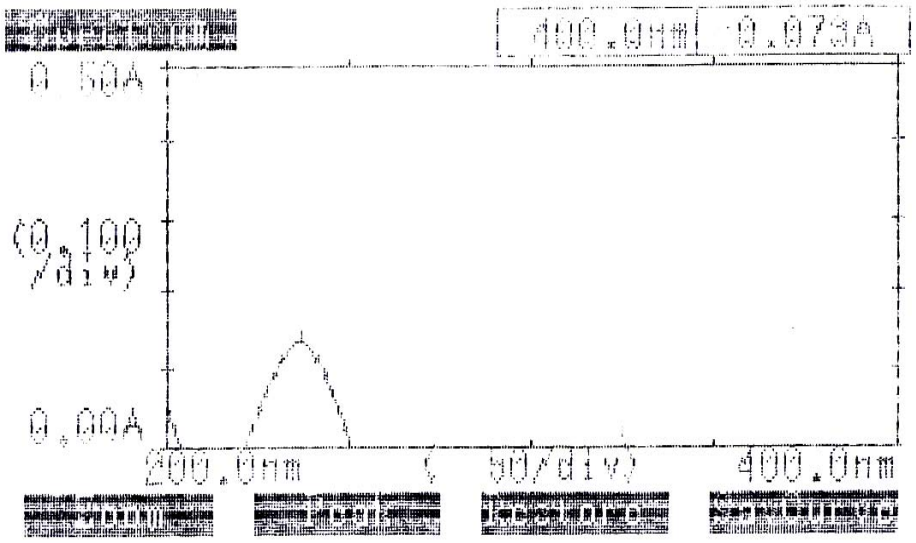


FIG 29: ULTRA VIOLET SPECTROSCOPY OF PHA SAMPLE EXTRACTED FROM *RHIZOBIUM MELILOTI* 14

that absorbs the incident radiation. By substituting a wide variety of materials of organic origin in the beam of the radiating material, it is readily observed that certain wavelengths are found to be associated with changes in the structures of the absorbing molecules. The resulting curves can be 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. These frequencies are also dependent upon the spacial interrelationship of the atoms contained in the molecular unit. Infrared spectra can thus provide a total simultaneous chemical analysis (Kaniz et al, 2000). Polyhydroxybutyrates as well as medium chain lengths PHAs have also been rapidly detected by FTIR (Hong et al 1999). The IR spectrum obtained for PHA samples from *Rhizobium meliloti* 14 is shown in Fig 30. The IR spectra obtained showed characteristic absorption bonds for esters and the presence of C=O and C-O were obtained at 1724 cm^{-1} and 1281 cm^{-1} respectively (Misra et al, 2000). Apart from this a peak at 1377 cm^{-1} was seen which is due to the CH_3 or methyl bending. Peaks due to methyl stretching were also observed at 2975 cm^{-1} and 2926 cm^{-1} . CH_2 or methylene group was observed at 1450 cm^{-1} and methine or CH peak was at 3434 cm^{-1} . An increase or shift to left or higher frequency of 1739 cm^{-1} was seen in some samples. This peak has been interpreted as to the presence of higher alkanoates. PHA_{MCL} at 1740 cm^{-1} , (HB + mcl HA) at 1732 cm^{-1} have been reported (Hong et al, 1999). But *Rhizobia* are known to accumulate only short chain length PHA such as

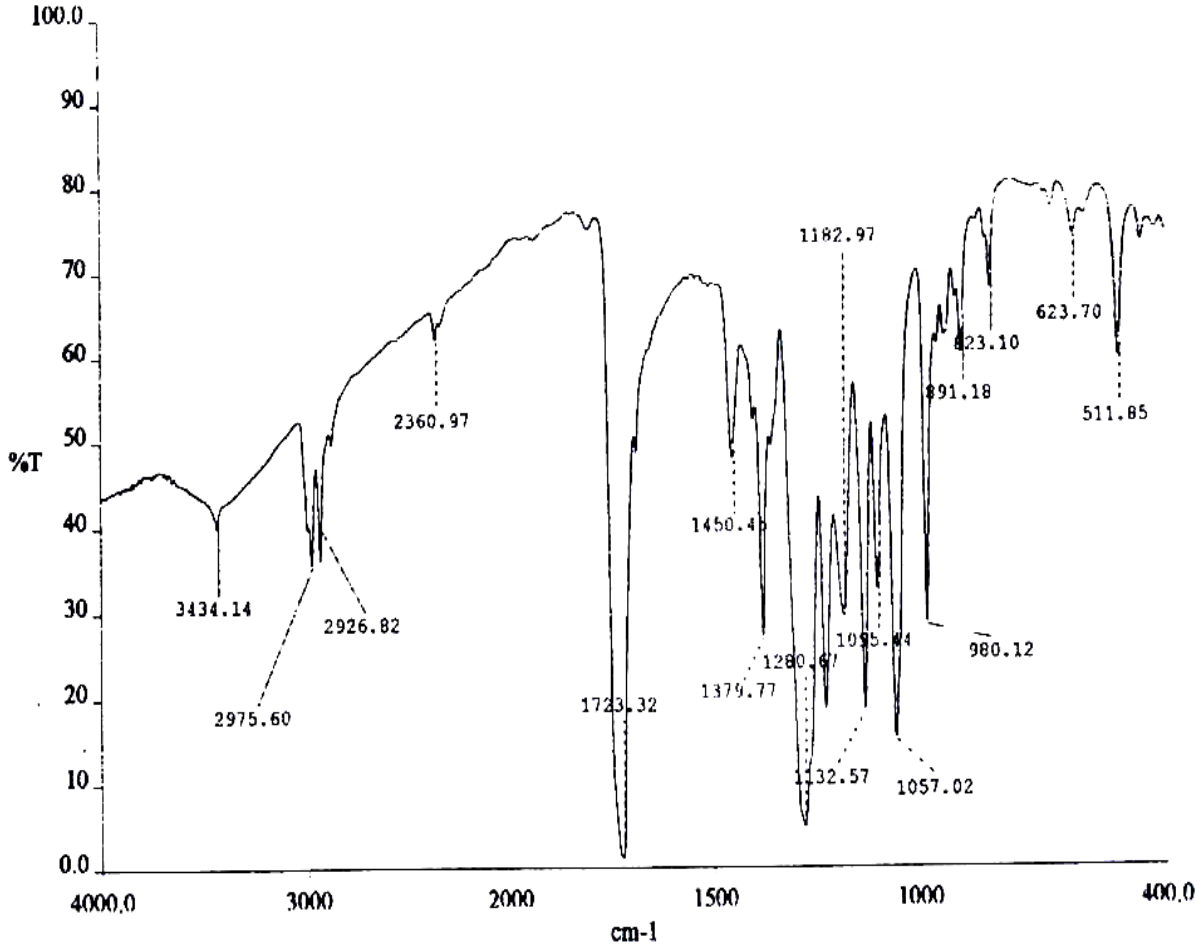


FIG 30: INFRARED SPECTROGRAPH OF PHA SAMPLE EXTRACTED FROM *RHIZOBIUM MELILOTI* 14

butyrate and valerate. Hence the shift could be because of a possible conjugation with the ester single-bonded oxygen. Such conjugation interferes with possible resonance with carbonyl group leading to an increase in absorption frequency for the C=O bond. This was seen along with decrease in the frequency of the C-O stretch to 1215 cm^{-1} . The presence of a strong peak at 2400 cm^{-1} was probably due to OH of carboxylic acid such as poly β–hydroxy butyric acid along with the ester in the sample. The shift to higher frequency was interpreted as said above, since further studies confirmed only the presence of PHB and PHV in the sample. Strong absorption was observed at 1720cm^{-1} due to C=O stretching vibration 1180 cm^{-1} characteristic of C-O stretch at 3000 cm^{-1} and 1460 cm^{-1} C-H stretching and bending respectively. A band at 1380 cm^{-1} showed the presence of methyl group. Intensity of absorption at 3000 cm^{-1} in relation to intensity at 1720 cm^{-1} was longer. Absorption at 3000 cm^{-1} indicates longer aliphatic chains. I R spectroscopy revealed the possible presence of a copolymer such as polyhydroxyvalerate in the sample.

Gas chromatography is a very efficient method for quantitative estimation as well as characterization of PHA. Fig 31 shows the GC graphs. GC confirmed the presence of another polymer or a copolymer, which eluted out at higher retention time. The butyrate methyl ester eluted at 9.5 min and valerate methyl ester at 12.81 min. GC also showed that the copolymer (hydroxyvalerate) was about 3% of the total polymer content.

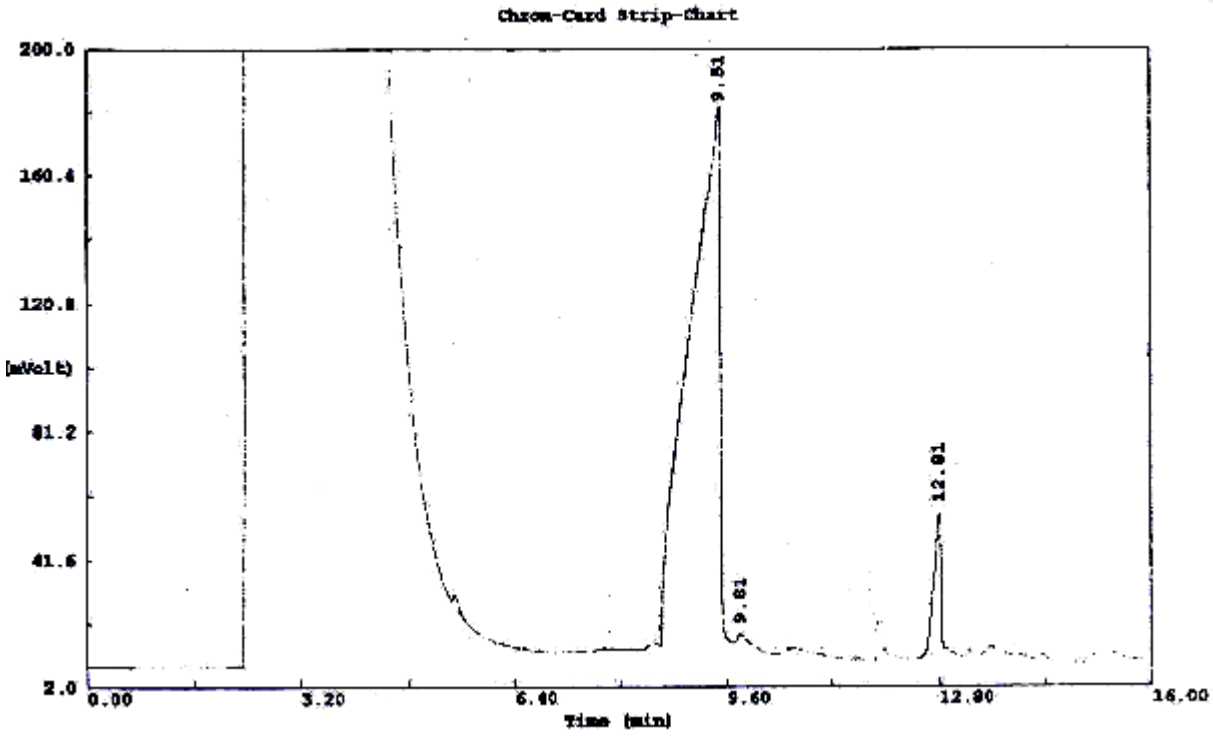


FIG 31: GAS CHROMATOGRAPHY OF PHA SAMPLE EXTRACTED FROM *RHIZOBIUM MELILOTI* 14

GCMS is a useful technique to determine and understand the molecular structure of any compound. The methyl esters obtained after methanolysis of the sample showed fragmentation patterns in GCMS that enabled to define the structure of PHA obtained from *Rhizobium meliloti* 14. The molecular fragments obtained are as shown in (Fig 32)

The major molecular fragmentation obtained were as follows m/e 117 ($C_5H_9O_3$+), m/e 103 ($C_4H_7O_3$+), m/e 87 ($C_4H_7O_2$+), m/e 74 ($C_3H_6O_2$+), m/e 61 ($C_2H_3O_2$+), m/e 43 (C_2H_3O), m/e 59 ($C_2H_3O_2$) (C_3H_7O+), m/e 75 ($C_3H_7O_2$+). The α-cleavage reaction resulted in the loss of the alkoxy group from the ester to corresponding acylium ion and was observed at m/e 43, m/e 71, m/e 85, a second useful peak observed from the loss of the alkyl group from the acyl portion of the ester molecule which appeared as m/e 59 and m/e 87, m/e 117 m/e103 (Eversloh et al, 2001; Findlay and White, 1983). The other fragment ions seen as a β-cleavage reaction to methyl esters (McLafferty rearrangement) was m/e 74. Other rearrangements of the alkyl positions of the molecule in which a hydrogen atom from the alkyl portion is transferred to the carbonyl oxygen of the acyl portion of the ester results in fragments of m/e 61, m/e 75. The results confirm that the PHA extracted from the sample contains 3-hydroxy functional group and the presence of methyl esters of hydroxy butyrate and valerate.

The peaks at m/e 103 and m/e 74 define the 3-hydroxy functional structure (Lee et al, 1995a). The molecular fragments of 3-hydroxy functional

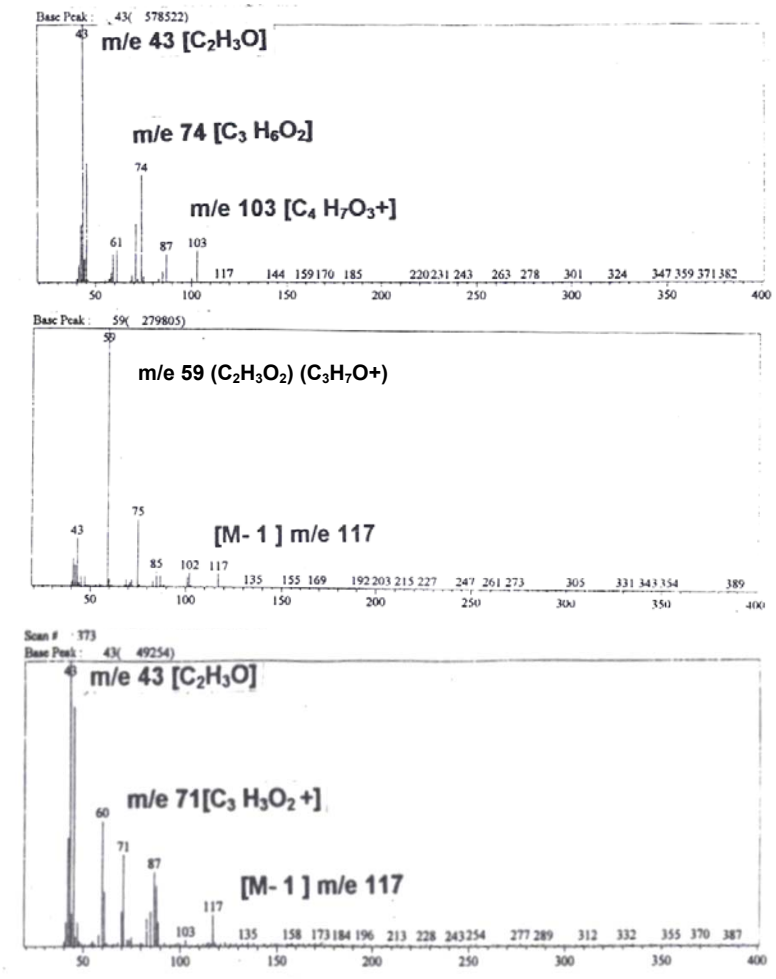


FIG 32: GC-MS OF PHA SAMPLE EXTRACTED FROM *RHIZOBIUM MELILOTI 14*

group are m/e 103, m/e 74, m/e 71, m/e 61, and m/e 43. The molecular ion-related peaks were rather weak because of high energy electron impart.

NMR spectroscopy is a valuable non-destructive method for monitoring polymer formation and degradation and has the advantages of accuracy, speed and sensitivity (Yan et al, 2000). NMR has been used to investigate various aspects of PHA like monomer composition, cellular content, conformational analysis, monomer linkage sequence, copolymer analysis and PHA metabolic pathway studies (Jacob et al, 1986). NMR has been used effectively to characterize the structure of polyhydroxyalkanoates (Choi and Yoon, 1994; Hori et al, 1994; Rodrigues et al, 1995; Matsusaki et al, 2000; Labuzek and Radecka 2001; Lee et al, 2001). The ^{1}H NMR spectrum of PHA (Fig 33) showed three groups of signals characteristic of PHB: a doublet at 1.29 ppm (B4) which is characteristic of methyl group, a doublet of a quadruplet at 2.5 ppm which is attributed to methylene group (B2) and a multiplet at 5.28 ppm characteristic of a methyne group (B3). A triplet at 0.9 ppm (V5) and a methylene resonance at 1.59 (V4) and methyne resonance at 5.5 (V3) indicated the presence of valerate in the polymer. This was comparable to reported data (Tombolini and Nuti, 1989; Tan et al, 1997) The concentration of HV was 3% which corresponds with the GC data. The intensity of carbonyl carbon (peak B1) resonance, methine (peak B3) carbon resonance, methyl carbon resonance (peak B4) and methylene carbon resonance (peak B2) are given in the spectrum (Fig 33 A). The absorbance in ppm is 169.143, 67.656, 40.864 and 19.787 for carbonyl, methine, methylene

