STRATEGIES FOR ENHANCED PRODUCTION OF POLY HYDROXY BUTYRATE AND ITS BLENDING FOR NANOCOMPOSITE PREPARATION

A Thesis submitted to the Lovely Professional University

For the Award of

Doctor of Philosophy

In

Biotechnology

By

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(December 2015)

DECLARATION

I declare that the thesis entitled **"STRATEGIES FOR ENHANCED PRODUCTION OF POLY HYDROXY BUTYRATE AND ITS BLENDING FOR NANOCOMPOSITE PREPARATION"** has been prepared by me under the guidance of Dr. Anand Mohan, Associate Professor of Department of Biotechnology, Lovely Professional University**.** No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Madhuri Girdhar has prepared her thesis entitled **"STRATEGIES FOR ENHANCED PRODUCTION OF POLY HYDROXY BUTYRATE AND ITS BLENDING FOR NANOCOMPOSITE PREPARATION"** for the award of PhD degree of the Lovely Professional University**,** under my guidance. She has carried out the work at the Department of Biotechnology, Lovely Professional University.

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ACKNOWLEDGEMENT

Firstly I would like to give glory to God almighty for providing me enough patience, zeal and strength that enabled me to complete this work successfully. I would like to thank almighty for his grace and help in all my endeavours and for bringing me this far in my academic career.

My heartfelt thanks and all salutations certainly goes to my guide and mentor of my research career Dr. Anand Mohan for his constant inspiration, inestimable motivation, uncompromising regulations, professional constructive guidance, along with freedom to accelerate and complete the work on schedule. His continuous support during the writing period of the thesis and the pain he took to sort of my mistake was encouraging.

My sincere gratitude is reserved for my co-supervisor Dr. Ajay Sharma, Assistant Professor in Department of Chemistry, Lovely Professional University, for his inspiring guidance, kind co-operation and continuous help in all academic matters.

I express my sincere gratitude to Prof. Dr. Neeta Raj Sharma, Head of School, Department of Biotechnology, for extending me all the facilities to accomplish my task and continuously motivating me.

I acknowledge with gratitude to all the HOD's and faculties of School of Biotechnology and Biosciences, for their selfless suggestions, during the course of the present study. I am also thankful to Dr S. M. Bhatt for critical review of thesis manuscript and software support.

I would like to acknowledge the cooperation and encouragement of all my Lab-mates Mr. Anupam, Deepika, Kamal kant, Harshil, Roshni, Geetanshu, Neelam, Navjot, Praveen, Deep Chand, Parneet, Neha and Kajal who have been my true companions, during my stay at LPU.

I would also like to thank Division of Academic Affairs (DAA) for continuous guidance in matter of regulations, formats and scheduling dates of progress report submission. Special thanks goes to Dr. Jasdeep Dhami (Former Co-ordinator of Ph.D programme) and Dr. Anoop Beri (Current Co-ordinator of PhD programme), Mr. Bubble Kumar for guiding and encouraging me during my thesis submission.

I am also thankful to the staff of Smitha Lab, Indian Institute of Technology, New Delhi; Staff of Central Drug Research Institute, Lucknow, Staff of Soil testing Laboratory at Punjab Agriculture University, Ludhiana and Staff of Sophisticated Analytical Instrumentation

facility at Punjab University, Chandigarh for their timely help in providing the instrumentation facility. I am also thankful to Mr. Kuldeep Singh, Mr. Gajender, Mr. Satish Mishra, Mr. Amandeep, Mr. Mukesh and all the Lab staff of Department of Biotechnology for their constant support during my research work.

Special words of thanks to my dearest friends for their continuous loving support, encouragement and for being all time with me in all my endeavours Manjula, Swati, Abhilasha, Garima, Swarnendu and Abhinav. They never left me alone; they were always there when I wanted them.

In the journey of life there are many people to whom you can express thanks and can be grateful for their help but there are some to whom you cannot be able to thank throughout your life for what they have done for you, that is your family. My parents who, I will say have contributed directly to my career from class I till now. The continuous zeal of my parents to always see me ahead in life, to always excel in my educational career is the only reason that I am writing the acknowledgement of this PhD thesis. This is the greatest ever gift I can give to my parents and that's why this thesis is dedicated to them. The love, affection and support of my mother have been always with me whenever I go. She has continuously prayed for my well being and my success. The strength and determination of my father to work out the unworkable tasks for me has been unparalleled. My brother Parth has always a continuous supporter of mine, sometimes the hard work he does in his studies inspires me a lot. My blessings are always with him for his career.

Finally, I would like to acknowledge the Research and Development (R&D) Department of Lovely Professional University for sanctioning Internal Project Grant of 35000/ INR as a financial support during my research work.

Madhuri Girdhar

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PREFACE

Plastics are used in current sphere of everyday life. The replacement of plastic are called as bioplastics which are highly biodegradable and biocompatible. Polyhydroxybutyrate is an important polymeric biomaterial with many properties resembling plastic.

There are numerous applications of PHB which can be further increased by blending technology through the creation of nanocomposites based on PHB matrix and related industrially important polymers.

Major hindrance in these works lie in costly production of PHB and dearth of suitable nanocomposites.

Both of the above described areas have been investigated for cost effective production of PHB and suitable nanocomposite production.