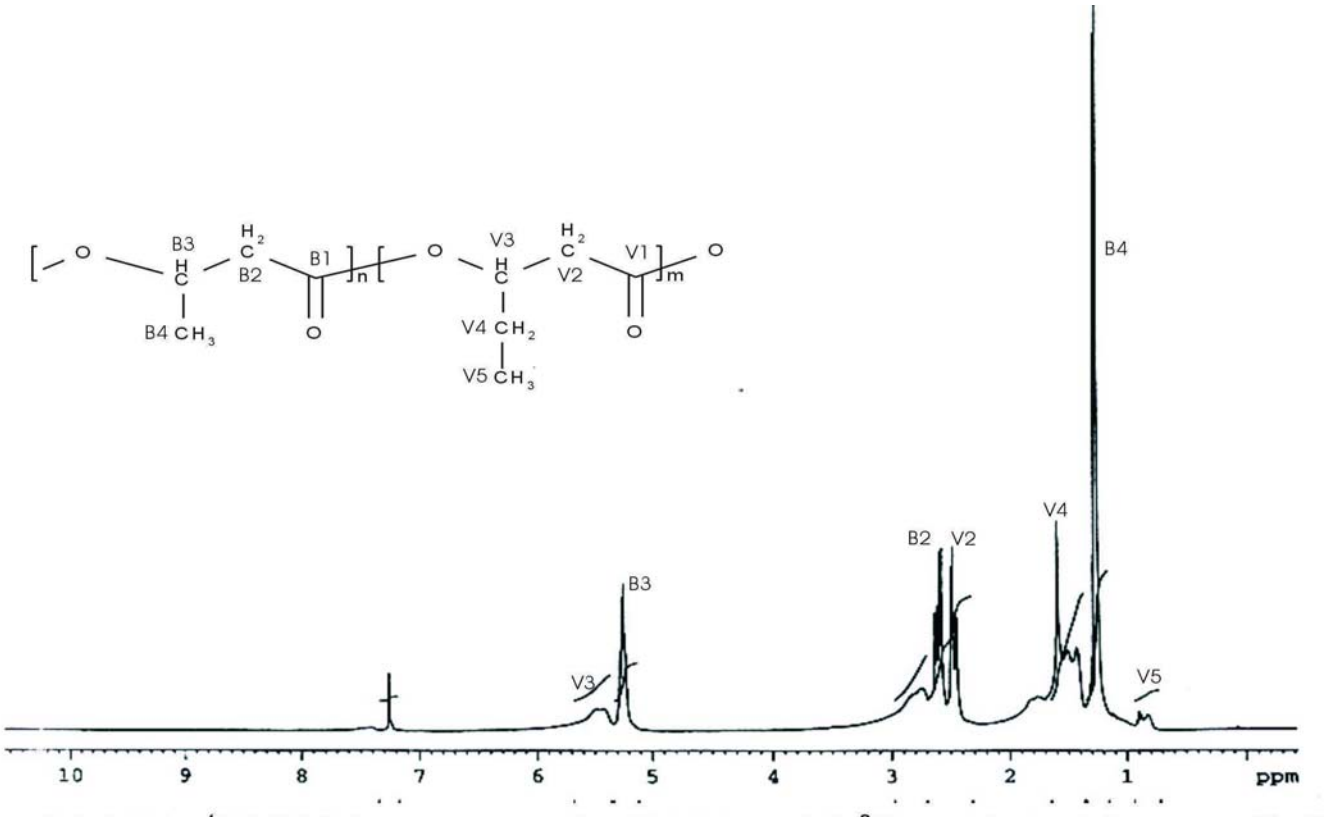


FIG 33: 400-MHZ ^{1}H NMR SPECTRUM IN $CDCL_3$ AT 30^{O}C IN *RHIZOBIUM MELILOTI* 14

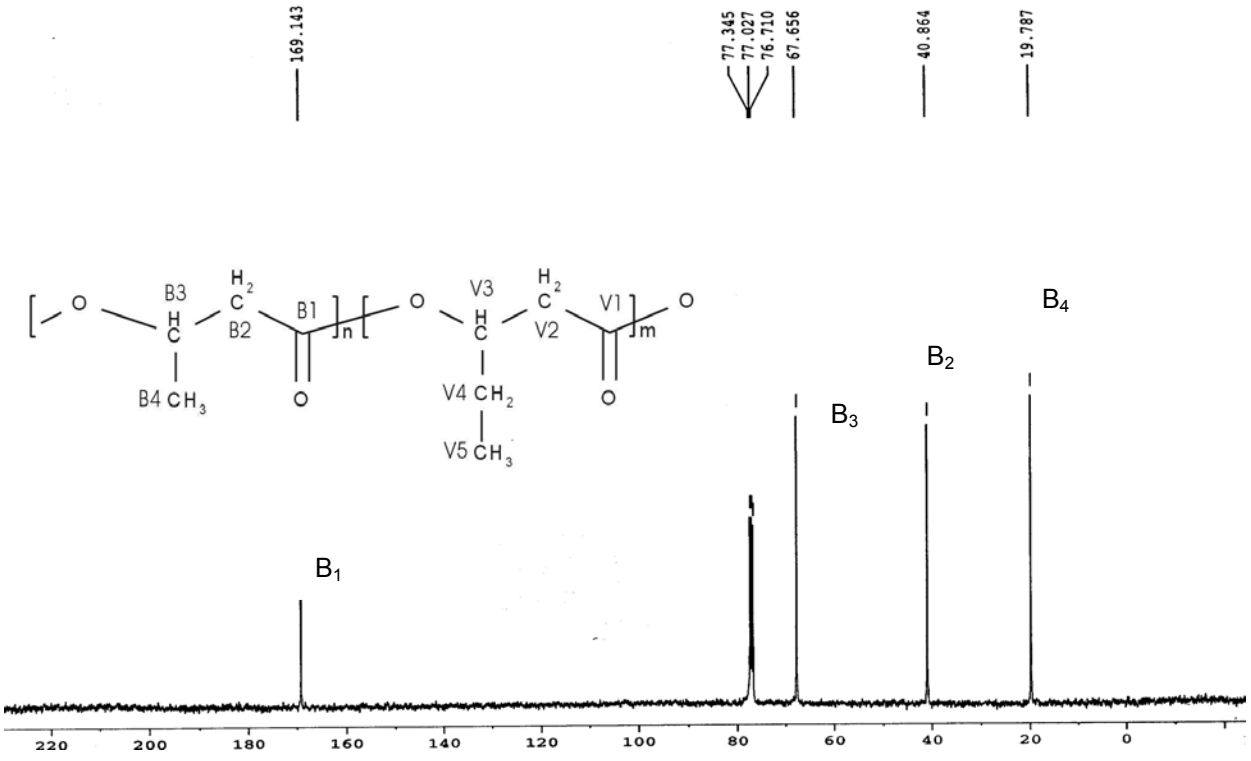


FIG 33A: 400-MHZ ^{13}C NMR SPECTRUM IN $CDCL_3$ AT 30^{O}C IN *RHIZOBIUM MELILOTI* 14

and methyl resonance respectively (Table 28). The data was comparable with reported values (Doi et al, 1986; Eversloh et al, 2001; Kamiya et al, 1989). The ^{1}H NMR results confirmed the presence of valerate in the polymer.

Studies on the DSC (Fig 34) gave details about the melting temperatures of the samples. This was compared with the temperatures of standard PHA, which confirms the presence of a copolymer. The standard P (HB-co-HV) with 5% valerate from Sigma showed a melting temperature (T_m) value of 160^{o}C and the standard PHB showed a peak value of 176^{o}C. PHA samples from *Rhizobium meliloti* 14 showed a peak value of 170^{o}C (Fig 34). PHB with 3 mol% HV is known to melt at 170^{o}C (Van der Walle et al, 2001). Melting temperature is an important feature since the temperature at which the polymer degrades has to be higher than the temperature at which it melts. Lower T_m will make the polymer better for a varied number of applications. Incorporation of the hydroxyvalerate monomer units into polyhydroxybutyrate will decrease the T_m of the polymer significantly, but there is no change in its thermal stability. This allows the thermal processing of the polymer as a melt without the risk of thermal degradation (Van der Walle et al, 2001). Hence a decrease of 6^{o}C in the melting temperature is of great significance. It also confirms the presence of valerate in the polymer extracted from *Rhizobium meliloti* 14. Also the PHA sample used for analysis was extracted from a simple fermentation medium containing sucrose as a carbon source. The molecular weight of PHAs depends on the organism, source of carbon and also the down stream processing.

TABLE 28: CHEMICAL SHIFTS OF CARBON ATOMS IN ^{13}C NMR

Carbon atom	Absorption (ppm) of PHA	
	Std PHA	***R meliloti* 14**
Carbonyl carbon	169.134	169.143
Methine carbon	67.654	67.656
Methylene carbon	40.869	40.864
Methyl carbon	19.787	19.787

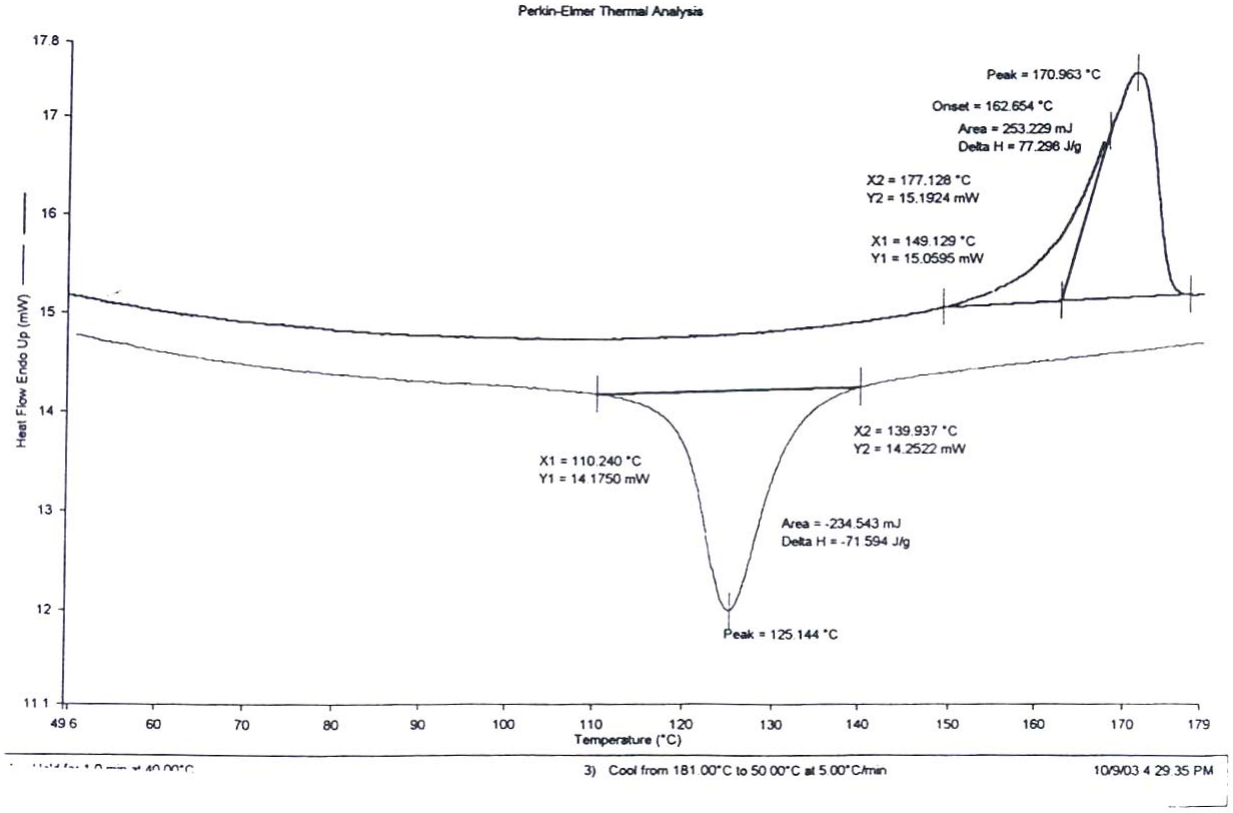


FIG 34: DIFFERENTIAL SCANNING CALORIMETRY SHOWING MELTING TEMPERATURE OF PHA SAMPLE EXTRACTED FROM *RHIZOBIUM MELILOTI* 14

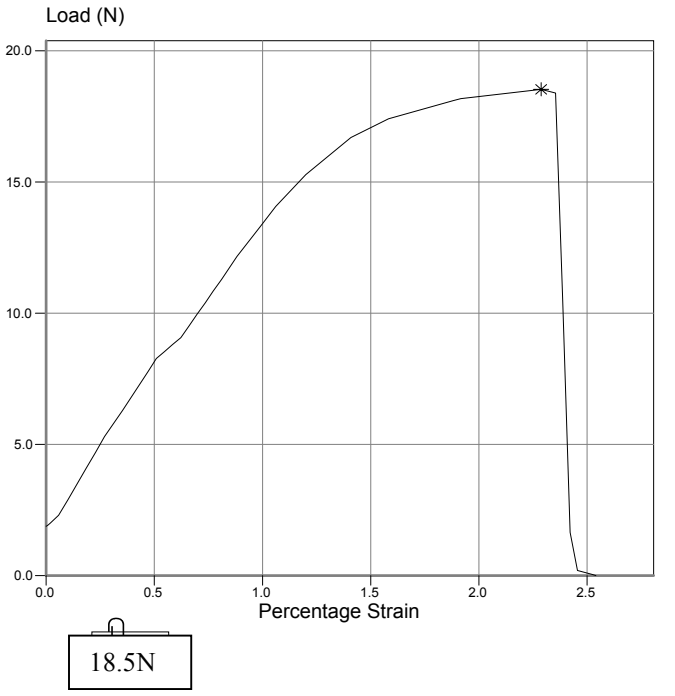
Molecular weight was measured by viscometry and the viscosity average molecular weight of the polymer was 5.63 x 10^5. The intrinsic viscosity was 4dl/g.

The solution of the polymer was casted on to clean glass plates. Clear and transparent films could be easily peeled off from the glass plate (Plate 11). Preliminary experiments also showed that the polymer could be heat-sealed. These films were used for tensile strength, water vapour permeability and oxygen transmission rate studies.

Tensile strength is a measure to what extent the material stretches before breaking. The solution cast films of PHA extracted from *Rhizobium meliloti* 14 were examined for its tensile strength and elongation to break. Tensile strength of the film was 36.9 MPa and was comparable to polypropylene (38MPa) when the film could bear a load of 16.9 N. and The maximum load taken was 18.5 N and the tensile strength at this load was 41.1 Mpa (Fig 35). The extension to break (%) was 1.29%.

The water vapour permeability of the films was 30 g/m^2/day and oxygen transmission rate was calculated as 476.37 (cc/m^2 /day /atm 65% Rh and 27^{o}C). Relatively low oxygen diffusivity has been reported for PHB films and this property is considered important for the film to be used as a food packaging material (Eggink et al, 1994). The low water permeability is significant for applications of this film as a packaging material.

PLATE 11: FILM FROM PHA EXTRACTED FROM *RHIZOBIUM MELILOTI* 14



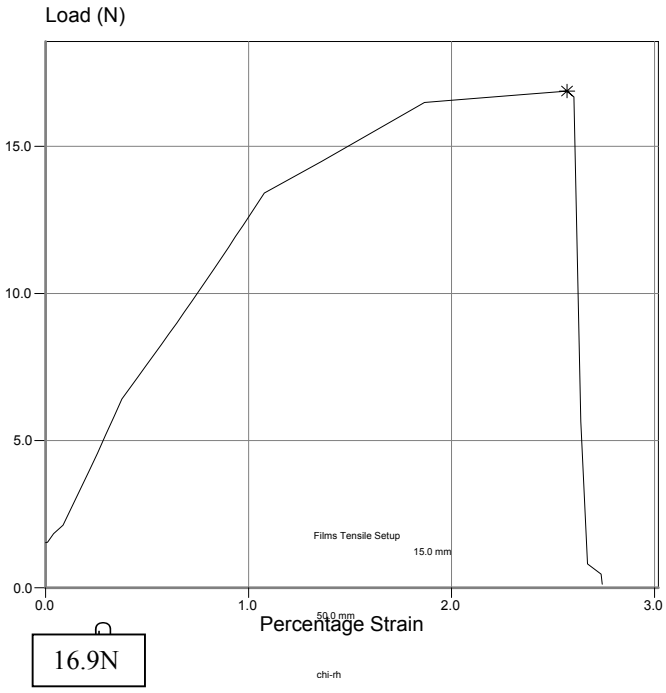


FIG 35: TENSILE STRENGTH OF PHA FILMS FROM *RHIZOBIUM MELILOTI* 14

Synthesis of PHA in the *Rhizobium meliloti* 22 (mutant strain)

It has been reported that the structure and composition of polymer produced in bacteria is generally dependent upon the type and concentration of carbon substrates used in the culture medium (different type of copolymers are produced by the co feeding C substrates in various combinations). In the present experiment analysis of the polymer produced by *Rhizobium meliloti 22* by GC DSC and NMR (Fig 36) indicted that the bacterium can synthesize poly-β-hydroxy butyrate –covalerate in the presence of sucrose alone and the concentration of HV was about 3.11 mol%. No noticeable change in the polymer composition could be found between parent and the mutant strain (*Rhizobium meliloti* 22). DSC studies of the polymer extracted from *Rhizobium meliloti* 22 however, showed two shoulder peaks showing a melting temperature of 165.37^{o}C and 167.53^{o}C apart from the major peak at 169^{o}C (Fig 36).

5.3 CONCLUSION

The polymer extracted from *Rhizobium meliloti* 14 was characterized as polyhydroxybutyrate with 3% valerate. The melting temperature of the polymer was 170^{o}C. A significant decrease of 6^{o}C was observed when compared to polyhydroxybutyrate sample obtained from Sigma. Decrease in the melting temperature is significant, as it is required for the thermal processing of the polymer. The viscosity average molecular weight of the sample was 5.63X10^{5}. The extracted polymer could be easily casted into films. The solution cast films

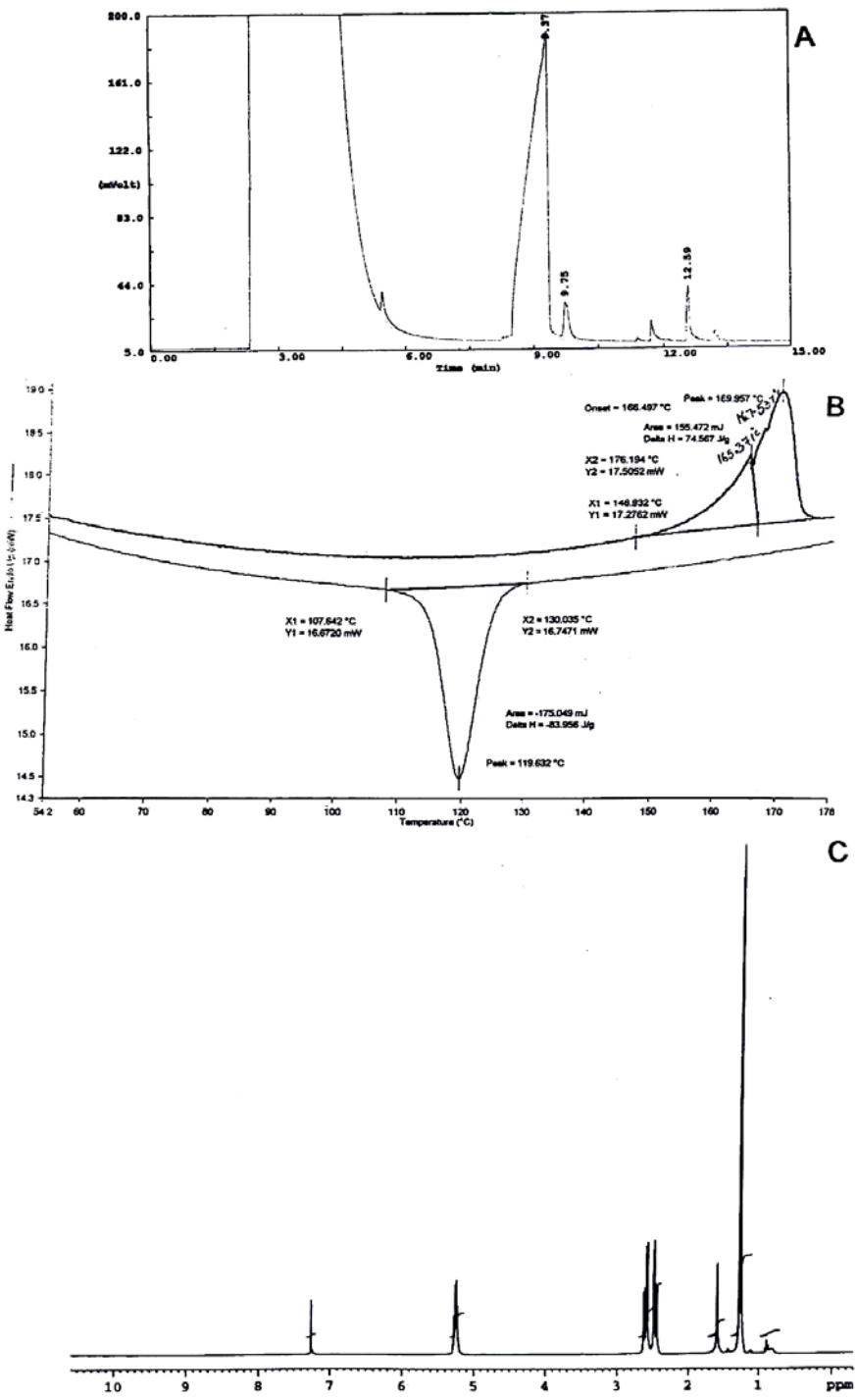


FIG 36: GC (A), DSC (B) AND NMR (C) ANALYSIS OF PHA FROM *RHIZOBIUM MELILOTI* 22

could be air-dried and they peeled off the glass plates into thin transparent clear films. The tensile strength of the film was comparable to polypropylene. Tensile strength of the films ranged between 36.9 MPa and 41.1 MPa and it could take a load of 18.5 N. WVTR and OTR of the film was 30 g/m^2/day and 76.37 (cc/m^2 /day /atm 65% Rh and 27^oC) respectively. In conclusion, the polymer obtained from *Rhizobium meliloti* 14 can be efficiently developed into a packaging film. Further, inputs based on knowledge of polymer chemistry and food packaging can improve the film and can be developed as a food packaging material.

Chapter 6

Production and Characterization of Copolymers

Introduc

6.0 INTRODUCTION

There are two important factors limiting the commercialization of polyhydroxyalkanoates. The major limiting factor is the cost of the carbon substrate utilized for PHA production. It has been calculated that 3 tonnes of glucose is required to get 1 tonne of PHA (Page, 1992). Second limiting factor is the material property of the polymer produced. Although the P (3HB) homopolymer triggered the commercial interest in PHA, naturally occurring P (3HB) does not have sufficient material properties for practical application (Tsuge, 2002). PHB homopolymer is highly crystalline and stiff material. It is brittle and has poor elastic properties.

On the contrary the copolymers have better advantage over PHB. The control of copolymer is important because the physical and mechanical material properties of the copolymer depend on the fraction of the 3-hydroxyvalerate. Shampoo bottles made from Biopol had a cap made of copolymer with low mol% of HV (~ 5%) for purposes of rigidity and to accommodate the thread and form a suitable closure. In contrast, 10 to 20 mol% HV was used for the shampoo bottle body to make it durable and flexible (Aldor and Keasling, 2001). It was seen that *Rhizobium meliloti* 14 produced PHB-co-HV in very low quantities in the presence of sucrose. Hence attempts were made to improve copolymer production.

6.1 MEDIA AND METHODS

Various carbon substrates were used as co-substrates in the PHA production medium containing sucrose as main carbon source for PHA copolymer production. All the experiments were carried out for 72 h in shake flasks at neutral pH and 200 rpm in PHA production medium described in page 109.

Oils and fats

i. Rice bran oil, pongamia oil, mustard seed oil, soy oil, palm oil, groundnut oil, castor oil and ghee were procured from local market and used as additional carbon sources at a concentration of 2-5 g/l in the PHA production medium containing 15-20 g/l of sucrose.

Fatty acids and organic acids

i. Fatty acids such as stearic acid, palmitic acid, adipic acid, octanoic acid and glycerol were used in the media as carbon sources at 2 g/l.

ii. Fatty acids such as stearic acid, palmitic acid, oleic acid and linoleic acid were added at 3 g/l after 36 h of fermentation. Sucrose in the medium was initially 15 g/l. Another 5 g /l of sucrose was added later along with the acids after 36 h of fermentation.

iii. 1,1.5 and 2 g/l of sodium propionate was used as sole carbon source in PHA production medium. 1.5 g/l of sodium propionate was used as additional carbon source along with 20 g/l of sucrose.

iv. Organic acids such as sodium butyrate, malic acid, pyruvic acid, citric acid, succinic acid and acetic acid were used at 20-g/l concentration. Initially the organic acids were added at 5 g/l and 15 g/l was added after 36 h of growth. 15 g/l of sucrose used at 0 hour and 5g/l was added after 36 hours.

v. 2,4,6,8 and 10 g/l of succinate was used along with sucrose (20 g/l) in PHA production medium.

Estimation of PHA

Cells were harvested after fermentation and PHA was estimated by hypochlorite method and by gas chromatography as described earlier (Material and Methods, page 103 and Chapter 5, page 232)

6.2 RESULTS AND DISCUSSION

Use of economic substrates and additional carbon sources were analyzed based on two aspects. Firstly the capability of the organism to utilize the carbon source and production of PHA and secondly production of a copolymer such as valerate by the organism as a result of incorporation of the co-carbon source. Keeping these factors in view various oils, fats, fatty acids, organic acids and other economic substrates were used as carbon source for enhancement of copolymer production by *Rhizobium meliloti* 14.

Effect of sucrose on copolymer production by *Rhizobium meliloti* 14

It was confirmed that *Rhizobium meliloti* 14 produced about 3% of HV when grown in the presence of sucrose as the sole carbon source (Fig 31, Chapter 5). No additional precursors of HV had to be added. Biosynthesis of PHB-Co-HV from structurally unrelated carbon sources such as glucose and fructose have been reported (Lee et al, 1995a). It is known that succinate an intermediate of the TCA cycle, is abstracted and then converted to propionyl-CoA via methyl malonyl-CoA pathway by reactions catalyzed by methylmalonyl-COA mutase and methylmalonyl-COA decarboxylase or via methyl malonyl-CoA: oxaloacetate transcarboxylase. Succinyl-Co A is also converted to propionyl-CoA via succinyl-CoA decrboxylase (Fig 5c). Alternatively it can be hypothesized that HV production in *Rhizobium meliloti* 14 was from the amino acids. It was observed that the final pH of the culture after fermentation was usually 5. This

could be due to the excretion of amino acids by *Rhizobium meliloti* 14 into the broth. Excretion of alanine, glutamic acid, aspartic acid, 2-oxoglutarate and γ-hydroxybutyrate into the medium has been reported in *Rhizobium etli* (Encarnacion et al, 1995). It is reported that addition of amino acids such as threonine, isoleucine and valine into culture medium increased the molar fraction of 3-hydroxyvalerate in *Alcaligenes* SH-69 (Yoon et al, 1995). Propionyl CoA was synthesized by the cells themselves from intermediates of amino acid metabolism. There is a possibility of excretion of some of the amino acids essential for PHA production by *Rhizobium meliloti* 14, which may be further, assimilated to yield a copolymer. However the cause for acidic pH in the fermentation broth was not examined. Since there is a considerable amount of copolymer production in *Rhizobium meliloti* 14 in the absence of any other precursor carbon substrate, the synthesis can be explained based on the above facts.

Effect of oils and fats on PHA production

Various oils and fats were used as sole carbon sources (2-5 g/l) in the PHA production media for enhancement of copolymer production. Biomass levels of *Rhizobium meliloti* 14 were highest with mustard seed oil (0.39 g/l) and the lowest was with soy oil (0.32 g/l). Ghee (fat) was used as sole carbon source and the PHA yield was 0.11g/l. However it was observed that the use of oils as sole carbon sources was not significant in terms of productivity (Fig 37). Hence oils

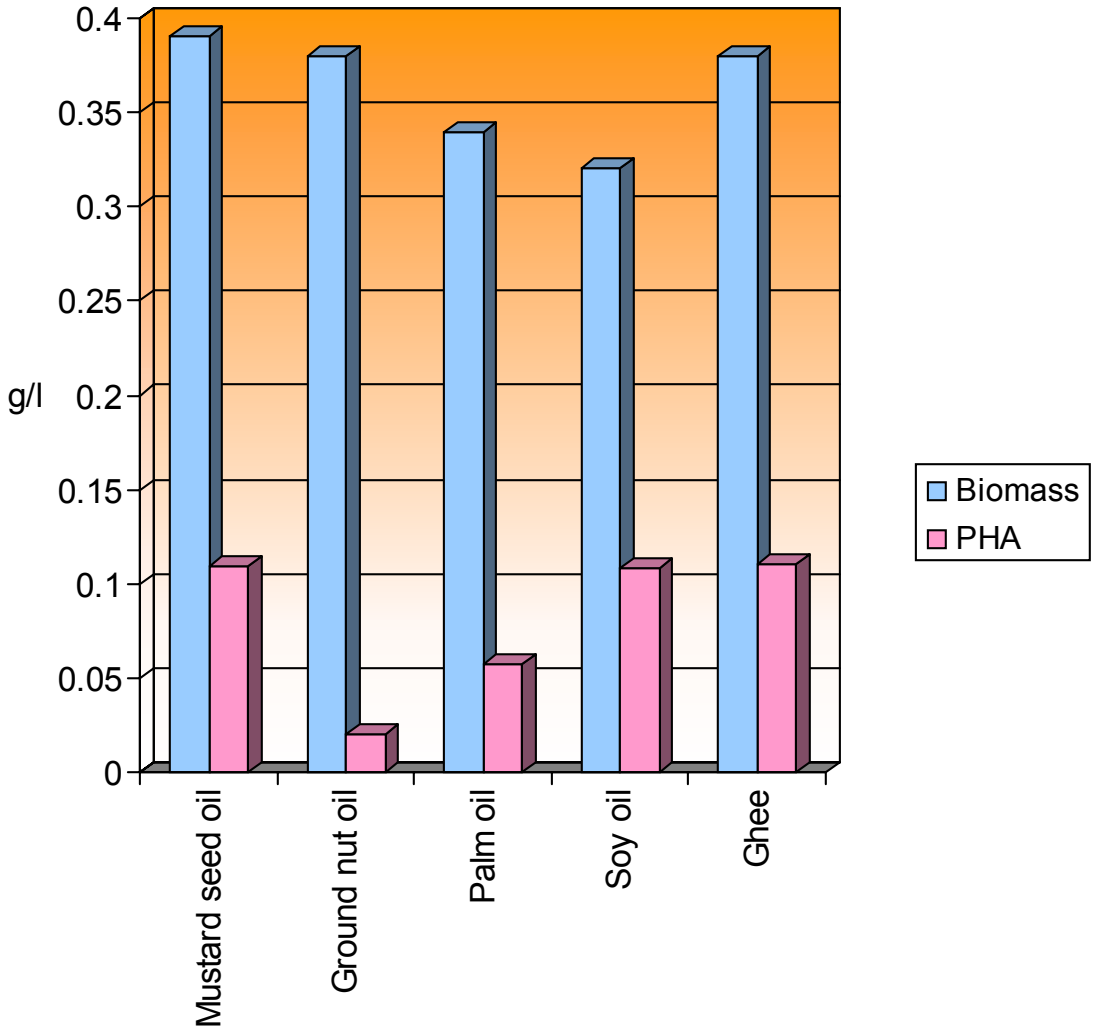


FIG 37: EFFECT OF OILS AND FATS AS CARBON SOURCES ON GROWTH AND PHA PRODUCTION OF *RHIZOBIUM MELILOTI* 14

were used at 2g/l (wt / v) levels along with 20 g/l of sucrose in the medium. This increased the biomass and PHA levels significantly (Fig 38). Highest PHA yields were obtained with rice bran oil (3.6 g/l), pongamia oil (2.4 g/l) and groundnut oil (1.8 g/l). Highest biomass was obtained with rice bran oil (4.1 g/l), pongamia oil (3.5 g/l) and palm oil (3.0 g/l). Gas chromatographic analysis of the PHA samples of the culture grown on rice bran oil, pongamia oil, groundnut oil and palm oil showed PHB: PHV ratio of 81: 19, 90: 10, 95: 5 and 95: 5 respectively (Fig 39). PHA yield in rice bran medium was 69.7% of the biomass. The highest HV content was obtained when rice bran oil was used as additional carbon source. Rice bran oil is also an economic substrate for PHA production. Rice bran contains 65-80% of triglycerides and, 3-10% of free fatty acids and 15-45% of diglcerides and monoglycerides. Rice bran is produced at 5,00,000 tons annually in India. Hence it is also an economical substrate.

Plant oils and animal fats have been used as substrates for polyester production. It s been reported that soybean oil and palm oil yield a PHB content of 33% and 40% respectively in Alcaligenes. The polyester produced in oils and fats did not yield any copolymer in Alcaligenes (Akiyama et al, 1992). But *Rhizobium meliloti* 14 was able to utilize these substrates and produce copolymers. Plant oils are reported as poor supporters of bacterial growth. The highest PHA content of 50% in large-scale production has been reported in *Aeromonas hydrophilla* (Tsuge, 2002). *Rhizobium meliloti* 14 did not utilize oils as sole carbon sources for copolymer production. This could be because of low

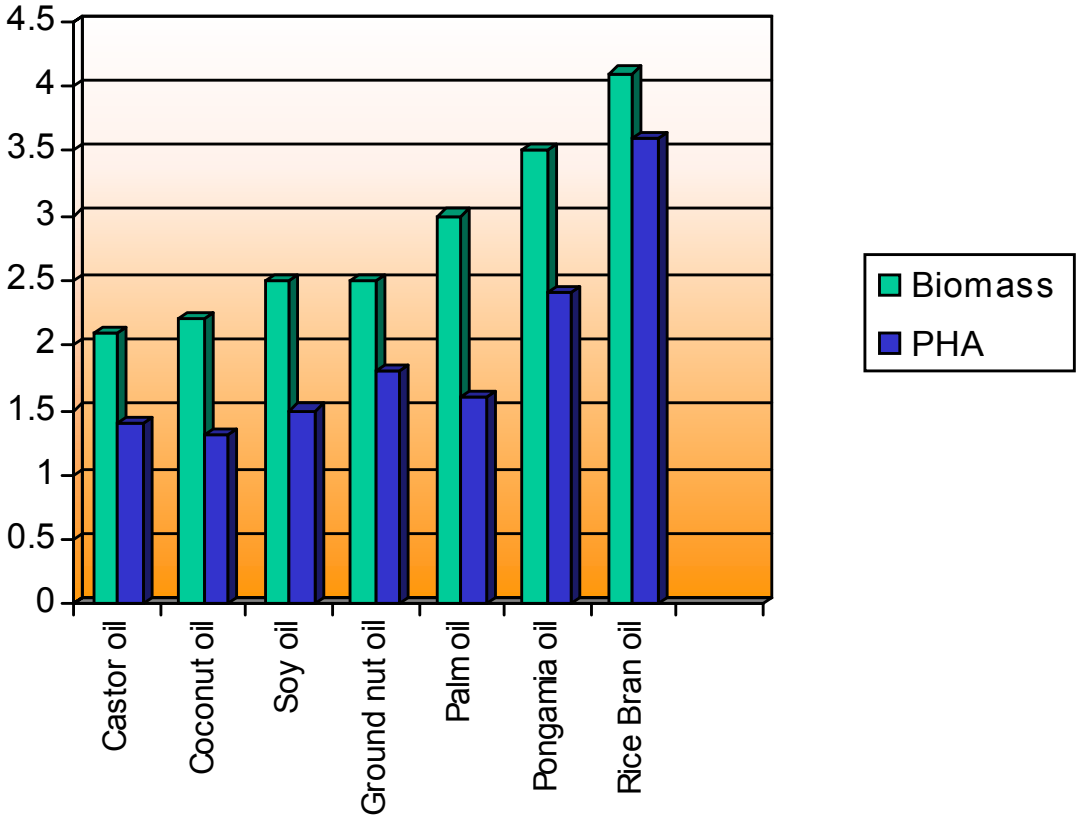


FIG 38: EFFECT OF ADDITIONAL CARBON SOURCE SUCH AS OILS (2 g/l) ALONG WITH SUCROSE (20 g/l) ON GROWTH AND PHA PRODUCTION OF *RHIZOBIUM MELILOTI* 14

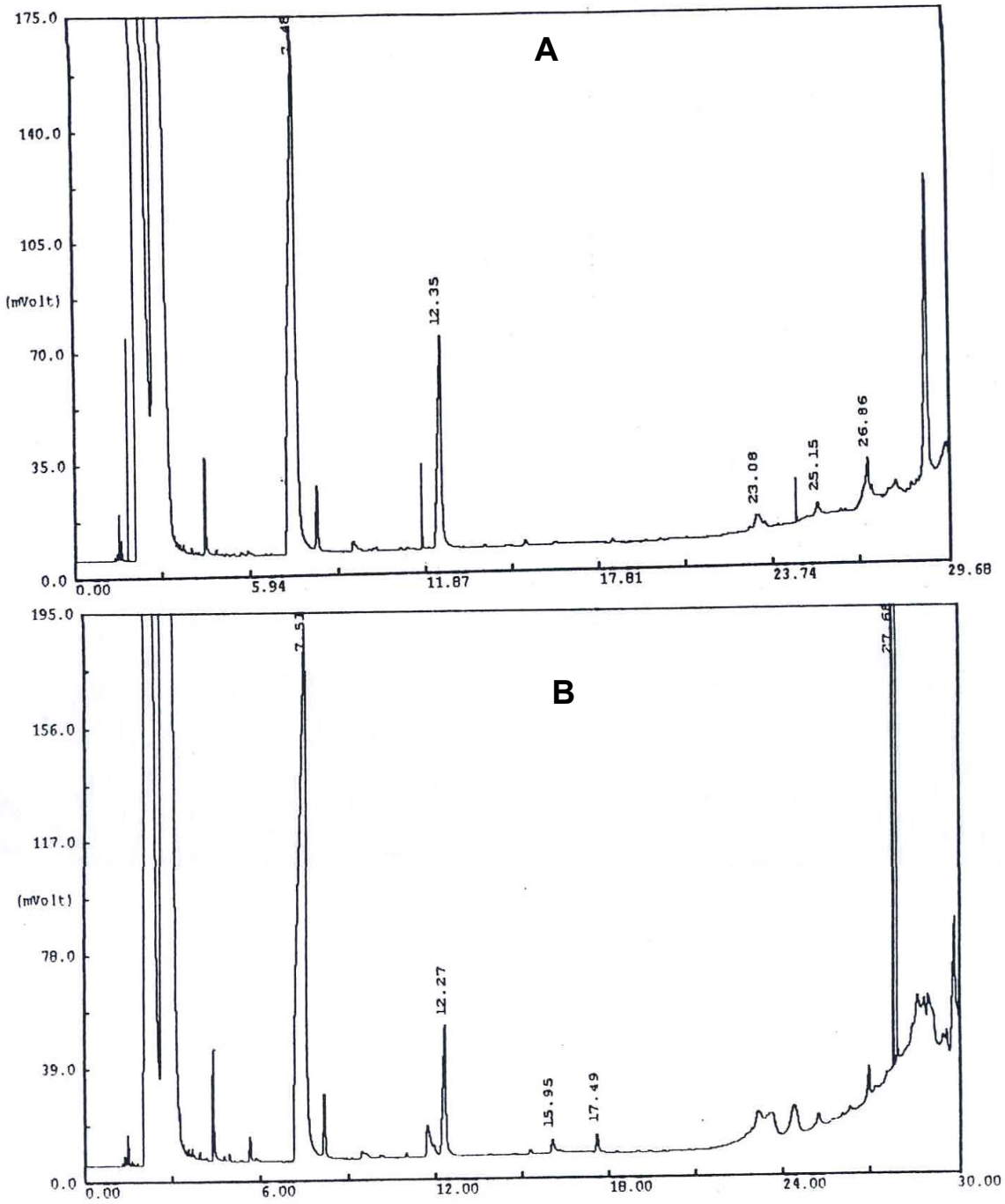


FIG 39: GAS CHROMATOGRAPHY OF PHA SAMPLES EXTRACTED FROM *RHIZOBIUM MELILOTI* 14 GROWN ON RICE BRAN OIL (A), PONGAMIA OIL (B).

uptake of these oils in the organism. The biomass levels were very low and hence no significant accumulation of PHA was seen. But the yields were significant when these oils were used as additional carbon sources along with sucrose (Fig 38). Ricinoleic acid, which is the major constituent of castor oil, contains 12-hydroxystearic acid (C_{18}). PHA yield was 66% (dry wt of biomass) when *Rhizobium meliloti* 14 was grown on castor oil as an additional substrate and the HV content was 2%. Biosynthetic pathway from 12-hydroxystearate has been proposed (Akiyama and Doi, 1993). However since *Rhizobium meliloti* 14 produces nearly 3% HV in sucrose grown medium the HV content in castor oil grown cells was not of much signiificance. The levels of PHA accumulation reached 72% (dry wt of biomass) when groundnut oil was used as an additional carbon source. It was observed that copolymers of hydroxyvalerate could be produced in *Rhizobium meliloti* 14, when substituted with fatty acids containing even numbered carbon atoms.

Fatty acids and organic acids

Fatty acids such as stearic acid, palmitic acid, octanoic acid, adipic acid and glycerol were used as sole carbon sources for PHA production in *Rhizobium meliloti* 14. The organism did not grow when octonoic acid and adipic acid were used. The pH levels reached 8.5 when adipic acid was used in the medium. Growth in stearic acid, palmitic acid, and glycerol was comparably better with 0.55 g/l, 0.31g/l and 0.2 g/l of biomass respectively. Yields of PHA were significant in stearic acid with 0.30 g/l and PHB: PHV was 98:2. There was no

polymer production when glycerol was used. Glycerol is known to support growth in *Rhizobium* (Stowers, 1985). Glycerol metabolism yields pyruvate, which is used via the TCA cycle. When glycerol was used as sole carbon source most of the carbon is utilised for biomass and hence no PHA accumulation was seen. Use of fatty acids as additional carbon sources along with sucrose increased the biomass and PHA levels (Table 29). The highest PHA yield was obtained with stearic acid 3.2 g/l. But the percentage of valerate in the polymer remained at 2%. Use of linoeliec acid in the medium resulted in 3% of valerate. Stearic acid has to be degraded via the β-oxidation pathway resulting in metabolites with fewer carbons and this has to become the precursors for PHA. However, comparably low amounts of valerate were seen in stearic acid grown cells with respect to the sucrose grown control.