In the current research, investigations have been carried on:

- Isolation and screening of PHB producing strains from agriculture and saline environment.
- Strain improvement protocols for isolated strains and standard strains of PHB production through mutagenesis.
- Optimization of media and large scale fermenter production of bacterial extracted PHB from improved strains and its characterization for quality.
- Exploration of pure form of PHB for production of nanocomposite biomaterial through mixing methods and its characterization through various nanomaterial characterization techniques.

This work will be helpful in production of low cost PHB material and applications in sutures production, 3D organ printing, Scaffolds making, biocompatible surgical threads, tissue engineering etc.

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INTRODUCTION

1.1: Plastics

Over the past century of industrial growth and anthropogenic development leads to profound dependency on plastics and plastic based products. Plastic has light weight, flexible and the most desired material utilized in day to day life. Synthetic plastics have come to origin and have gained importance since 1940s, from then have replaced glass and various other materials including metals in applications such as domestic, environmental and industrial (Lee, 1996; Poirier *et al*., 1995; Lee *et al*., 1991; Cain, 1992). There are array of ways in which plastics are utilized by human population *viz.,* packaging material, strengthened materials, electrical and electronics plates, chips, construction material, medical devices, bottles, sports materials and leisure products (Muthu *et al.,* 2012; Lajeunesse, 2004). Reliability on plastics depends on their excellent thermostability and other mechanical properties which enhances its durability (Rivard *et al*., 1995).

The molecular weight of plastic is 50,000 to 1, 00,000 Da. The high molecular weight of synthetic plastic increases its resistant to natural degradation and its persistence in soil for a longer duration (Atlas, 1993). The synthetic plastic is recalcitrant and xenobiotic to the environment and its was estimated to be accumulated in soil at the rate of 25 million tons/year (Lee *et al.,* 1991).

Global manufacturing of plastic for producing big automobiles to small packaging material is about approximately 260 million metric tons per year (Lazarevic *et al*., 2010; Khardenavis *et al.,* 2007). The European researchers have investigated and estimated that the synthetic plastics accumulation in the environment leads to an increase its value from 50000 to 100000 tons and it was also analysed that the value will surpass in the future years (Wolf *et al*., 2005). The observations of the current scenario states that if at this speed, the petroleum based products could be used in the coming years; it will take only 60-80 years for its complete utilization (Khare and Deshmukh, 2006). Synthetic plastics i.e. polypropylene and polyethylene are produced exclusively from non-renewable fossil based resources. The need of petroleum based fossils for the production of conventional plastics, in the time period of

2010-2015 is likely to be exceeds 300 million tons. Both production and after life remediation of plastic generates enormous amount of waste which is difficult to decompose and also generates green house gases along with other kind of health hazard problems in humans (Chen and Patel, 2012).

Plastic is defying any attempt of disposal approach i.e. recycling and burning as it is easily accumulated in landfills and oceans. Researchers investigated in USA and analysed that in the year 2006, the percentage of plastic material in landfills were found to be 12% (Khare and Deshmukh, 2006).

The adverse environmental impact and problem of solid waste management caused by the synthetic plastics, increases the demand of an alternative material that posseses comparable physical and chemical properties paralleled with conventional synthetic plastics (Patwardhan *et al.,* 2004). Growing ethical, legal and social issues in the European countries has banned various non-degradable plastics in various type of different consumer products, due to which the demand and development of biodegradable plastic has increased rapidly (Leaversuch, 1987).

In western world, a major part of fossil fuels were utilized by chemical industries for the production of plastics and synthetic polymers (Eggersdorfer *et al.,* 1992). In contemporary world petroleum based plastics are the main source of energy.

Several attempts were made for the recycling of plastic, but due to the problems associated with human health issues, the approaches were not proven successful. Relying on the limited and non-renewable energy sources, is one of the major problem. These non-degradable plastics are further accumulating in the environment which has become major global concern and cause of people's apathy (Andrady, 2011). Petroleum based non-renewable resources are diminishing because they are continuously consumed to meet the increasing demands of industries.

In the world, production of energy contributed 93% of fossil reserves, whereas only 7% are utilized by the chemical manufacturing industries including solvents and plastics (Eggersdorfer, 1992). The overall consumption of fossil fuel is influenced if a small part of

synthetic plastics are replaced with the biodegradable polymers produced from renewable resources. In US countries, the scientist estimated that the demand of bio-base polymers rise more than 15% per year to 200 million tons valued at $845 million in 2012 (PFFC, 2008). The use of biodegradable plastics could reduce this impact, because it is completely degraded by microorganisms (Gregory, 2009). The area of "Plastics produced from biological sources" is increasing rapidly due to the increase in information about continuous depletion of fossil reserves. The methods of biological conversion, for the production of bio-based plastics are currently given more concern by various researchers and scientists. The development of bio-based plastics which are further degradable should be adopted, because the petroleum based plastics are non-sustainable. The production of polymer is completely a biological process in which various natural materials i.e. including different fatty acids, starch, various sugars, different types of proteins and cellulose can be consumed by environmental microorganisms as a raw material and converted into various monomers. The bio-based polymers have unique properties and applications, which is a focal area of research by polymer scientists, medical experts and engineers. These biodegradable plastic is basically a product of multidisciplinary research efforts. The advent in application of biodegradable plastics could thus, significantly contribute to solve the menace caused by waste disposal and environmental pollution.