Various organic acids were used as sole carbon sources. Propionate (sodium propionate), butyrate (sodium butyrate), pyruvic, succinic, malic, fumaric and citric acid were used in the medium. Propionate is known to be the precursor of copolymers such as hydroxyvalerate (Lee et al, 1994; Lee et al, 2000; Kobayashi et al, 2000). Propionic acid was used as a sole carbon source at 1, 1.5 and 2-g/l level in the medium (Fig 40). Growth of the organism was low and the PHA contents decreased with increase in propionic acid in the medium. To date, most attempts at producing PHB –co- HV copolymer with different compositions in Recombinant *E coli* and *Ralstonia eutropha* have used

TABLE 29: EFFECT OF FATTY ACIDS ON GROWTH AND PHA PRODUCTION IN *RHIZOBIUM MELILOTI* 14

Fatty acids (3g/l) + Sucrose (20 g/l)	Biomass (g/l)	PHA (g/l)	PHB : PHV
Stearic acid	5.3	3.286	98:2
Oleic acid	4.2	1.05	
Palmitic acid	4.9	2.891	99:1
Linoleic acid	3.5	0.735	97:3
Octanioic acid	-	-	-

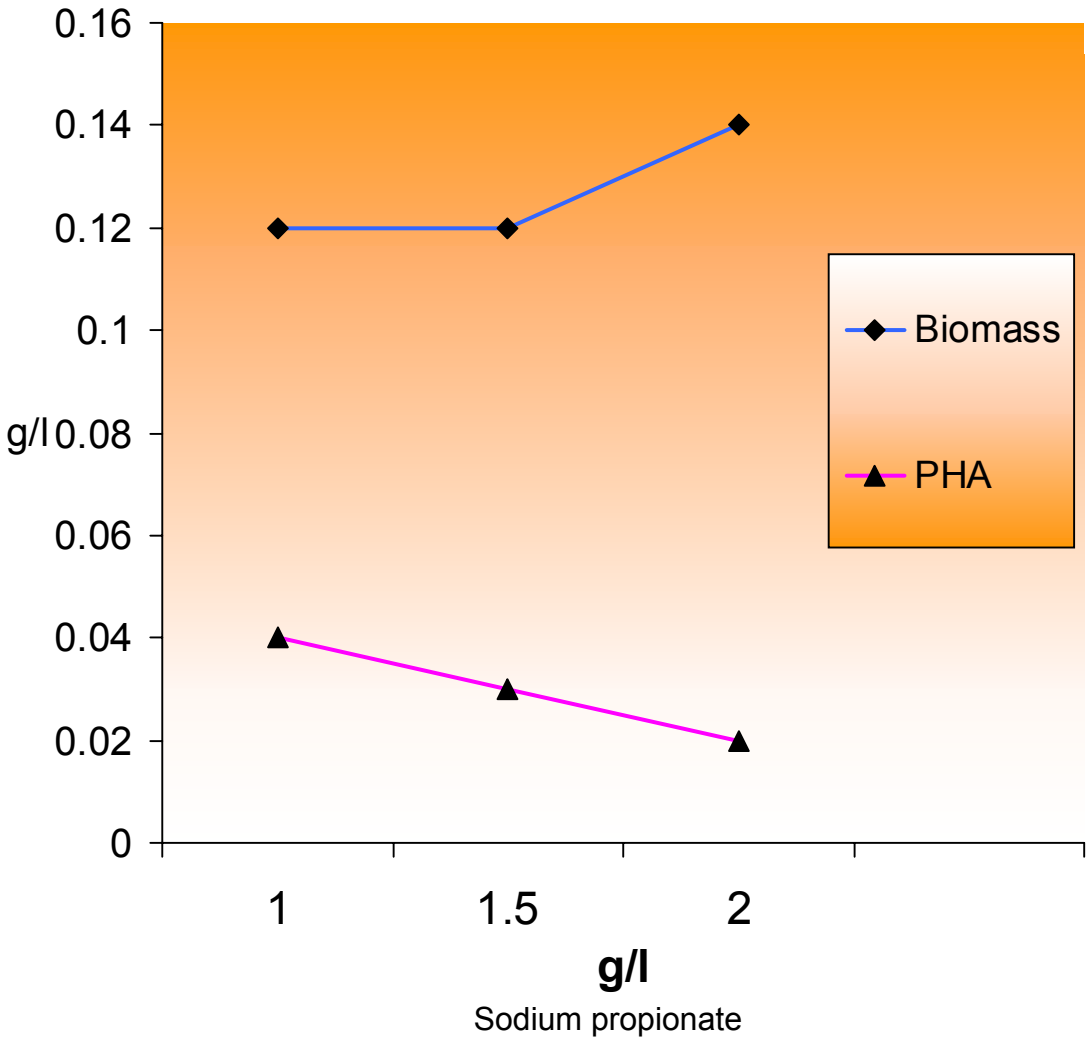


FIG 40: EFFECT OF SODIUM PROPIONATE ON PHA PRODUCTION AND GROWTH OF *RHIZOBIUM MELILOTI* 14

the strategy of varying propionate concentration to vary the HV fraction. Organic acids at higher concentrations inhibit growth of the organism. Inhibition of propionic acid (Page et al, 1992a) is known to be stronger than acetic, butyric valeric and lactic acid (Kobayashi et al, 2000). The toxicity of the volatile acids is attributed to their undissociated lipophilic molecules that penetrate freely the cell membrane, dissociate and acidify the cytoplasm. As a result, the gradient of protons through the membrane cannot be maintained, and the production of energy and transport system dependent on the gradient are decoupled. The dissociation also induces an anion accumulation, resulting in increased internal osmotic pressure of cells. In response to the accumulation of fatty acids microorganisms release free energy via ATPase and expel protons out of cells in order to maintain the proton gradient. This results in overall decline of microbial activity including acid utilization rate, growth rate and yield (Yu et al, 2002). Hence acids were used along with sucrose as additional carbon sources in the medium. PHA yield when propionic acid was supplemented with sucrose was 4.3 g/l which was similar to the control levels. However, the biomass levels in the propionate supplemented medium was higher (6.5 g/l) (Fig 41). The HB: HV content in the polymer was 95.2: 4.7 as analyzed by gas chromatography.

Various organic acids such as sodium butyrate, pyruvic, citric, succinic, fumaric and malic acid were used as both additional and sole carbon sources and their importance in copolymer production was studied. The yields of PHA were 72%, 73% and 74% when pyruvic, succinic and acetate were used. Gas

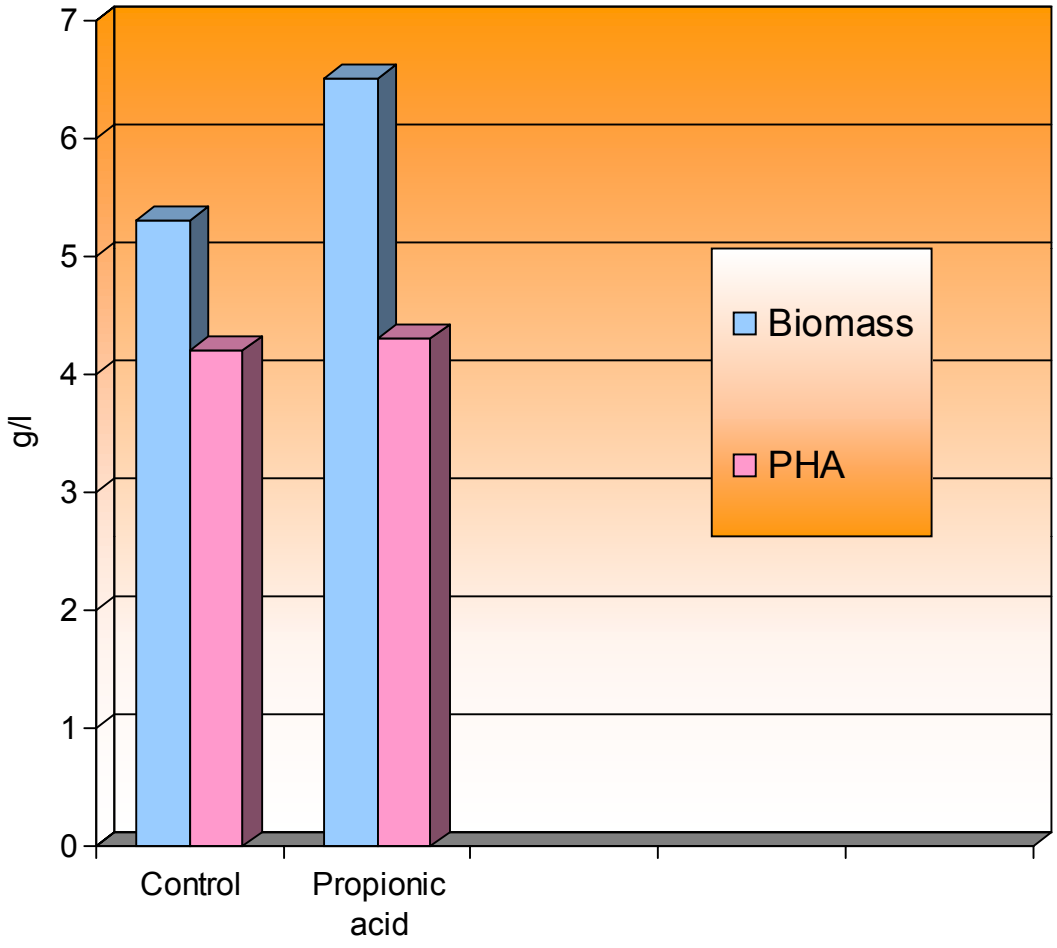


FIG 41: EFFECT OF PROPIONIC ACID AS AN ADDITIONAL CARBON ON GROWTH AND PHA PRODUCTION IN *RHIZOBIUM MELILOTI* 14.

chromatography studies showed that production of highest HV (12%) (Fig 42) content was seen when pyruvic acid was used (Table 30). Pyruvic acid is the precursor for acetyl-CoA and an increase in acetyl-CoA will favour PHA synthesis. Addition of pyruvate as carbon source might limit the availability of reducing equivalents for the TCA cycle. This might promote the cell to synthesise PHA. Excess of acetyl-CoA also favours the fatty acid synthesis pathway via malonyl-CoA, which might lead to the accumulation of hydroxyvaleryl-CoA and hydroxyvalerate in the cell. Supplementing sodium butyrate also increased the HV content. GC analysis of the PHA samples extracted from cells grown on sodium butyrate showed 5.3 mol % of HV.

Succinate is known to stimulate growth in *Rhizobium* (Stowers, 1985). Although addition of succinate is *Rhizobium* is reported to give highest growth rate, in *Rhizobium meliloti* 14 the biomass levels did not increase to great extent when succinate alone was provided. Therefore sucrose (20 g/l) was used along with 2, 4, 6 and 10 g/l of succinic acid in the medium. The PHA yields increased to 77% (2.8 g/l). Sucrose uptake also increased with increase in succinate concentration (Fig 43) (Biomass 4.3g/l and PHA 3.1g/l). Catabolite repression by succinate has been reported in *Rhizobium meliloti* (Ucker and Signer, 1978). But no catobolite repression by succinate was observed here. Further observations regarding diauxic growth was not done as gas chromatographic analysis showed that there was no copolymer accumulation even after 72h of fermentation. pH gradually increased with increase in succinate levels from 5.5 to 8 in these

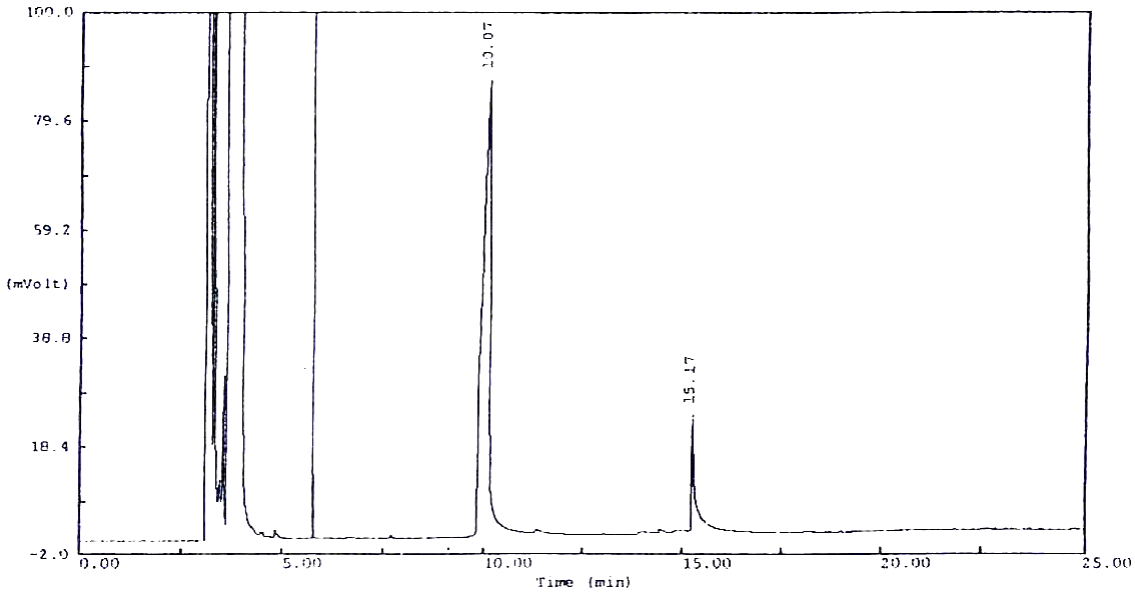


FIG 42: GC ANLYSIS OF PHA SAMPLE FROM *RHIZOBIUM MELILOTI* 14 GROWN ON PYRUVIC ACID AS CO-SUBSTRATE.

TABLE 30: EFFECT OF ORGANIC ACIDS ON COPOLYMER PRODUCTION.

Organic acids	Biomass (g/l)	PHA (g/l)	PHB:HV
Pyruvic acid	4.8	2.7	88 : 12
Citric acid	3.7	3.0	97 : 3
Succinic acid	4.3	3.1	100 : 0
Fumaric acid	3.5	2.3	98 : 2
Malic acid	3.3	2.1	100 : 0
Control	4.7	3.8	97 : 3

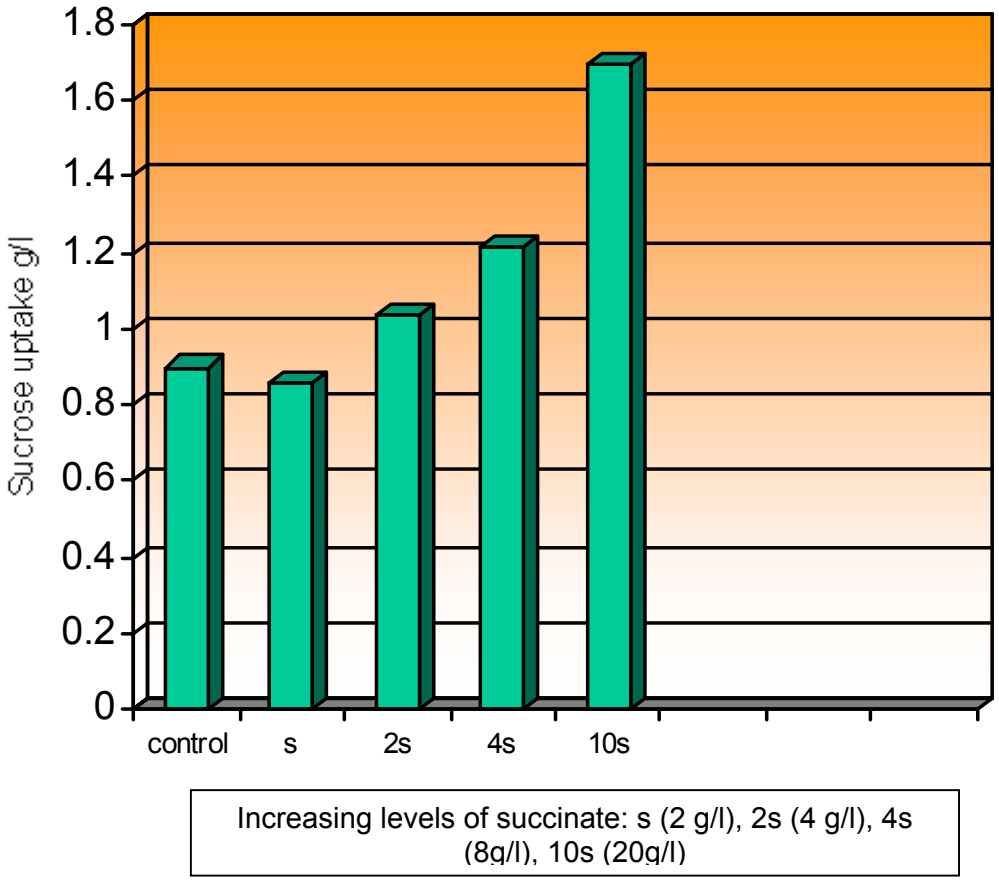


FIG 43: SUCROSE UPTAKE IN *RHIZOBIUM MELILOTI* 14 WHEN GROWN WITH SUCCINATE AS CO SUBSTRATE.

experiments. High alkalinity was also seen in cultures grown in only succinate with no sugar supplementation. Lack of pH maintenance in shake flask could be the reason for poor growth and yields of PHA in *Rhizobium meliloti* 14 when grown on succinate. This could also be responsible for lack of HV content in the polymer. The optimum levels of succinate seemed to lie in between 2 and 4 g/l with 20 g/l of sucrose, wherein the pH was maintained at neutral level (6.9). It is reported that the initial high acid concentrations depressed cell activity and reduced the acid utilization rate (Yu et al, 2002). At low acid rates (when succinate was used as sole carbon source) the cell activity again declined because of limitation of carbon substrates. Thus both lead to poor biomass and PHA levels. Use of optimised level of succinate (lower than 2 g/l) could be beneficial for stimulating PHA synthesis in *Rhizobium meliloti* 14.

6.3 CONCLUSION

Rhizobium meliloti 14 did not require addition of extra carbon substrates to induce copolymer production. 3% of valerate was seen in the polymer extracted from the cells grown in medium containing sucrose as sole carbon source. PHB: PHV content of 81: 19 and 69.7% of PHA yield was seen when the medium was supplemented with rice bran oil. Rice bran oil is also an inexpensive source of carbon. PHB: PHV content was 90: 10, 95:5, and 98:2 when *Rhizobium meliloti* 14 was grown in medium containing pongamia oil, palm oil and stearic acid respectively. PHA yield was 72 % with PHB: PHV content of 88:12 when

pyruvic acid was added in the medium as a co-substrate. Overall the organism was capable of synthesizing PHA with high valerate content. This is highly significant, as increase in the valerate content will improve the material properties of the polymer.

Chapter 7

Extraction and Purification of Polyhydroxyalkanoates

7.0 INTRODUCTION

Since PHAs are accumulated intracellularly, the development of an efficient recovery process including cell disruption is indispensable to reduce its total production cost. Separation methods such as centrifugation, filtration, flocculation are routinely used for separation of cells in the fermented culture broth. Some of the well-known methods of PHA extraction are done using solvents (Ohleyar, 1995; Noda, 1998; Kurdikar et al, 2000), hypochlorite, dispersions of hypochlorite and solvent, mixtures of enzymes (Holmes and Lim 1990; Ramsay et al, 1994), surfactants and chlelating agents (Ramsay et al, 1992; Kessler et al, 2001). In this chapter a modified method of cell lysis for PHA extraction has been worked out.

7.1 MATERIALS AND METHODS

Materials

a) Solvents and chemicals: Chloroform AR grade, dichloromethane AR grade, acetone AR grade, diethyl ether AR grade, absolute ethanol, sodium hypochlorite (5% chlorine), sodium hydroxide and ammonium hydroxide were obtained from Ranbaxy chemicals.

b) Surfactant : Triton X 100

c) Chelating agent: Ethylenediaminetetraacetic acid

Medium and culture conditions

The medium used for inoculum contained the following components (g/l), $Na_2 HPO_4\ 2H_2O$, 4.4; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4\ 7H_2O$, 0.2; sucrose, 10; yeast extract, 0.5; pH 7.0. 10% inoculum was prepared at 30 ^{0}C and 250 rpm for 24 h. The production medium was prepared similar to the inoculum medium but without yeast extract. The concentration of sucrose was increased to 20 g/l. PHA production was carried out in 100 ml of the above medium taken in 500-ml Erlenmeyer flasks. Incubation was performed at 250 rpm and 30^{0}C for 72 h.

Molecular weight

Molecular weight analysis was carried out as described earlier (Chapter 5, page 234).

Extraction methods

Extraction by sodium hypochlorite

Extraction by sodium hypochlorite was carried out as described earlier (Materials and methods, page 103).

Extraction using Chloroform

Cells were collected by centrifugation (6000 rpm for 20 min at room temperature) and were washed with sufficient acetone for 10 min. Acetone dried cells were held in 5 volumes of chloroform and left overnight at room

temperature. Clear chloroform layer was obtained by filtering with glass wool. This was again centrifuged at 4000 rpm at 10^{o}C to obtain a clear solution of PHA in chloroform. PHA was then precipitated with hexane (5 vol.) (Choi and Lee, 1999).

Solvent extraction of PHA from cells was also done using soxhlet apparatus as described earlier (chapter 5, page 230). Chloroform was used as a solvent.

Recovery of PHA with dispersions of hypochlorite solution and chloroform

PHA containing cells were collected by centrifugation (6000 rpm for 20 min at room temperature). These cells were treated with dispersion of hypochlorite and chloroform in the ratio of 1:1 and kept for digestion for 1 h at 37^{o}C. The digested cell material was then recovered by centrifugation at 6000 rpm for 10 min. Centrifugation resulted in three separate phases. The upper phase was that of hypochlorite solution, the middle phase contained non PHA cell material and undisrupted cells, and the bottom phase was chloroform containing PHA (Hahn et al, 1994). The bottom phase was carefully removed by pipetting or by using a separating funnel. PHA was recovered by precipitation with hexane.

PHA extraction with surfactant and chelating agents

Biomass containing PHA was collected by centrifugation (6000 rpm for 20 min at room temperature). Cells were suspended in known quantity of water. 0.6% of triton X 100 and 0.06% of EDTA were added to the cells and pH was adjusted to 13 with 1 N NaOH solution and they were kept at 50^{o}C for 10 min. They were then centrifuged at 6000 rpm for 10 min. Digested cell material was washed with acetone and dried. The precipitate containing PHA was further purified by dissolving in chloroform and precipitated with hexane (Chen et al, 2001a).

Alkaline extraction

Biomass containing PHA was collected by centrifugation (6000 rpm for 20 min at room temperature). The precipitated cells obtained were resuspended in a known quantity of water. pH was adjusted to 11 with ammonium hydroxide or sodium hydroxide and kept at 50^{o}C for 10 min. The digested cell material obtained was then washed thoroughly with water and then with acetone. The precipitate containing PHA was further purified by dissolving in chloroform and precipitated with hexane (Choi and Lee, 1999).

Extraction by enzymes

PHA containing cells PHA was collected by centrifugation (6000 rpm for 20 min at room temperature). The cells were suspended in a known quantity of

water and thermally treated at 80^{o}C for 10 min and cooled. A proteolytic enzyme such as protease (Novozyme) was used for extraction of PHA. Protease (22500 units / ml) was added in the range of 0.5 ml of enzyme to 10 ml of substrate (biomass) containing 1% suspension of biomass (on dry weight basis) and was incubated at 40^{o}C, for 2 ½ h.

Extraction of PHA using cell lytic culture

Isolation and purification of cell lytic culture

Rhizobium meliloti 14 was grown on the PHA production medium (Materials and methods, page 109). Cells were collected by centrifugation of 100 ml of 72 h-culture broth. The cells were suspended in distilled water and the volume was made upto 100 ml. Agar (1.5%) was added to this and sterilized. This was poured on to sterilized plates. Various soil samples were collected for isolation of a lytic culture. These were serially diluted and 0.1 ml was spread on the agar plates containing killed *Rhizobium meliloti* 14 cells. Plates were incubated at 30^{o}C and observed for clearance zones upto 48 h.

The cultures that grew well on the agar medium containing heat killed *Rhizobium meliloti* 14 cells were picked up. These cultures were further grown on medium containing *Rhizobium meliloti* 14 cells as carbon source. The colony that produced a significant clearance zone was identified as the lytic culture.

Identification of Lytic culture

Microscopic observation

Microscopic observation of the lytic culture was done by light microscopy as well as phase contrast microscopy. Unstained preparations of the culture were observed initially by light microscopy. Both stained (Simple staining, See Materials and methods, page 107) and unstained preparations were observed under phase contrast microscopy.

Cover slip Culture

A sterilized cover slip was carefully inserted at an angle of about 45^{o}C into nutrient agar medium in a petri dish, until about half of the coverslip was in the medium. The isolated lytic culture was inoculated along the line where the medium meets the upper surface of the cover slip. After 10 days at 25^{o}C, the cover slip was carefully removed. The orientation of the cover slip in the medium was noted and was placed on a glass slide. Noting the orientation of the cover slip was to distinguish between substrate mycelium and aerial mycelium. Substrate mycelium was observed on the area of the cover slip that was covered with the medium and the aerial mycelium on the area of the cover slip above the medium. This distinction between the substrate and aerial mycelium is an important character in the identification of actinomycetes (Williams et al, 1968).

Growth of cell lytic culture

The lytic culture grew well on nutrient agar medium (Materials and methods, page 109). Inoculum of the cell lytic culture was prepared on the following medium:

Composition	**g/l of distilled water**
Na_2HPO_4 $2H_2O$	4.4
KH_2PO_4	1.5
$(NH_4)_2SO_4$	1.0
$MgSO_4$ $7H_2O$	0.2
Sucrose	10
Yeast extract	1.5
(pH 7.0)	

Inoculum was prepared at 30^{o}C and at 150-rpm incubated to 24 h.

Growth associated lysis

Rhizobium meliloti 14 was grown on the PHA production medium (Materials and methods, page 109). At the end of the cultivation period the culture broth was heated to 80^{o}C for 10 min and cooled. Decanting the medium under sterile conditions separated cell lytic culture described above. The pelleted cells were transferred at 40-mg level (dry weight basis) to every 100-ml of inactivated *Rhizobium meliloti* 14 culture broth under sterile conditions. Aerobic

cultivation was carried out further without additional supplementation of nutrients at 30^{o}C, 150 rpm for 24h. After growth, the cell lytic culture formed pellets and was separated by simple filtration through glass wool (Fig 44). PHA was isolated from other cellular materials by mixing with a solvent such as chloroform. One volume of chloroform was added to 4 volumes of hydrolysed culture broth and stirred for 10 min at room temperature. The bottom phase of the chloroform layer was separated and dried at 50^{o}C to obtain PHA.

Alternatively, a mixture of surfactant and chelating agent (Triton X 100, 0.6% and EDTA, 0.06%) was added to the hydrolysate and incubated at 50^{o}C for 10 min at pH 6 - 7. The hydrolysed broth was centrifuged at 7000 rpm for 15 min to isolate the polymer. The PHA sediment was dewatered at low temperature to complete dryness.

PHA content was also determined gravimetrically by sodium hypochlorite hydrolysis (Materials and methods, page 103).

Lysis by enzyme / culture filtrate

Rhizobium meliloti 14 was grown on the PHA production medium (Materials and methods, page 109). At the end of the cultivation period (72 h) the culture broth was heated to 80^{o}C for 10 min and cooled.

The lytic culture was grown as described under growth associated lysis. The culture supernatant was collected by decanting the broth under sterile conditions. The crude culture filtrate obtained was used at 1 volume level to

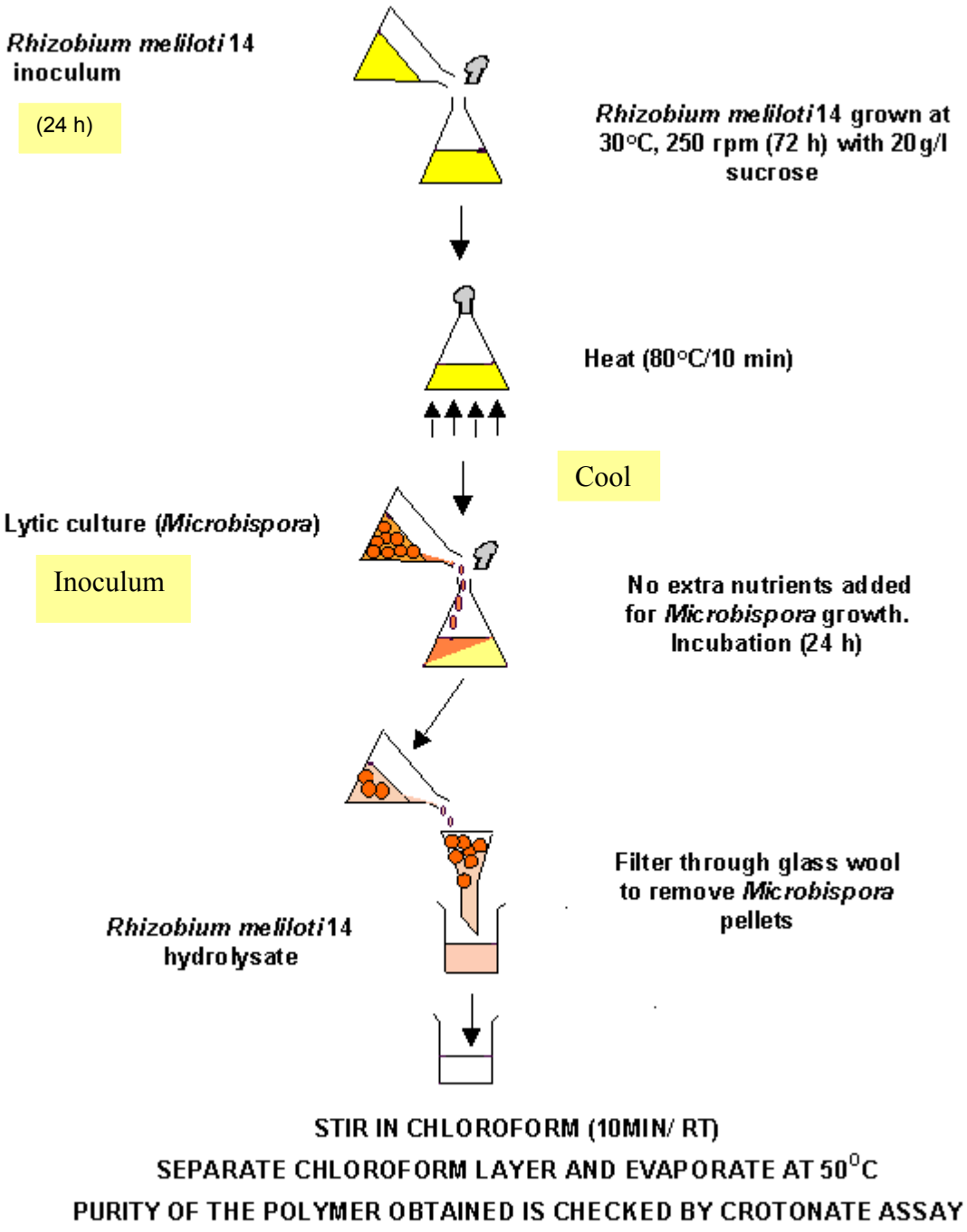


FIG 44: MICROBIAL METHOD OF EXTRACTION OF POLYHYDROXYALKANOATES

hydrolyze 3 g/l of thermally inactivated biomass of *Rhizobium meliloti* 14 (Fig 45). The hydrolysis was carried out at 50^{o}C for 3 h and PHA was isolated using chloroform as described above under growth associated lysis.

Lytic activity assay

Rhizobium meliloti 14 biomass was thermally inactivated at 80^{o}C for 10-min and suspended in phosphate buffer. Optimization of different pH (at 50^{o}C), different temperature (at pH 8), enzyme concentration and concentration of biomass for lysis and time required were tested using the lytic culture filtrate. Lytic activity was determined by hydrolyzing *Rhizobium meliloti* 14 cells of known dry weight (3 mg/ 5 ml) with 48-72 h old clarified culture filtrate (0.5 ml) of cell lytic culture at 50 ^{o}C, pH 6-7 for 1 h. O.D was measured at 620 nm and activity was calculated as 1000 X (initial OD at 620 nm - inal OD at 620 nm) (Triveni and Shamala, 1999).

Crotonoate Assay of Enzyme extracted PHA

Crotonoate assay was done as described earlier (Chapter 5, page 231).

Characterisation of PHA extracted using lytic enzyme

Characterization of the extracted polymer was carried out using Infrared spectroscopy, gas chromatography and differential scanning calorimetery, as already described in chapter 5, pages 231, 232 and 234.

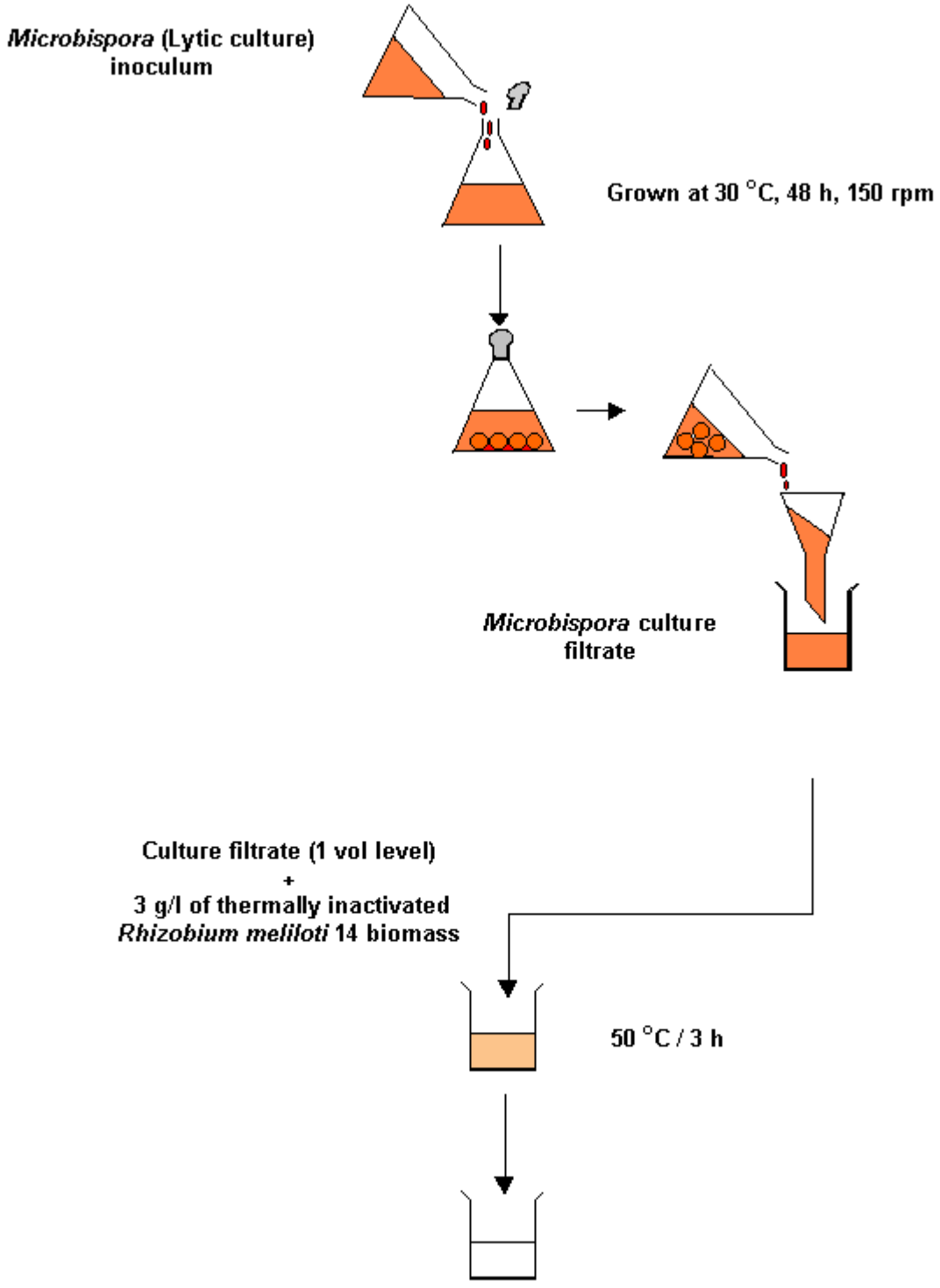


FIG 45: EXTRACTION OF POLYHYDROXYALKANOATES BY LYTIC ENZYME

Scanning electron microscopy of enzyme treated biomass

Scanning electron microscopic analysis of the lytic enzyme treated biomass was carried out as described in chapter 1, page 120.

7.2 RESULTS AND DISCUSSION

Extraction by sodium hypochlorite

Extraction by sodium hypochlorite was very efficient as it gave 96% pure sample (Table 31). Extraction by sodium hypochlorite causes severe degradation of PHB resulting in a 50% lower molecular weight polymer compared to that of intact cellular PHB (Ramsay et al, 1992). Though the method is simple and effective, generation of chlorine containing solution at the end of extraction process is also a serious limitation for its use. Dispersions of sodium hypochlorite and chloroform at 50% sodium hypochlorite in chloroform gave yields similar to that of sodium hypochlorite alone. When hypochlorite is used for the differential digestion of non-PHA cellular material chloroform is used to draw the polymer out. Here chlorinated wastes are generated in large quantities and handling is not easy. There is also degradation of the polymer during treatment (Hahn et al, 1993). Gas chromatographic studies show degradative changes in peak area of PHB and HV. Samples extracted by sodium hypochlorite showed (viscosity average) molecular weight of 7.39×10^5.

TABLE 31: EXTRACTION OF PHA FROM *RHIZOBIUM MELILOTI* 14 CELLS USING DIFFERENT METHODS OF CELL HYDROLYSIS

Methods of hydrolysis		**Extraction system**	**Dry wt of *R meliloti* biomass (g)**	**PHA recovered (g)**	**Purity of PHA (%)**
Control		Chloroform	1	0.03	66
Control		EDTA+Triton	1	0.16	68
Sodium Hypochlorite		Chloroform	1	0.47	96
***Microbispora* growth**					
Period (h)	Biomass (mg %)				
24	40	Chloroform	1	0.21	90
		EDTA+Triton	1	0.49	90
48	110	Chloroform	1	0.17	94
		EDTA+Triton	1	0.41	92
72	160	Chloroform	1	0.08	90
		EDTA+Triton	1	0.07	90
***Micobispora* Lytic enzyme**		Chloroform	1	0.22	86
		EDTA+Triton	1	0.47	92

Extraction of PHA by solvents

Chloroform extracted PHA samples from *Rhizobium meliloti* 14 were 66% pure. Although solvent extraction maintains the quality of the polymer this method requires large quantities of toxic and volatile solvents. High concentration of polymer can be very viscous and large amounts of solvents are required for processing (Ramsay et al, 1994). Large-scale use of solvents is harmful and separation of insoluble cell material after solvent extraction is difficult and 100% extraction is not possible (Choi and Lee, 1999). But extraction by solvents does not degrade the polymer during extraction. Gas chromatography of solvent extracted samples showed the presence of 2.5% of PHV in the sample. Hence is a useful method compared to extraction by sodium hypochlorite.