1.2: Biodegradable plastics

Ephemeral commercial goods are produced frequently through synthetic and semi synthetic polymers, which are being used in various industries and different types of environment based applications (Lee *et al.,* 2011; Poirier *et al.,* 1995). The attractive features of synthetic polymer are convenient and useful for human life. The non-degradable nature of synthetic polymer creates a major waste burden on the environment. The development of natural polymers or biopolymers solves the problem of waste management and also helps in the preservation of limited petroleum reserves. The environmental friendly, non-toxic and renewable nature of biopolymer, increases its applications in the area of environmental science. Bioplastics can act as a potential material to displace this ever-increasing waste of plastic and equally can be utilized in all applications. Merit of bioplastic over synthetic plastic, is its degradability with time and in certain cases non immunogenic nature which can be utilized for designing of medical implants and devices. Bioplastics consists of thermostable polyesters and are an important class of advanced biomaterial. Biopolymers have an advantage for being degradable under normal environmental conditions and their physio-

chemical properties are similar to synthetic polymers. Currently, biodegradable polymers such as Polylactic acid (PLA), Polyhydroxyalkoanates (PHA) and Polyhydroxybutyate (PHB) and cellophane have created a lot of interest for scientists and researchers. Biodegradable natural polymers are synthesized by various microbial species (Saharan *et al.,* 2007). Intracellular accumulation of granular molecules inside the microbial species occurs during unfavourable or starvation conditions (Witholt and Kessler, 2002). Due to the unique properties, members of PHA's family could be utilized in number of applications and have great prospective in the future (Saharan, 2012).

Repeating monomeric units are joined together through covalent bonds and defined as polymers. PHAs are basically defined as 3-hydroxy fatty acid molecule monomers that are attached end to ends with each other to form straight polymeric PHA molecule. 103-104 monomers join together to form the final polymeric structure. Byrom (1994), firstly described about granules of the PHA material which were about 8 to 13 per cell and reported that they have a diameter in the range of 0.2 to 0.5μm in the bacterium *Alcaligenes eutrophus*. PHA synthesis were reported to occurs inside bacteria during nutrient imbalance conditions, as higher carbon ratio with reduced nitrogen along with phosphorus and oxygen (Anderson and Dawes, 1990; Chen, 2009). PHA has been reported to be produced by almost 30% of soil thriving bacteria (Wu *et al.,* 2000). Most bacteria observed in sludge and in extreme environments are also known to produce PHA. Almost 90 genera of photosynthetic bacteria, archaea bacteria, lower eukaryotes, aerobes and anaerobes are capable of producing bioplastic (PHAs), along with PHB which is almost 80% of total produced PHA (Kumaravel *et al.,* 2010). The composition of monomers present, their physiochemical properties, size and structure depends on the organism producing the PHA material. PHA has different classes which are described on the basis of variance of groups and their position on the chain. PHAs could be defined as homopolymer or co-polymer and have almost 150 different constituents hydroxyalkanoate (HA), 3-hydroxybutyrate (3HB), 4-hydroxybutyrate (4HB), 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HHx), 3- hydroxydecanoate (3HD), Short chain-length (SCL), and Medium chain-length (MCL) (Loo and Kumar, 2007; Kadouri *et al.,* 2005; Berlanga *et al.,* 2006). These PHAs are biodegradable in nature and can be produced by utilizing renewable resource which provides cost effectiveness as compared to conventional synthesized plastics. Bacteria could also degrade PHA at a faster rate of 3–9 months through their own synthesized PHA depolymerases making PHA totally biodegradable (Jendrossek,

2001). The production cost of PHAs is a major drawback. The utilizing of low cost substrates can help in resolving the drawback of high cost production (Jiang *et al.,* 2008).

Properties of pure PHB could be compared well to commonly used bulk plastics, e.g. polypropylene and polyethylene. PHB is a unique natural biopolymer, which incorporates exceptional properties (Hrabak, 1992): (i)Thermoplastic or Elastomeric processability; (ii) Resistant 100% to water and moisture; and (iii) 100% biodegradability and biocompatible. The unique properties utilize the biodegradable plastics in several applications that are paralleled to the synthetic plastics and a suitable method for new waste-management strategies.

Renewable carbohydrates are being utilized by the researchers for the production of PHA. Bio-waste materials can be used effectively for PHA biosynthesis and this can prove to be a proficient strategy for enhanced cost subsiding production along with overcoming of disposal problems (Koller *et al.,* 2005a).