Extration of PHA by enzymes

Commercial enzyme such as protease from Novozyme was also tried. PHA recovered was 56.7 % based on the dry weight of the biomass used. Use of commercial enzymes also involves high cost. A separation process for PHA production using enzymes was reported (Holmes and Lim, 1990) and this process was also used in commercial production of PHB by BIOPOL. But there are limitations to this process. The biomass suspension has to be centrifuged and buffered before digestion. Based on cell wall composition a mixture of commercial enzymes needs to be used. The action of the enzymes and different enzyme mixtures to be used has to be optimized (Choi and Lee, 1999). The PHA recovery by enzymatic digestion of *Rhizobium meliloti* 14 was about 90%, which

would require further investigation. The enzyme extracted samples showed a molecular weight (viscosity) average of 1.7×10^6.

Extraction of PHA by surfactants and chelating agents

PHA from *Rhizobium meliloti* 14 cells after treatment by surfactants and chelating agents was 68% pure (Table 31). Surfactants such as SDS, Triton X and Tween and chelating agents such as EDTA have also been used in PHA extraction (Baptist, 1962; Ramsay et al, 1994; Chen et al, 2001a). Surfactants denature cell membrane protein and leads to easier disruption of cells. They also solubilize non-PHA cell materials so that they can be separated easily from PHA. Chelating agents destabilize outer cell membrane by forming complexes with divalent cations to aid the breaking of the cells (Chen et al, 2001a). EDTA (ethylenediaminetetraacetic acid) is a chelating agent included in buffers to chelate Mg+, which will reduce deoxyribonuclease activity because of the Mg+ requirement of the enzyme. SDS is a detergent that denatures proteins and solubilize lipids in membranes leading to cell lysis. But PHA recovery by SDS is expensive and it causes pollution problems resulting in high disposal cost. Triton X was tried as a surfactant and was found to be very effective on *Rhizobium meliloti* 14 cells. Purity of the samples is important as the quality of PHA affects its performance. But native PHA is bound by phasins, which are granule associated proteins. Hence extraction by surfactants alone did not suffice for

proper recovery of PHA from *Rhizobium meliloti* 14 cells. The per cent recovery was however insignificant compared to other methods.

Alkaline Extraction of PHA

Alternatively alkaline extraction of *Rhizobium meliloti* 14 cells was tried for alkaline lysis. At alkaline pH there is hydrolyzing and saponifying of proteins and lipopolysaccharides (Chen et al, 2001a). A pH range from 10 to 11.5 was found to be effective for cell lysis in *Rhizobium meliloti* 14 (Fig 46). PHA yield was 57% of dry weight of biomass when ammonium hydroxide was used for lysis and 17.6% of dry weight of biomass when sodium hydroxide was used. Alkali extraction causes less degradation of PHA during recovery and but there is the disadvantage of long time, high temperature incubation requirements. However, it is cheaper compared to other chemical digestions tried for PHA recovery. Although NaOH has been reported as a better and cheaper alkali for PHA extraction (Choi and lee, 1999), ammonium hydroxide was found to be more effective for extraction of *Rhizobium meliloti* 14. Samples extracted from ammonium hydroxide showed a molecular weight (viscosity) average of 1.3×10^6.

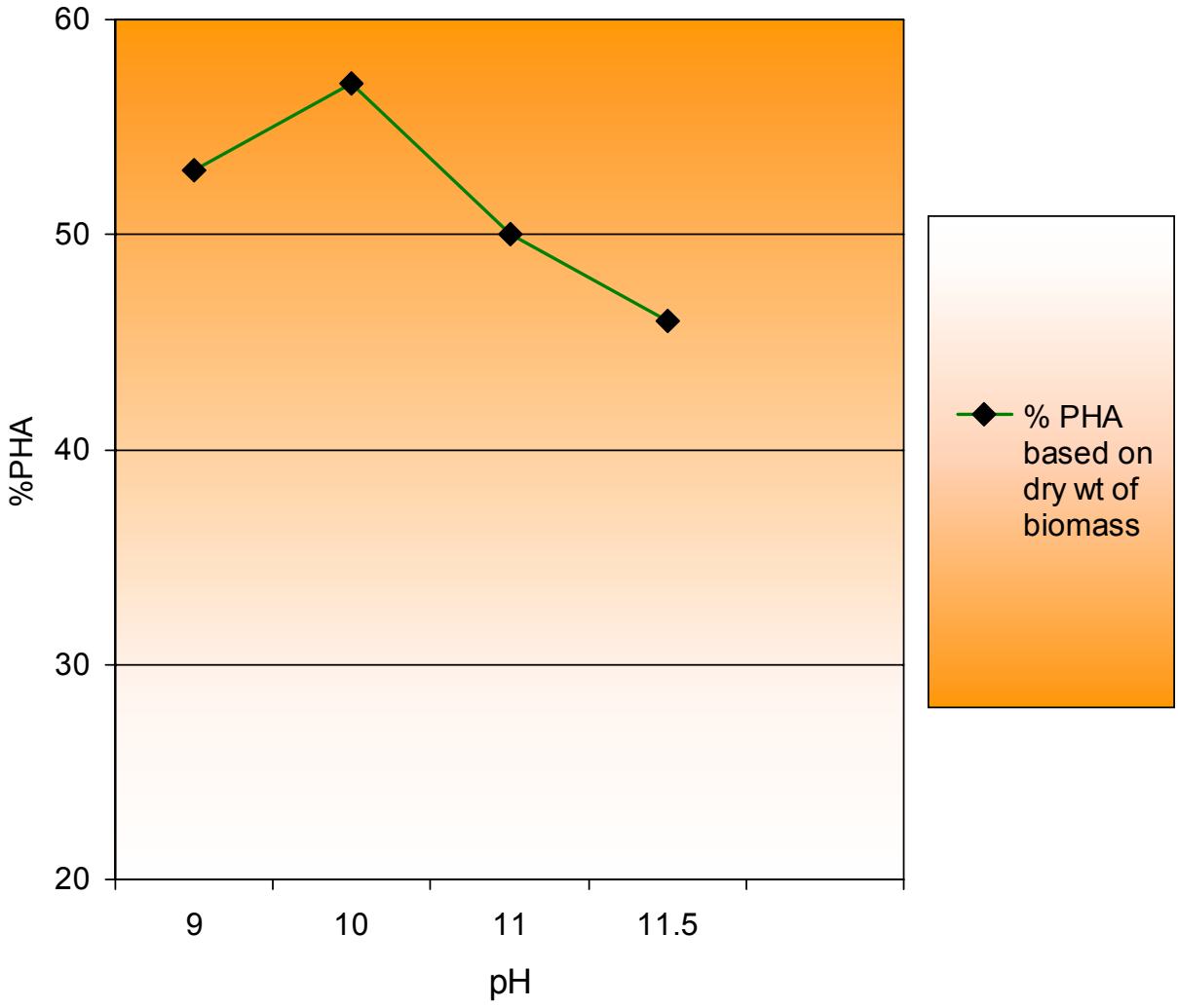


FIG 46: EFFECT OF ALKALINE pH (USING NH_4OH) ON EXTRACTION OF PHA FROM *RHIZOBIUM MELILOTI* 14

Microbial method for PHA Extraction

A simple and effective microbial method of extraction of polyhydroxyalkanoates from bacterial cells has been developed. In this method lytic enzyme of an actinomycete culture has been used to lyse *Rhizobium meliloti* 14 cells.

Identification of lytic culture

Various soil samples were used to isolate a cell lytic culture. The culture that showed a significant clearance zone on the plates containing heat killed *Rhizobium meliloti* 14 cells was selected amongst other cultures, which were screened for lysis. The culture was aerobic and grew well at 25-30^{o}C. Growth on slant was matty, thick and firm. In the liquid medium the culture grew as small pellets when *Rhizobium meliloti* 14 cells were used as a substrate in the medium (Plate 12). The presence of aerial and substrate mycelium was observed by cover-slip culture method (Plate 13 and 14). Substrate mycelium was stable and with septa dividing the substrate hyphae into long cells. The aerial mycelium showed the presence of conidia (Plate 15). Based on the morphological characters as well as microscopic observations, the culture was identified as an actinomycete and genus was ***Microbispora***. Actinomycetes are primarily soil inhabitants and are involved in the degradation of organic matter. Hence they possess a well-developed enzyme system in order to survive in the environment. The *Microbispora* culture was capable of utilizing *Rhizobium meliloti* 14 cells as carbon source and was found to grow well on it.

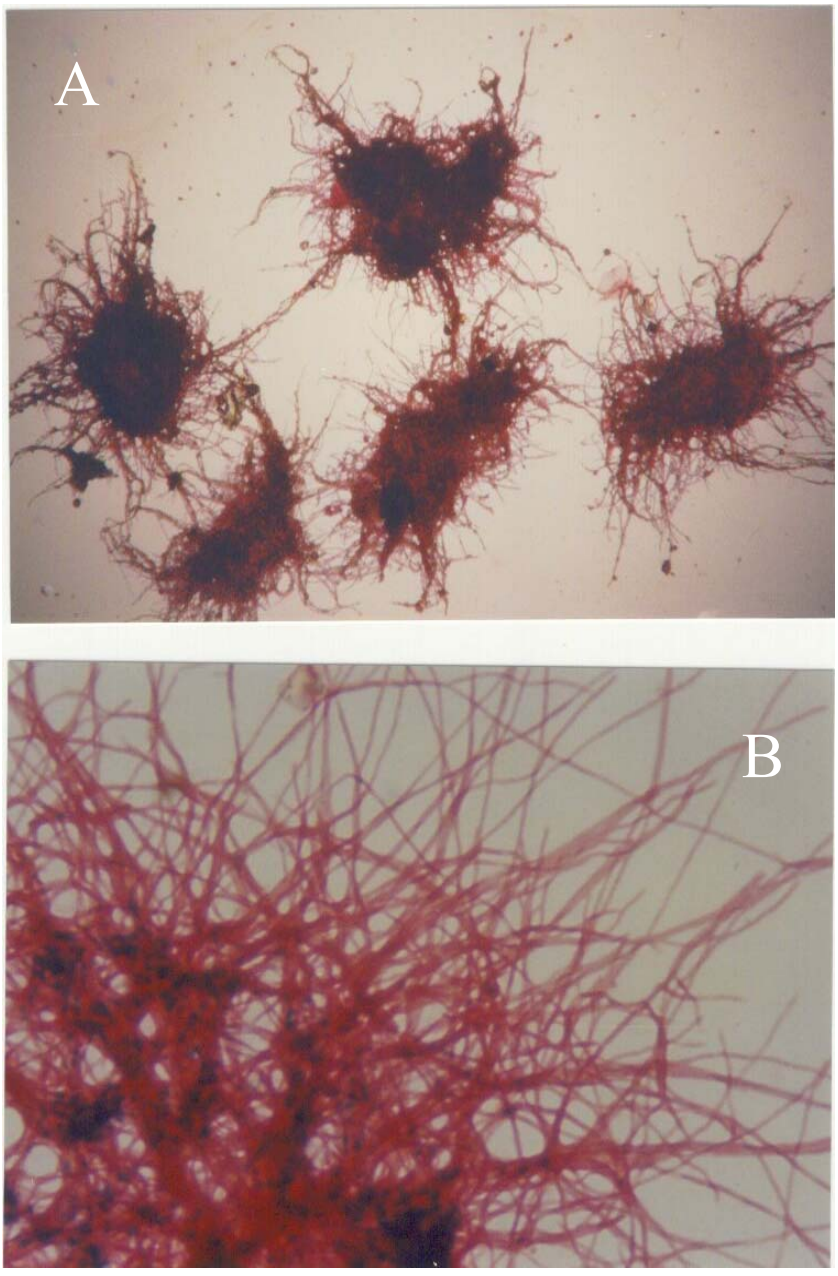


PLATE 12: *MICROBISPORA* CULTURE GROWN ON *RHIZOBIUM MELILOTI* 14 CELLS
A: 40X MAGNIFICATION
B: 100X MAGNIFICATION

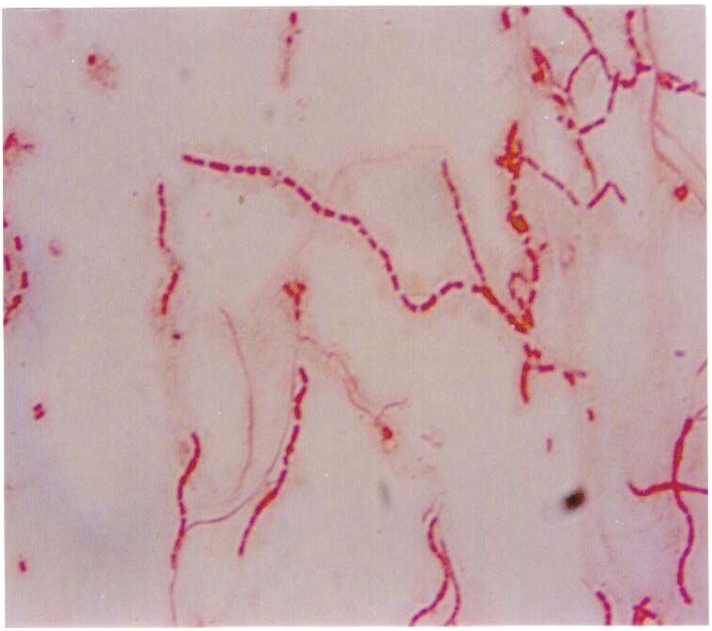


PLATE 13: SUBSTRATE MYCELIUM OF *MICROBISPORA* OBSERVED ON COVER-SLIP

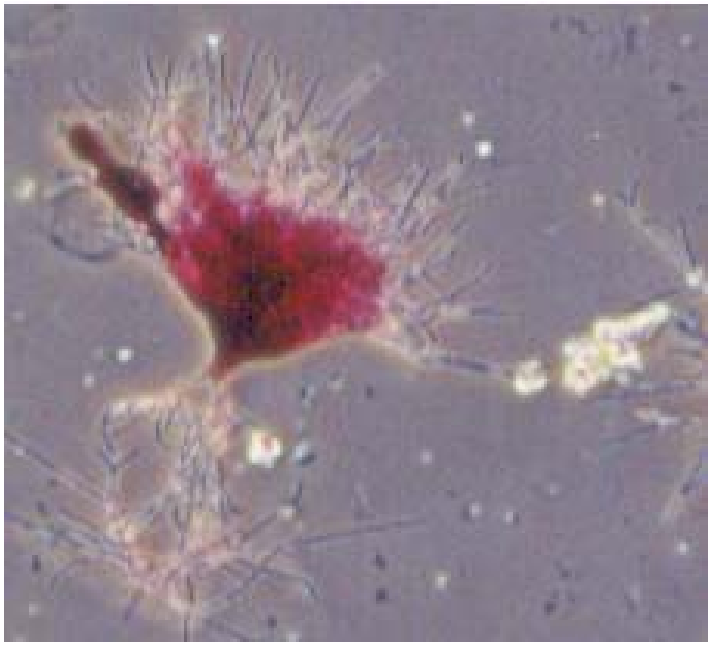


PLATE 14: *MICROBISPORA* CULTURE SHOWING SUBSTRATE AND AERIAL MYCELIUM

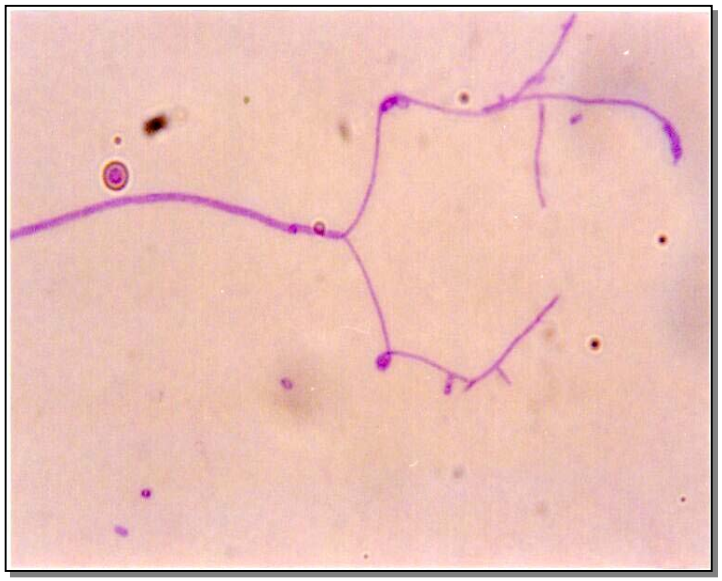


PLATE 15: *MICROBISPORA* SHOWING CONIDIA FORMED ON THE AERIAL MYCELIUM.

It was observed that *Microbispora* could use the *Rhizobium meliloti* 14 cells as sole source of nutrient.

Extraction of PHA by Cell Lytic enzyme from *Microbispora* sp

Microbispora digested the *Rhizobium meliloti* 14 cells and released PHA into the medium and could be observed under the phase contrast microscope (Plate 16 and 17). The only requirement in this method was a prior thermal inactivation of PHA containing cells along with the culture medium at 80^{o}C. Digestion of cell materials was by growing a lytic culture or by using its culture filtrate, and isolation of the released polymer was by the use of a solvent such as chloroform. Here the lytic culture, was grown directly in the broth having inactivated PHA containing *Rhizobium meliloti* 14 cells to release PHA and isolation of released PHA was by dissolving in chloroform. The chloroform solution was then evaporated and the polymer was obtained. Although extraction with hypochlorite gave around 96% purity, there is severe reduction in the molecular weight of the polymer. PHA extracted from hypochlorite extraction is also known to be more crystalline and brittle (Ramsay et al, 1992). With solvent extraction the purity was 66% and with only surfactant and chelating agents 68% (Table 31). With the cell lytic culture the yields were 90-94%. A 48-h old culture gave better yields than 24 or 72 h culture. This is because after 48 h, growth of the lytic culture increased, which also finally resulted in degradation, and utilization of the polymer released (Plate 18). This also suggests that there is a

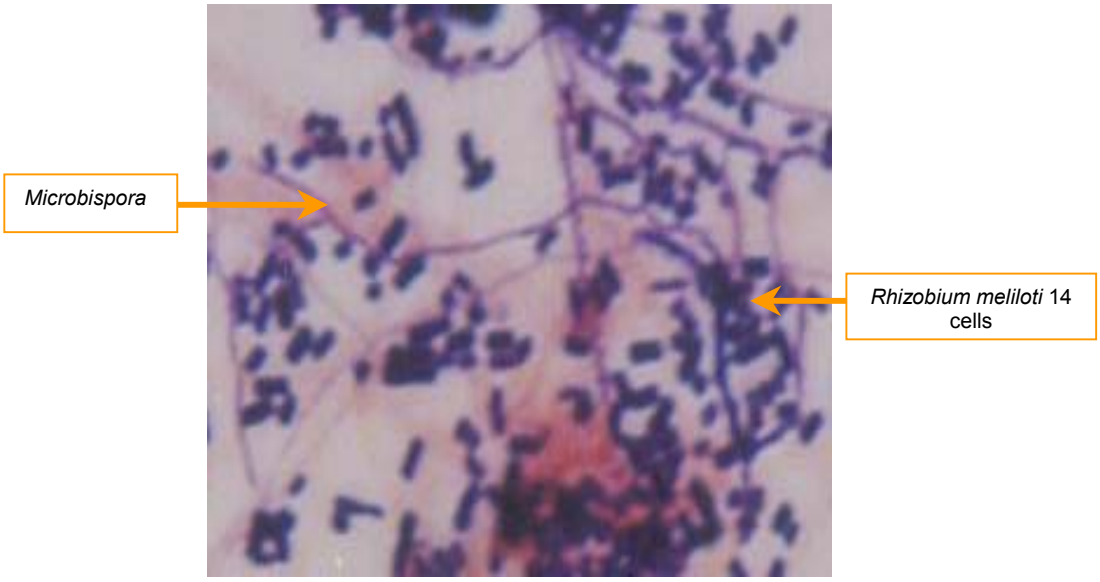


PLATE 16: *MICROBISPORA SP* CELLS GROWING ON *RHIZOBIUM MELILOTI* 14 AS A SUBSTRATE

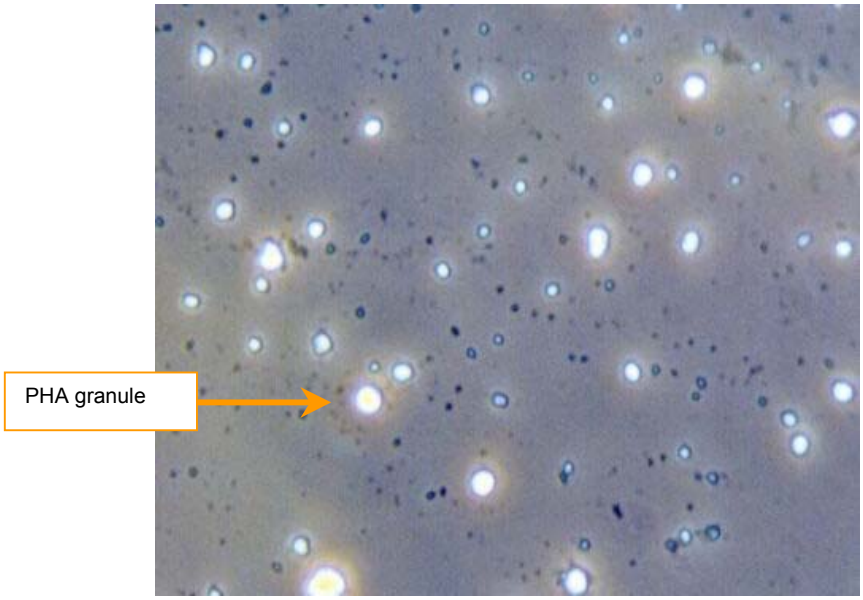


PLATE 17: PHA GRANULES RELEASED INTO THE MEDIUM FROM *RHIZOBIUM MELILOTI* 14 AFTER GROWTH OF *MICROBISPORA* (40 X MAGNIFICATION)