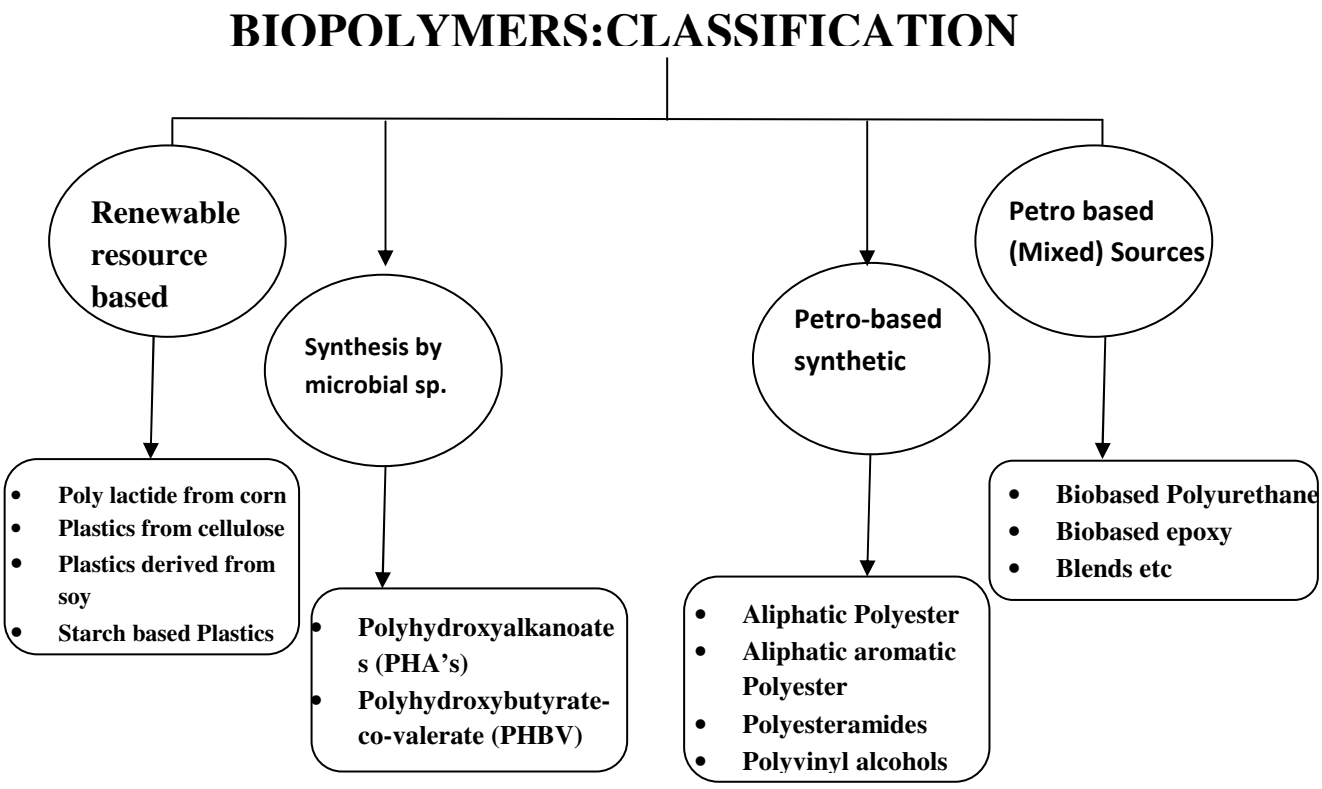


Figure 1.1: Classification of different biopolymers

1.3: Production of PHB a valuable Biodegradable Plastic from PHA class

The production of PHB polymers is an interesting area of research because these polymers can be synthesized through bacterial fermentation by utilization of renewable resources and other carbohydrate sources. PHB is recovered by various methods and bacteria synthesize them as energy and food reserve in granular form. This accumulation of PHB material in bacteria is basically a response mechanism towards variance in nutrient environment, where excess of carbon sources are used and limitation of other nutrient i.e. nitrogen, phosphorous etc., this physiological condition can be utilized for the production so excessive yields are achieved (Du and Yu, 2002b; Steinbüchel, 1991; Yu, 2001; Byron, 1994).

The PHB production requires utilization of carbon and nitrogen substrates, synthetic lab media like M9 media, DSC-97 (as synthetic lab medium) for purpose while natural sources such as palm oil, sugar cane, pea shell can also be used as biowastes material.

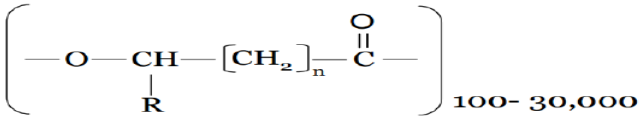
PHB becomes an encouraging substitute for the replacement of non-biodegradable plastic. It is biodegradable polymer which is produced in majority among the members of PHA family. It is highly biodegradable and biocompatible material that can serve multiple purposes *viz.,* firstly as a suitable material for reducing environmental pollution caused by use of non-degradable plastics; secondary use as a new type of biomaterial that can be utilized in formation of various biomedical products through enhanced blending processes. The uses and applications become seamless in commercial purposes *viz.,* packaging, paper coating, fertilizer, compost bag, controlled drug releases, bone fixation parts, tissue engineering products, 3 D organ printing and other varied product.

Development of various applications has taken place which includes biodegradable plastics which is environment friendly for packing applications, biomedical applications, body implants, guided nano drug release systems and bone tissue engineering. On the basis of these developments, microbial PHA became the basis of wide range of industrial applications which ranges from various fermentation products, advanced materials development, medicinal applications, and biofuel production along with fine chemicals. Numerous applications in this field are initiating intensive research globally. All over the world, loads of companies are involved for the commercialization of these developments especially in China, Korea and Japan (Chen, 2009a).

1.4: Structural description of PHB

PHB was first observed in 1926 in the bacteria *Bacillus megaterium* (Lemoigne, 1926). It was described as a storage material of bacteria which could be further degraded by depolymerases of bacterial cell and metabolized to produce carbon and energy as soon as the supply nutrient is restored. (Byrom, 1994).

PHAs is composed of R(-)-3-hydroxyalkanoic acid monomers which varies to butyric acid in case of PHB with C3 to C14 carbon atoms along with various saturated or unsaturated and straight or branched chain with aliphatic or aromatic side groups (Doi *et al.,* 1992; DeSmet *et al.,* 1983). The molecular weight of the polymer ranges at 2×10^5 to 3×10^6 daltons, depending upon the type of microorganism and the growth environment (Byron, 1994).



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)
	R = methyl	poly(4-hydroxyvalerate)
n = 3	R = hydrogen	poly(5-hydroxyvalerate)
	R = methyl	poly(5-hydroxyhexanate)
n = 4	R = hexyl	poly(6-hydroxydodecanoate)

Figure 1.2: Structure of PHA's: R-group and 'n' indicates various members of PHA's family (Ojumu *et al.,* 2004)

PHA's can be differentiated into two distinctive units depending on the length of the monomeric side-chains. Short-chain-length PHA polymers are made of 3-hydroxyalkanoic acids along with repeating monomer units of 4–5 carbons, comparatively medium-chain-length PHA polymers composed of repeating monomer units of C6-C14 carbons.

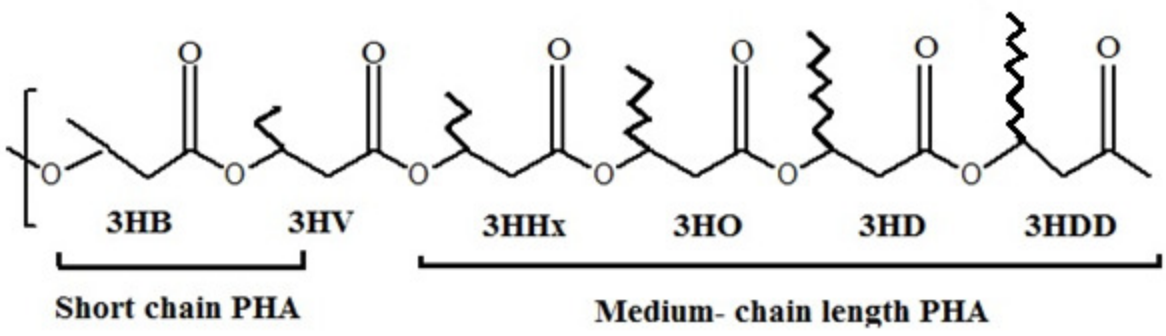


Figure 1.3: Different chain length of PHA's

PHA synthesis has high level of substrate specificity accepting only 3-hydroxyalkanoates (3HAs) of a specific range of carbon length (Anderson and Dawes, 1990).

There is a high level of flexibility in biosynthesis of PHA which makes possible for designing and producing related biopolymers with attractive physical properties and numerous applications which can range from stiff to brittle plastic or rubbery polymers (Anderson and Dawes, 1990).

1.5: Biosynthesis of PHB

Polyhydroxybutyrate accumulates in cells as separate granules, varying in size and number per cell varying depending on various species of organism. Granules can be seen as contrasting refractive inclusion through electron microscopy. The microorganisms accumulating PHA are very easy to identify by staining with dyes such as Sudan black or Nile blue (Schlegel, 1970; Ostle and Holt, 1982).

PHB granules are produced in metabolic pathway of microorganism and deposited as intracellular water-insoluble inclusions. Glucose remains the primary substrate for PHB production (Rehm *et al.,* 2001). During the pathway glucose is converted in to pyruvate and the reaction is catalyzed by various types of enzyme and ions. In this pathway, one molecule of glucose are oxidized into CO_2 and water to generate approximately 32 moles of ATP. Due to this, approximately 2870 kJ or 686 kcal energy is liberated. Pyruvate is converted into Acetyl CoA and which is further converted into Poly-β-hydroxybutyrate (PHB) through three step process. The biosynthesis of PHB is carried out by three enzyme, namely as 3-ketolthiolase (phb A), acetoacyl-CoA reductase (phb B) and PHA synthase (phb C) (Loo and Kumar, 2007; Madison *et al.,* 1999).

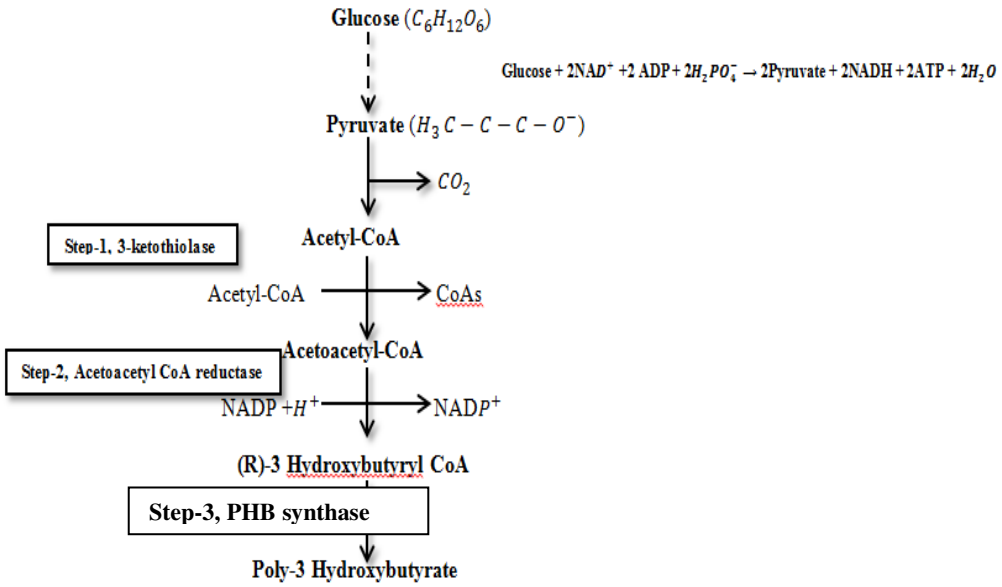
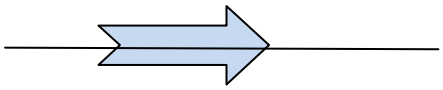
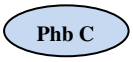
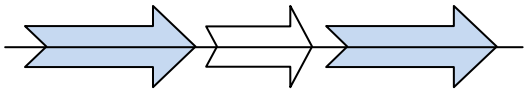
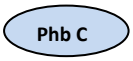
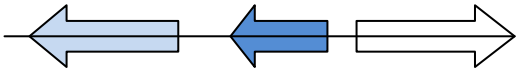
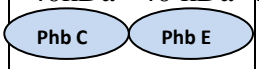
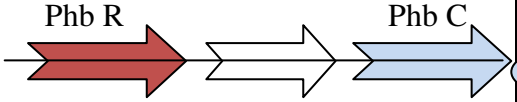
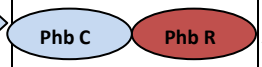