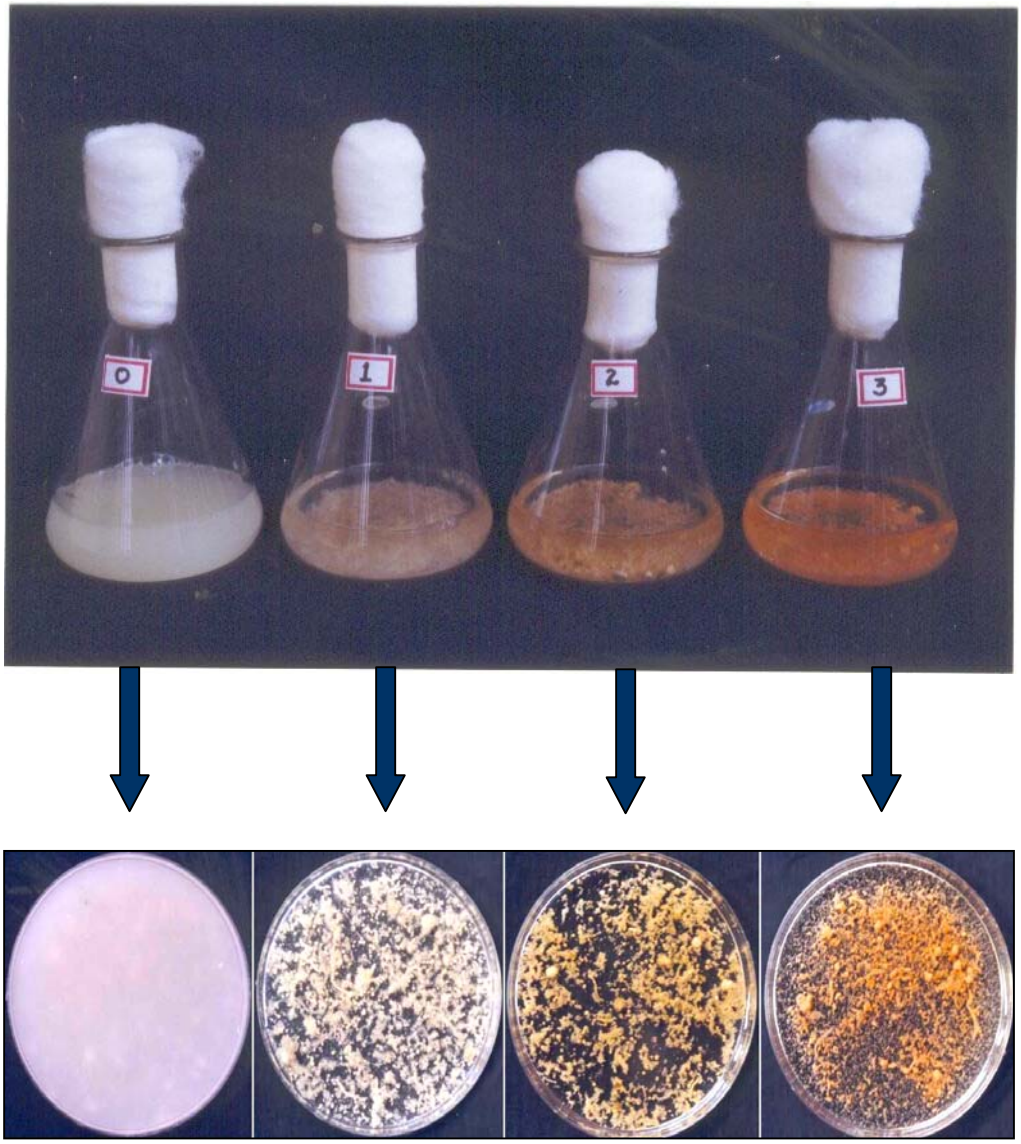


PLATE 18: *MICROBISPORA* CULTURE GROWING ON *RHIZOBIUM MELILOTI* 14 CELLS AT (0) 0 H, (1) 24 H, (2) 48 H, (3) 72 H

mixture of various enzymes being released by *Microbispora.* This method was also simple and less time consuming and not hazardous compared to hypochlorite extraction. Here minimization of solvent use for the extraction of polymer has been achieved. This method is a biological method of cell hydrolysis of PHA containing bacteria, where the lytic culture was grown without additional nutrient supply directly in the PHA broth, without buffering, centrifugation or resuspension of the cells. Separation of lytic culture was easy and was done by filtration only.

Culture filtrate (crude) was also efficiently used for hydrolysis of bacterial cells. PHA of 86% purity was obtained on purification by chloroform. The culture filtrate was found to contain maximum activity of 350 u at 50^{o}C for 1h (Fig 47). A ratio of 2:1 of culture filtrate and biomass was found to contain highest lytic activity of 491 U, whereas 1.5:0.5 and 1:2 gave 396 and 150 U respectively (Table 32). Microscopic examination revealed that a 3-h treatment of *Rhizobium meliloti* cells by crude lytic enzyme degrades all cell material at 50^{o}C (Plate 19). Scanning electron microscopic analysis of enzyme treated biomass of *Rhizobium meliloti* 14 cells, showed degradation of cell (Plate 20). There seems to be an increase in the cell porosity and the hence the cell shrinks due to leakage of all cell material. This was observed even in light microscopy (Plate 19) where the heat-inactivated cells of *Rhizobium meliloti* 14 appear empty. Only the remnants of the cell wall, which takes up the stain, can be observed.

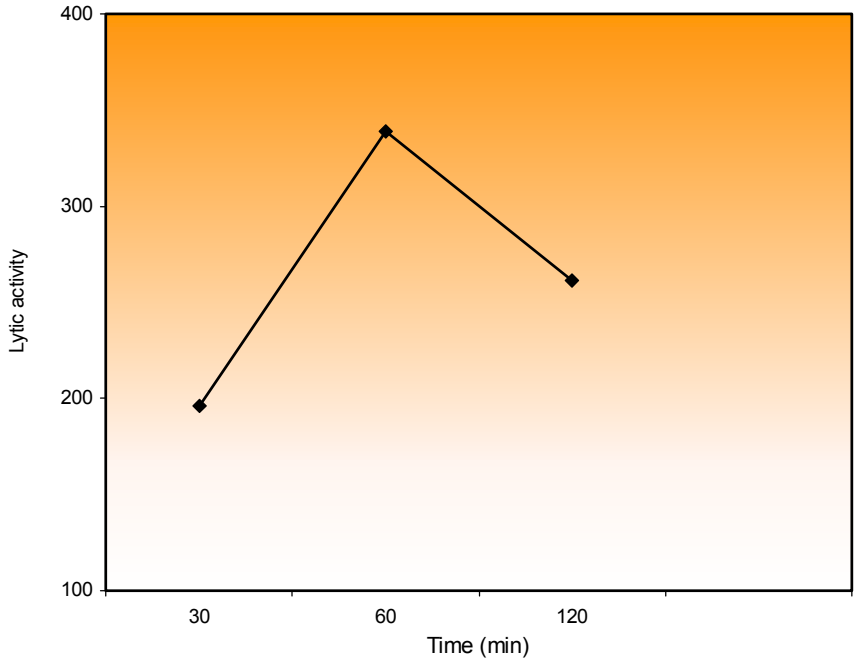
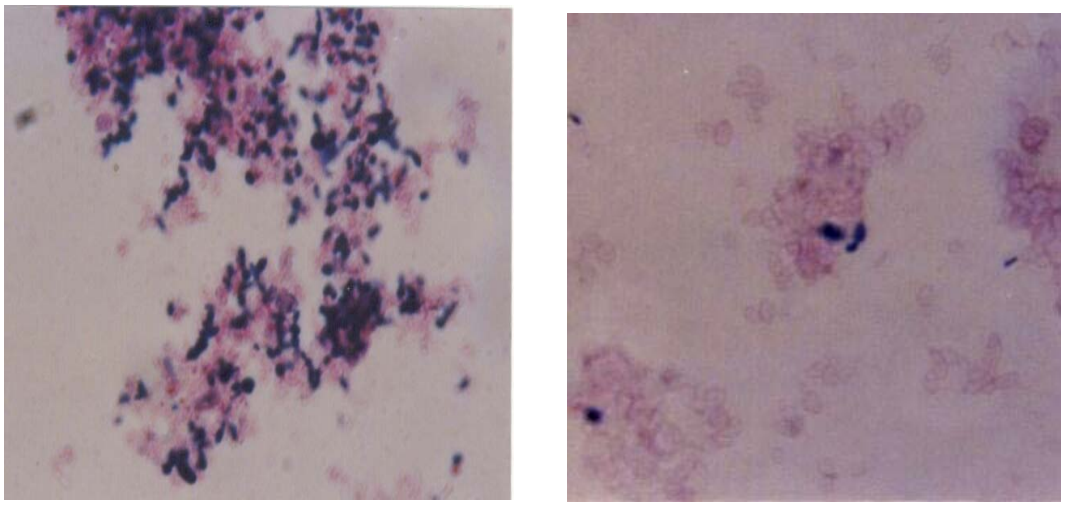


FIG 47: OPTIMIZATION OF LYTIC ACTIVITY OF *MICROBISPORA* CULTURE FILTRATE ON *RHIZOBIUM MELILOTI* 14 CELLS AT 50^{O}C

TABLE 32: LYTIC ACTIVITY OF *MICROBISPORA* CULTURE FILTRATE ON PURIFIED BIOMASS OF *RHIZOBIUM MELILOTI* 14 (AT 50^{O}C AND pH 8) AT DIFFERENT CULTURE FILTRATE: BIOMASS RATIOS

Ratio of Culture filtrate : Sample Treated at 50^{o}C for 1h	**Lytic activity [1000 X (initial OD at 620nm - final OD at 620 nm)]**
2:1	491
1.5:0.5	396
1:2	150



A B

PLATE 19: LYSIS OF *RHIZOBIUM MELILOTI* 14 CELLS BY *MICROBISPORA* LYTIC ENZYME AT (A) 0 h AND (B) 3 h

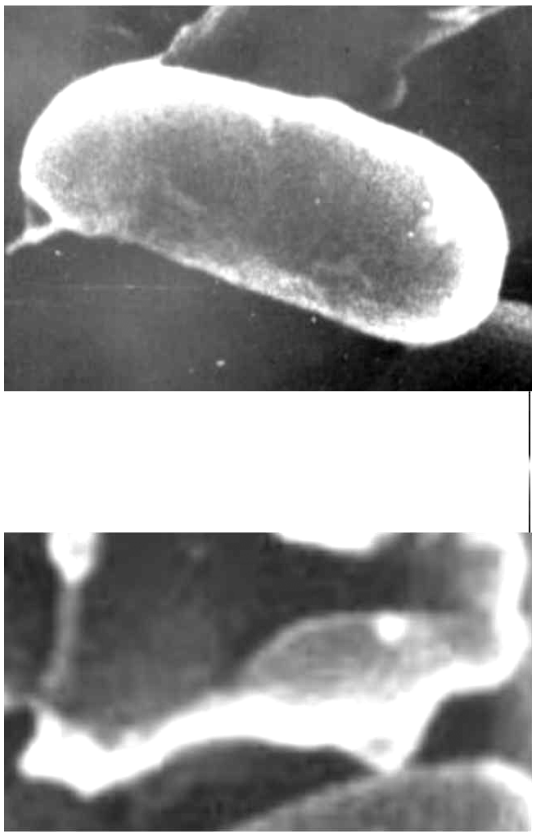


PLATE 20: SCANNING ELECTRON MICROGRAPH OF (A) *RHIZOBIUM MELILOTI* 14(CONTROL) (B) LYTIC ENZYME TREATED *RHIZOBIUM MELILOTI* 14 CELL

Use of surfactant and chelating agents for purification after hydrolysis by crude culture filtrate resulted in 92 % pure PHA (Table 31). More PHA and proteins are released when surfactant and chelating agents are used simultaneously (Chen et al, 2001a). However this purification was not necessary when growth associated hydrolysis was adopted.

The quality of PHA was also analyzed by Infrared red spectroscopy and gas chromatography. The extracted sample was found to be pure. Absence of peak at 1650cm^{-1} (corresponding to C=O of amides associated with proteins) and absence of vibrations near 2928cm^{-1} (characteristic of N-H of proteins) were seen in the graphs (Fig 48). This suggests that the PHA extracted after the enzyme extraction was pure and free of any associated cell material such as protein. Gas chromatographic analysis showed the presence of polyhydroxyvalerate along with butyrate in the enzyme-extracted sample. 1.4% of valerate was observed in the samples which could not be seen in sodium hypochlorite extracted sample. The DSC melting curves show two peaks (Fig 49). The major peak was at 169^{o}C and a small shoulder was seen at 171^{0}C. The polymer obtained could be a blend or mixture of two polymers. Bacterially synthesized polymers are neither random or block copolymers. They are interpreted as mixture of two random copolymers (Kamiya et al, 1989). Enzyme extraction may ensure the release of less prominent units in the polymer, which may not be properly extracted by other methods.

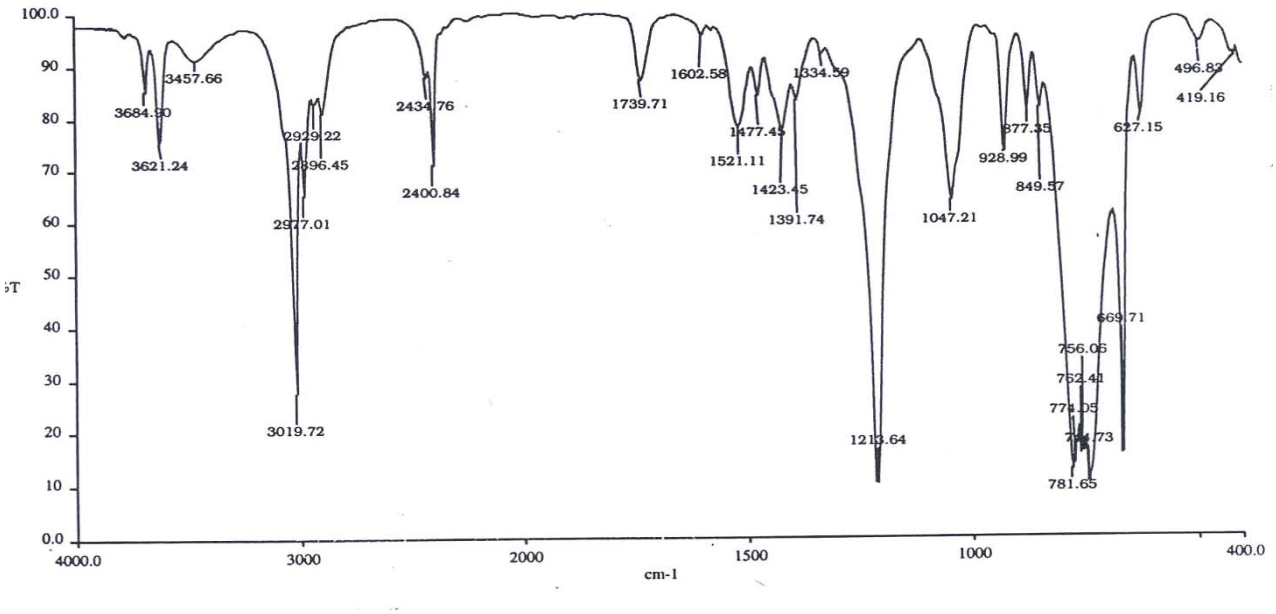


FIG 48: INFRARED SPECTROGRAPH OF ENZYME EXTRACTED PHA SAMPLE FROM *RHIZOBIUM MELILOTI* 14

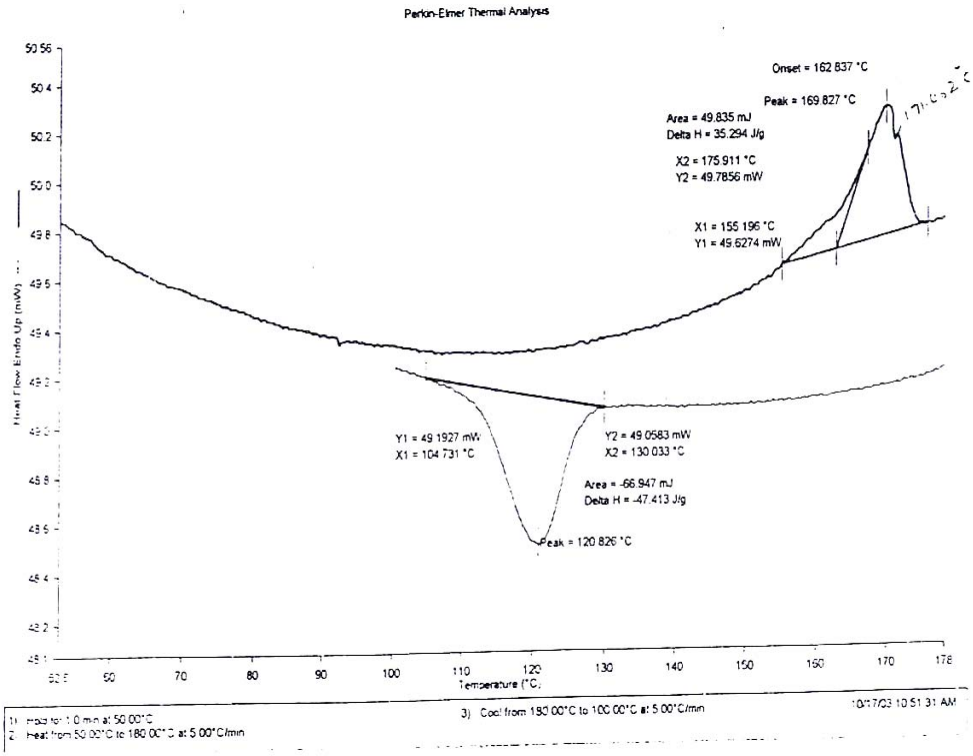


FIG 49: DIFFERENTIAL SCANNING CALORIMETRY SHOWING MELTING TEMPERATURE OF ENZYME EXTRACTED PHA SAMPLE FROM *RHIZOBIUM MELILOTI* 14.