Figure 1.4: Biosynthesis of PHA from Glucose.

PHB can be synthesized both by chemical reactions and biological approaches as well (He *et al.,* 1999; Kemnitzer *et al.,* 1993). High molecular weight of polyhydroxybutyrate is produced by bacterial fermentation process (biological method) as compared to chemical based methods. Biosynthesis of PHB does not allow high level of control over the monomeric structures to be incorporated in the final PHB polymers; the specificity of PHB polymerase (or PHB synthase) will basically influence the monomers incorporated in the polymers (Chen *et al.,* 2004). Biosynthesis of PHB is basically carried out by microorganisms growing in aqueous environments containing nutrient resources such as starch, glucose, sucrose, fatty acids, and also nutrients contained in waste water under 30–37°C and normal atmosphere pressure. The synthesis could be considered as environment friendly and sustainable, as compared with non biodegradable plastic produced from petroleum which are non-renewable resource being depleted quickly.

PHB synthase genes (Phb C) are described in four groups as class I, II, III, IV respectively depending upon their substrate specificities and subunit compositions. The two subunit Pha C and PhaE (~40 kDa) or PhaR (~22 kDa), they compose class III and IV synthase (Lu *et al.,* 2008).

Table 1.1: There are four classes of PHB Synthases, PhbC (Rehm *et al.,* 2003)

Class	PHA synthase genes	Subunit	Species	Substrate
I	Phb C 	~60-73 kDa 	*Cupriavidus nector*	$3HA_{SCL}$-CoA(~C3-C5) $4HA_{SCL}$-CoA,$5HA_{SCL}$-CoA,$3MA_{SCL}$-CoA
II	Phb C1 Phb C2 	~60-73 kDa 	*Pseudomonas aeruginosa*	$3HA_{MCL}$-CoA (~>C5)
III	Phb C Phb E 	~40kDa ~40 kDa 	*Allochromatium vinosum*	$3HA_{HCL}$-CoA (3HAMCLCoA[~C6-C8], 4HA-CoA,5HA-CoA
IV	Phb R Phb C 	40 kDa~22kDa 	*Bacillus megaterium*	$3HA_{HCL}$-CoA

1.6: Microbial production of PHA by wild type strains

PHB has been isolated from soil bacterium. There are variety of strains that have been reported to produce PHB e.g. *Alcaligenes latus, Bacillus megaterium* (Ojumu *et al.,* 2004) *Pseudomonas oleoverans, Ralstonia eutropha, Aeromonas hydrophila* and *Pseudomonas stutzeri* (Chen, 2009). Salt stress conditions also influence the quantity and the quality of PHB produced by *Cupriavidus nector* strain (Passanha *et al.,* 2014)

1.7: PHB from Halophiles

Halophilic microorganisms can be utilized for the PHB production system. They have advantage of capability to grow optimally at high salt concentrations (Quillaguamán et al. 2010). High concentrations of salt prevents the growth of nonhalophilic microorganisms, which allows for an optimal process where strict sterile conditions are not required reducing the overall costs. The fermention technology includes sterilized equipments and culture media, which requires a huge cost expenses. By using Halophiles, the fermentation process becomes cost-effective (Quillaguaman *et al.,* 2010).

Major Halophilic strains synthesizing PHB are *Haloferax mediterranei, Halopiger aswanensi s,Heloferax alexandrines* (Tekin *et al.,* 2011). Halomonas spp., have been the candidates for production of diverse products used in various industrial fields (Quillaguamán *et al., 2010).*

1.8: Enhanced production of PHB through media optimization and strain improvement

Optimization of different parameters i.e. media, temperature, aeration, pH, incubation time, different carbon sources, nitrogen sources and NaCl concentration become highly important in the process of high PHB production (Flora *et al.,* 2010). Elsayed *et al.,* 2013 worked on *Azomonas macrocytogenes* and enhanced the production of PHB through modifying medium concentration of glucose and potassium nitrate. Enhancement was reported from 24% to 42% of total cell dry weight. In the same way many more workers have reported enhancement in PHB production through optimization of above defined key parameters. Enhancement of PHB production can be carried out by reducing culture time, increasing cell density and finally overall PHB content has to be taken into consideration. High-cell density cultures increase productivity and reduce the cost of downstream process and wastewater treatment. In order to achieve a high cell density and PHB yield, batch cultivation with continuous supply of fed has been the most popular fermentation system (Kirithika *et al.,* 2011).

Fed-batch culture has been the most popular culture system to achieve a high-cell-density and PHB content (Suzuki *et al.,* 1986; Kim *et al.,* 1992; Lee, 1994; Kim *et al.,* 1994). However, besides more complicated operation and increased susceptibility to contamination, fed-batch cultivation relies on high cell dry weight accumulation leading to low growth rates before induction, which sometimes prolongs the time needed for production (Shokri *et al.,* 2004). Comparatively for batch cultivation, bacteria can be inoculated in a stirred tank bioreactor under certain predetermined basic conditions (temperature, aeration, pH etc.) going through various growth phases (lag phase, exponential phase, stationary phase and decline phase). At the end of fermentation process, microbial cells are collected. The fermentor becomes ready for another batch after cleaning and sterilization process. Batch culture offers certain advantages making it comparable to fed-batch culture. It could be utilized for different fermentation reactions and can be sterilized which minimized the risk of infection or strain mutation. Batch culture also incurs high labour cost and extended idle time (Nielsen *et al.,* 1994).

Mutations, both chemical and physical, are major experimental protocols that have been used to improve industrial strains from long time. Few workers have worked for the enhancement of PHB production through mutagenic modification of strains. The common mutagenic agents that have been used for the purpose are UV radiations, Acridine orange, Ethyl methane sulphonate, Acriflavin etc. Many strains till date after mutagenesis experiments have been improved and have reported enhanced production of PHB eg. *Bacillus megaterium Y6*, *Bacillus subtilis K8* and *Bacillus firmus G2* (Katircioglu *et al.,* 2003).

1.9: Problems associated with Commercial Exploitation of PHB

Enhanced production options through various strategies can provide production at large scale but there are still certain problems associated with commercial exploitation of PHB. The problems are:

- Polyhydroxybutyrate is stereoregular isotactic polyster, which is completely biodegradable in nature. It has been analysed that bacterial PHB shows an outstanding crystallinity of over 80%, but have high brittleness and poor impact resistance at room temperature.
- The molten state of PHB is unstable and thus its processing window i.e. the melting temperature and the degradation temperature is very much close (Td±Tm). The narrow processing window makes it very difficult to work and thus is a major drawback associated with its commercial exploitation. Traditional thermal methods i.e. injection moulding and extrusion are for the processing of Bacterial PHB. Great efforts are often required to control the processing temperature precisely.
- The processing and production cost of PHB is very much high, which act as a barrier for its commercialization and restricts the manufacturer to increase its utility and as a substitute of synthetic plastics.

To overcome the above shortcomings and enlarge its practical applications, much work has been done to modify PHB, through physical blending. Physical blending is a simple, easily carried out, and effective method to modify PHB. By blending other polymers with PHB, materials with improved properties are often acquired depending on blend component and composition.

Out of all these blends degradable blends are of considerable importance, as with biocompatible PHB, many of the blend components also have biocompatibility. This feature provides the possibility of applying them in medical fields, such as surgical sutures and drug delivery along with applications in other commercial fields.

1.10: Nanocomposite

Nanotechnology is the science of 21st century promising greater technological development at nano level. Wide varieties of "nanomaterials" are being developed with restricted size and composition. The main objective of the studies is to improve the mechanical, electrical, physical, optical, and other related properties. Nanocomposites are multiple phase solid substance having one, two or three dimensions of less than 100 nm. It can involve porous media, gels, copolymers and colloids but is more appropriate for combination of matrix and nano-phase. They have different categories based on size (Kamigaito, 1991), less than 5 nm is used for catalytic activity, less than 20nm is for producing hard magnetic substance into soft, less than 50nm is for changes in refractive index while less than 100nm is for producing supermagnetic effects, etc.

They are abundantly found in nature, in abalone shell and in bones. Since mid-1950, organic clays of nano origin are employed in checking flow of solutions of polymer. In 1970, the clay composites became quiet common but the exact term nanocomposite was not being used for such improved materials (Theng, 1979).

1.11: Types Of Nanocomposite

1.11.1: Ceramic matrix nanocomposite

The major part in this is ceramic, which is a chemical substance belonging to the group of nitrides, oxides, silicates, borides and many more. There second component is a metal. Both these components, i.e., metal and ceramic are properly mixed with one another to form a nanoparticle. Such nanocomposite have enhanced optical, magnetic (Kruis *et al.,* 1998) and electrical features and are resistant to corrosion and possess various other protective properties (Zhang *et al.,* 2003). The best examples of these are of titanium oxide (ceramic) and copper (metal) (Effenberg *et al.,* 2001).

1.11.2: Metal matrix nanocomposite

They are reinforced and are either continuous or non-continuous in nature. The most used example is carbon nano tube. The metal matrix nanocomposite are having high tensile strength and electrical conductivity. Other examples include boron nitride and carbon nitride metal matrix composites (Bakshi *et al.,* 2010). One more type of nanocomposite have high energy, existing as mix of sol-gel having base of silica, which on mixing with oxides of metals and aluminium powder at nano scale leads to manufacture of super thermal substances (Ryan *et al.,* 2008).