7.3 CONCLUSION

A simple and effective microbial method of extraction of polyhydroxyalkanoates from bacterial cells has been developed. In this method lytic enzyme of an actinomycete (*Microbispora*) culture has been used to lyse *Rhizobium meliloti* 14 cells. The *Microbispora* culture grew directly on heat killed *Rhizobium meliloti* 14 cells and extra nutrients were not added for the growth of the lytic culture. The lytic culture formed pellets and it could be separated easily by filtration. PHA was released into the broth and was extracted by a minimum quantity of chloroform. PHA extracted by this method was 90-94% pure. Crude culture filtrate was also effective in lysing of *Rhizobium meliloti* 14 cells. In conclusion, the *Microbispora sp* produced lytic enzyme, which could solubilise, the bacterial cell material and hence release PHA.

Summary and Conclusion

Biodegradable polymers are slowly being adapted into various applications and are foreseen as alternatives to synthetic plastics. At present these polymers are used mostly in specialized applications including packaging. There is a need for practical ventures into biodegradable plastics rather than putting off the concept to the end till it becomes inevitable for its production. The initial setbacks in the field due to economic reasons should not stop science from advancing in this field. The need to develop natural alternatives is not only challenging but also is in the interests of the world as a whole. Any new input in the field will add another rung to the ladder towards materializing this concept.

Polyhydroxyalkanoates are polyesters accumulated in bacteria. These bioplastics are being used in various applications such as coatings, laminations and biodegradable inks. The properties of these biopolyesters range from thermoplastic elastomers to highly rigid stiff thermoplastics. As packaging materials polyhydroxyalkanoates have moisture vapour barrier properties similar to PET and polypropylene. They are biodegradable, biocompatible and are produced by microorganisms by utilizing renewable sources of carbon.

Rhizobia are known to accumulate PHA as a source of carbon and energy during conditions of nutrient imbalance. The production of PHA in *Rhizobia* has been studied in bacteroids with regard to its role in nitrogen fixation. There are few reports wherein PHA production is studied in free living *Rhizobia* with respect to development of bioplastics. Hence study of PHA production in *Rhizobia* was taken up. The members of the family *Rhizobiaceae* differ in the mode of

adaptations to stress and imbalanced nutrient conditions. They offer a variety of metabolic pathways directing carbon towards synthesis of biopolymers such as polysaccharides or polyhydroxyalkanoates. Though *Rhizobia* are strict aerobes they are also capable of adapting to microaerophilic conditions in free living state wherein they accumulate PHA. Polysaccharide synthesis is also a common feature in *Rhizobia*. It is interesting to study the intricacies of carbon metabolic traffic being directed either towards polyhydroxyalkanoate synthesis or polysaccharide biosynthesis in conditions of nutrient imbalance. This work aimed at studying the capability of a locally isolated strain of *Rhizobium* for production of polyhydroxyalkanoate and the characterization of the polymer to study its potential towards development as a biodegradable packaging material.

Rhizobia were isolated from various natural samples such as soil and root nodules of leguminous plants. Based on different staining techniques nearly 20 colonies were isolated as positive PHA producers from a batch of 150 purified strains. They were further grown in the Yeast Mannitol Agar medium along with standard strains and were examined for PHA production after hypochlorite extraction. PHA obtained was expressed in terms of % of biomass. **In the present study amount of PHA produced by the tested cultures varied from 5 - 65%.** The standard strains produced PHA in the range of 11-47%. Amongst all the cultures that were tested culture R14 showed promising PHA yields of about 65%. This was 10% higher than previously reported data and hence this

culture was further investigated. **Based on the morphological, biochemical and nodulation tests R 14 was identified as a strain of *Rhizobium meliloti* (*Sinorhizobium meliloti*) and designated as *Rhizobium meliloti* 14.**

An alternative technique such as polymerase chain reaction was used to develop a rapid detection technique for PHA producing bacteria. Primers were selectively designed based on gene of *Rhizobium meliloti* and *Alcaligenes latus* (both having Type I PHA sythases) whose β-ketothiolase sequences are known. β-ketothiolase and acetoacetyl-CoA gene sequences of *Rhizobium meliloti* and *Alcaligenes latus* were aligned. Based on the conserved region sequences primers LF and LR were designed for PCR amplification. **A total of 22 strains both PHA positive and PHA negative strains were tested by PCR using the designed primers. There was a significant difference in the banding pattern of PCR amplicons between producers and non-producers of PHA.** All the strains that amplified the expected PCR product were confirmed as PHA producers by hypochlorite extraction method. The PHA negative strains such as 905 (*R trifolii*), 2002 (*R japonicum*) showed non-specific amplification and strains 2228 and 2231 did not show any amplification. Two standard *R leguminosarum* strains showed a clear difference in their amplification. Strain 99 (*R leguminosarum,* IMTECH) which gave 41% PHA yield by extraction method was amplified to the expected PCR product. Whereas, strain 2005 (*R leguminosarum*, NCIM) which was PHA negative, showed nonspecific multiple amplifications. **The absence of any amplification by the PHA negative strains**

justified the use of these primers for effective identification of PHA producing *Rhizobia*. Identification of PHA producing organism was also carried out using Fourier transform infrared spectroscopy (FTIR). About 10 locally isolated bacteria were screened by FT-IR analysis. The prominent regions seen in many of the samples were bands at **1720 – 1740 cm^{-1} (C=O)**, 1280 cm^{-1} (C-O), 1375 cm^{-1} ($–CH_3$), 1455 cm^{-1} ($-CH_2$), and 3424cm^{-1} (OH). **By FTIR analysis it was possible to distinguish the good and poor producers of PHA.**

PHA production in *Rhizobium meliloti* 14 was studied in reported media such as Yeast mannitol broth, Tryptone Yeast extract medium and Rhizobium minimal medium, in order to understand the nutrient requirement of the organism for PHA production. *Rhizobium meliloti* 14 gave PHA yields upto 2 to 3.5 g/l with sucrose and mannitol as carbon sources respectively. Accumulation of PHA was absent in the medium containing only yeast extract and tryptone indicating that nitrogen deficiency is one of the prerequisites for PHA production. By studies on the growth kinetics (in shake flask experiments) of the organism growth pattern and PHA accumulation in *Rhizobium meliloti* 14 were ascertained. The organism reached stationary phase by 24 hours and PHA accumulation occurred during the log phase itself and increased in the stationary phase. Stationary phase was observed upto 72 h. Accumulation of extracellular polysaccharide occurred at 36 h and reached a maximum at the end of stationary phase. It was also observed that both PHA and extracellular polysaccharide were produced simultaneously. As the carbon source supplied would was also be utilized for

polysaccharide production, it was necessary to obtain a mutant which would be a negative polysaccharide producer.

Rhizobium meliloti 14 required an optimum pH of 7 and temperature of 30^{o}C at 200 rpm for good growth as well as PHA production. Maximum PHA yields was obtained at stationary phase of the culture although the organism accumulated PHA during the logarithmic phase also. High aeration in the medium decreased the PHA levels suggesting the probability of oxygen limitation favouring PHA accumulation. The organism did not require any complex nitrogen sources such as yeast extract for PHA production. However, yields of biomass increased considerably with the addition of yeast extract in the medium. Inorganic nitrogen sources such as nitrates, ammonium salts and urea were preferred for growth and PHA production. PHA yield in ammonium salts such as ammonium phosphate, ammonium acetate, ammonium sulphate and ammonium chloride was 57.8%, 57.6%, 40.7% and 43.7% respectively in medium containing mannitol. Other major elements required for growth and PHA production were magnesium and phosphorus. The most favoured carbon sources for PHA production were mannitol and sucrose. The yields of PHA in sucrose were higher when urea was used as the nitrogen source. Acidity in the medium seemed to favour PHA production rather than alkalinity. Uptake of phosphate was high at low carbon concentration and phosphate depletion in the medium seemed to favour PHA synthesis. A carbon and nitrogen ratio of minimum 105 was necessary for high PHA yields when sucrose and urea were used. The highest

percentage of PHA obtained was 70.7% with a yield of 2.27g/l. **In order to eliminate or reduce the production of exopolysaccharide, *Rhizobium meliloti* 14 was subjected to mutation.**

Mutagens such as Novobiocin and K14-10- (4^1-N-Piperidinobutyl–2-chlorophenoxazine)(PBCP) were used for mutation. The chemical PBCP used in the experiment to treat *Rhizobium meliloti* culture was used as a plasmid-curing agent. After repeated treatment of parent strain with PBCP followed by screening, a mutant, which was less mucoid compared to the parent strain, was isolated. **Mutation of *Rhizobium meliloti* 14 resulted in a strain that accumulated nearly 10 % more of PHA. The exopolysaccharide produced by the mutant was 50% less compared to the parent.** Concentrations of exopolysaccharide synthesized by parent (*Rhizobium meliloti* 14) and mutant (*Rhizobium meliloti* 22) were 2.8 and 5.7 gl^{-1} respectively. Plasmid profiles of *Rhizobium meliloti* 14 and *Rhizobium meliloti* 22 showed the lack of a high molecular weight plasmid in the mutant. This could be responsible for less polysaccharide production and high conversion of carbon into PHA in the mutant. A highest yield of 3.7 g/l of PHA was obtained in the mutant. **Carbon uptake was significantly higher in the mutant (*Rhizobium meliloti* 22).**

A thorough comparison of biomass and PHA yields by parent and the mutant strains were carried out in shake flasks based on Response surface methodology. **The technique was extended to assess the nutrient limitation conditions favorable for PHA accumulation in bacterial cells and to**

compare the nutritional performance of the mutant (*Rhizobium meliloti* 22) strain with that of parent (*Rhizobium meliloti* 14) simultaneously with limited experiment using central composite rotatory design (CCRD) experiments. The medium optimised for PHA production for *Rhizobium meliloti* 14 by RSM was sucrose (g/l) 51.58, urea (g/l) 0.65, inoculum (ml/l) 10 and K_2HPO_4 (g/l) 0.48. Maximum yields of PHA obtained by the parent strain was 3.0 g/l (biomass 6.23 g/l) in the medium containing urea as the nitrogen source. Highest yields obtained by the mutant strain was **5.66 g/l** (biomass 9.29 g/l). Efficiency of produciton of biomass and PHA was more in the mutant compared to the parent. **A higher yield in the mutant strain compared to the parent was due to decrease in the exopolysaccharide production. Phosphate appeared to be the limiting nutrient favouring PHA production in parent (*Rhizobium meliloti* 14). Mutant showed requirement for both phosphorus and nitrogen unlike parent. The quantitative yield of PHA and carbon conversion efficiency of the mutant was consistently higher compared to parent when grown in medium containing urea as the nitrogen source. The carbon conversion efficiency of the strains could go as high as 0.6 g /g of carbon utilized, subject to the fermentation conditions. The PHA yields in both the strains exceeded 80% and reached 85 to 89 % depending on the cultural conditions. The highest PHA yield ranged between 5.5 g/l to 6.5 g/l**. Biomass obtained ranged between 6.0 g/l to 9.0 g/l.

Highest yield of 91% of PHA was obtained in (batch) fermenter trials. But the quantitative yields were 1.62 g/l, which was, less than the yields obtained in shake flask experiments. It was concluded that further optimization of the conditions at fermenter levels are necessary for scale up.

Characterization of polyhydroxyalkanoates was done by various spectroscopic methods such as the infrared spectroscopy, gas chromatography, GC mass spectroscopy and nuclear magnetic resonance studies. The polymer extracted from *Rhizobium meliloti* 14 (grown on sucrose as carbon source) was characterized as polyhydroxybutyrate with 3% valerate. The purity of extracted sample was checked by ultra violet spectroscopy and was confirmed as 96% pure. The IR spectra obtained showed characteristic absorption bonds for esters and the presence of C=O and C-O were obtained at 1724cm^{-1} and 1281cm^{-1} respectively. Apart from this a peak at 1377 cm^{-1} was seen which is due to the CH_3 or methyl bending. Peaks due to methyl stretching were also observed at 2975 cm^{-1} and 2926 cm^{-1} . CH_2 or methylene group was observed at 1450 cm^{-1} and methine or CH peak was at 3434 cm^{-1}. I R spectroscopy revealed the possible presence of a copolymer such as polyhydroxyvalerate in the sample and gas chromatography confirmed the presence of another polymer or a copolymer. **Gas chromatography also showed that the copolymer was about 3% of the total polymer content.** The methyl esters obtained after methanolysis of the sample showed fragmentation patterns in GCMS that enabled to define the structure of PHA obtained from *Rhizobium meliloti* 14. The ^1H NMR spectrum of

PHA showed three groups of signals characteristic of PHB: a doublet at 1.29 ppm which is characteristic of methyl group, a doublet of a quadruplet at 2.5 ppm which is attributed to methylene group and a multiplet at 5.28 ppm characteristic of a methyne group. A triplet at 0.9 ppm and a methylene resonance at 1.59 ppm and methyne resonance at 5.5 ppm indicated the presence of valerate in the polymer. The concentration of HV was however only 3% which corresponds with the GC data. Carbon resonances in ^{13}C NMR was (in ppm) at 169.143, 67.656, 40.864 and 19.787 for carbonyl, methine, methylene and methyl resonance respectively. **The ^{1}H NMR results confirmed the presence of valerate in the polymer.**

From differential scanning calorimetry (DSC) melting temperature of the polymer was analyzed. The melting temperature was 170^{o}C. A significant decrease of 6^{o}C was observed when compared to polyhydroxybutyrate sample obtained from Sigma. Decrease in the melting temperature is significant, as it is required for the thermal processing of the polymer. The viscosity average molecular weight of the sample was 5.63X10^{5}. **The extracted polymer could be easily casted into films. The solution cast films could be air-dried and they peeled off the glass plates into thin transparent clear films. The tensile strength of the film was comparable to polypropylene.** Tensile strength of the films ranged between 36.9 MPa and 41.1 Mpa and it could take a load of 18.5N. WVTR and OTR of the film was 30g/m^{2}/day and 476.37 (cc/m^{2} /day /atm 65% Rh and 27^{o}C) respectively. No noticeable change was seen in the polymer

composition of the parent (*Rhizobium meliloti* 14) and the mutant (*Rhizobium meliloti* 22) strain. The polymer obtained can be efficiently developed into a packaging film. **Further, inputs based on knowledge of polymer chemistry (blending) and food packaging can improve the film and can be developed as a food packaging material.**

Use of economic substrates and additional carbon sources were analyzed based on two aspects. Firstly the capabilities of the organism to utilize the carbon sources and produce PHA. Secondly, production of a copolymer such as valerate by the organism as a result of incorporation of the co-carbon sources. Keeping these factors in view various oils, fats, fatty acids, organic acids and other economic substrates were used as co-carbon source for enhancement of copolymer production by *Rhizobium meliloti* 14. ***Rhizobium meliloti* 14 did not require addition of extra carbon substrates to induce copolymer production. 3% of valerate was seen in the polymer extracted from the cells grown in medium containing sucrose as sole carbon source. PHB: PHV content of 81: 19 and 69.7% of PHA yield was seen when the medium was supplemented with rice bran oil. Rice bran oil is also an inexpensive source of carbon. PHB: PHV content was 90: 10, 95:5, and 98:2 when *Rhizobium meliloti* 14 was grown in medium containing pongamia oil, palm oil and stearic acid respectively.** Various organic acids such as pyruvic, citric, succinic, fumaric and malic acid were used as both additional and sole carbon sources and their importance in copolymer production was studied. The yields of PHA

were 72%, 73% and 74% when pyruvic, succinic and acetate were used. **PHA yield was 72 % with PHB: PHV content of 88:12 when pyruvic acid was added in the medium as a co-substrate.** Supplementing sodium butyrate also increased the HV content to 5.3% in the cells. **Overall the organism was capable of synthesizing PHA with high valerate content. This is highly significant, as increase in the valerate content will improve the material properties of the polymer.**

Various methods of extraction were tried for extraction of polyhydroxyalkanoates. Efforts were towards easier down stream processing after fermentation. Extraction by sodium hypochlorite was very efficient and the purity of the sample was 96%. Chloroform extracted PHA samples from *Rhizobium meliloti* 14 were 66% pure. Gas chromatography of solvent extracted samples showed the presence of 3% of PHV in the sample. Samples extracted by sodium hypochlorite showed (viscosity average) molecular weight of 7.39×10^5. Commercial enzyme such as protease from Novozyme was also tried. PHA recovered was 56.7 % based on the dry weight of the biomass used. The enzyme extracted samples showed a molecular weight (viscosity) average of 1.7×10^6. PHA from *Rhizobium meliloti* 14 cells after treatment by surfactants and chelating agents was 68% pure. A pH range from 10 to 11.5 was found to be effective for cell lysis in *Rhizobium meliloti* 14. PHA yield was 40.32% of dry weight of biomass when ammonium hydroxide was used for lysis and 17.6% when sodium hydroxide was used. Ammonium hydroxide was found to be more effective for extraction of

Rhizobium meliloti 14. Samples extracted from ammonium hydroxide showed a molecular weight (viscosity) average of 1.3 x 10^6.

A simple and effective microbial method of extraction of polyhydroxyalkanoates from bacterial cells has been developed. In this method lytic enzyme of an actinomycete (*Microbispora*) culture has been used to lyse *Rhizobium meliloti* 14 cells. The *Microbispora* culture grew directly on heat killed *Rhizobium meliloti* 14 cells and no extra nutrients were added for the growth of the lytic culture. The lytic culture formed pellets and it could be separated easily by filtration. PHA was released into the broth and was extracted by a minimum quantity of chloroform. PHA extracted by this method was 90-94% pure. Crude culture filtrate was also effective in lysis of *Rhizobium meliloti* 14 cells. The *Microbispora sp* produced lytic enzyme, which could solubilise, the bacterial cell material and hence release PHA. Scanning electron microscopic analysis of enzyme treated biomass of *Rhizobium meliloti* 14 cells, showed degradation of the cell. There seems to be an increase in the cell porosity and hence the cell shrinks due to leakage of cell contents. This was observed even in light microscopy where the killed cells of *Rhizobium meliloti* 14 appeared empty. Only the remnant of the cell wall, which was stained, was observed. The quality of PHA was also analyzed by Infrared red spectroscopy and gas chromatography and was found to be pure and showed the presence of valerate in the polymer. **In conclusion, the *Microbispora***

culture used, produced lytic enzymes, which could solubilise the bacterial cell material and hence release PHA.

Overall, during this study a potent strain of *Rhizobium meliloti* has been isolated, characterized and has been cultivated in optimized medium to produce upto 85% of PHA in its biomass. The strain was found to produce PHB-CO-HV at 97:3 mol% on sucrose as carbon substrate and HV concentration could be increased by 3-6 folds by using rice bran oil, pongamia oil or pyruvic acid as co carbon substrate. A mutant which could produce higher concentration and with better carbon conversion efficiency has also been isolated. The carbon conversion efficiency of 0.6 g PHA /g of carbon was obtained. A simple and easier microbial method of PHA extraction has also been worked out. The results indicated that the bacterium has great promise for the production of PHA copolymer for packaging application.

FUTURE STUDIES

The yield and productivity of polyhydroxyalkanoates by *Rhizobium meliloti* 14 needs to be studied at fermenter levels. Scale up studies by batch and fed batch fermentations with two stage fermentation strategy may be done on the lines of increasing the biomass in the first stage and limiting the culture with a nutrient that favours PHA accumulation. High cell density fermentation with further improvement on the carbon conversion efficiency of the organism may be done to develop an economical process. Feeding strategies to improve

copolymer production can be carried out based on the end product requirements. As the bacterium is producing measurable amounts of PHV on sucrose itself it will be interesting to analyze the metabolic flux and biochemical pathways involved which can be successfully utilized for further manipulations to obtain PHA copolymer on sucrose itself. This is a very potential area of research for economic production of PHA copolymer. *Rhizobium meliloti* 14 appears to be endowed with great potentiality for exploitation as a bacterium for the production of PHA copolymer, which is a promising biodegradable packaging material of the future.

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