1.11.3: Polymer matrix nanocomposite

The addition of nanoparticles to a polymer matrix enhances the nature and features of the polymeric matrix and is termed as nano filled polymer composites (Manias and Evangelos, 2007). The improved material shows a high performance and stability along with novel physical properties, which were earlier absent in pristine polymer (Manias and Evangelos, 2007). Many investigations were carried out on the polyhydroxybutyrate and its co-polymers, in order to improve its short comings both at physical and mechanical levels. The PHB nanocomposite shows improved properties which are paralleled as compared with synthetic plastics and become commercialised easily. Example includes Polyhydroxy butyrate blend with montmorillonite (nanoclay) matrix (Zubirdikudis, 2007).

1.12: Blending of PHB and Polymer Nanocomposites

In material research, development of polymer nanocomposites could enhance the polymer applications in different fields of science and technology. Polymer based nanocomposites are considered as the next industrial revolution materials. The significant commercial applications of Polymer nanocomposite, provides an alternative to the synthetic plastic based materials (Camargo *et al.,* 2009).

Clay minerals are abundant natural material found on earth surface. Clay material have size less than 2 µm possessing defined properties of slip formation, rheology, ion exchangeable and plasticity within the nanosized silicate layers. Montmorillonite (MMT) consisting of 2:1 aluminosilicate layers, composed of an octahedral sheet of aluminium oxide sandwiched between the tetrahedral sheet of silicate layer having free cations present on its top. (Bailey,

1984; Brindley and Brown, 1980; Mittal, 2009) In the octahedral layer, aluminum atoms are replaced with other cations (e.g., magnesium, iron), which creates some charge defects in the structure and acts as an inorganic ion exchange material (Morgan, 2011). The high percentage of MMT is present in Bentonite clay. Modification done at chemical and structural level for raw bentonite clay makes possible the preparation of new nanostructural material which can be suitable for varied applications. The virgin material does not produce combined properties and synergestic effect, which could be produced by the blending of polymer-clay nanomaterial and would ultimately yield a better nanocomposite with improved properties.

There is plethora of methods available for production of polymer/clay nanocomposites by simple blending of inorganic clay with the polymer in order to get the required properties. (Lagashetty and Venkataraman, 2005). The method of in-situ-polymerization involves the polymerization of monomeric units in which inorganic material acts as a filler inside the swollen galleries of polymeric matrix, where solvent system plays a very important role (Ray and Okamoto, 2003), melt intercalation of polymers etc (Wu *et al.,* 2006; Pluta *et al.,* 2006; Thakur and Thakur, 2015).

For the preparation of nanofibres at nanoscale, the technique of Electrospinning has attracted the interest of many industrial and academic scientists. The estimated volume of polymer blends available world widely is approximately more than 7000,000 metric tons per year. It has been also analyzed that the average growth rate for the preparation of such kinds of improved material is 6-7% per year. The adjustment of cost-performance balance and altering the technology for the preparation of products, which are highly specialized for end use applications in the field of medicine, preparation of biodegradable scaffolds and sutures, controlled drug delivery, biosensors, photoconductors etc. All these possibility are offered by the Polymer blending technology (Zhu *et al.,* 2000; Berkland *et al.,* 2001; Messersmith and Giannelis, 1993; Mu and Feng, 2001; Oriakhi and Lerner, 1995; Rosca *et al.,* 2004), enhancement in the performance of resin's, improving specific properties, viz. tensile strength., and also solve the problem of waste management.

Polymer nanocomposites become a unique type of engineered material, in which the polymer thermal, mechanical, optical and electronic properties could be improved by the addition of inorganic particles (nanoclay as filler) at low loading rates. Due to nanoscale size of synthetic

inorganic particle, along with their high chemical activity and reactivity, they are hazardous to the environment as well as living organisms (Ray and Okamoto, 2003). Naturally derived nanoparticles such as cellulose nanoparticles from wood pulp (Mitra *et al.,* 2014) and Poly hydroxybutyrate from microorganisms are used for the preparation of nanocomposites. These methods could be useful in overcoming the major drawback caused by the synthetic inorganic nanoparticles. The resulting nanocomposite may have desirable properties and lesser environmental impact.

Polyhydoxybutyrate is a hydrophobic polymer and is one of the rarest example, which has unique properties i.e. completely biocompatible and biodegradable in nature (Jirage *et al.,* 2013). For the end use applications of PHB, blending technology is utilized to improve its properties both at thermal and mechanical levels. The biodegradable materials such as nanoclay, resins, other polymers acts as a filler and intercalated in the swollen galleries of polymeric matrix, the surface morphology is analyzed by the sophisticated scanning electron microscopy technique.

Blending provides numerous opportunities as it involves mixing of characters of parental material and different blends are achieved with increased application. Different blends and nanocomposites are achieved with the process. The surface morphology and group characteristics of the individual parents are important factors for the preparation of successful polymer based nanocomposites. Novel exciting materials with new combinatorial properties are expanding rapidly in the area of science and technology. These advance material possesses properties of their parental material which with combined interactions at nano levels can have numerous applications (Yu *et al.,* 2006).

Nanoscopic inorganic particles of size 10-100nm in at least one dimension, are uniformly diffused in the matrix of organic PHB polymers matrix, resultant into the formation of PHB based polymer nanocomposites (Scott *et al.,* 2004; Ray and Okamoto, 2003). Coventional filled polymers are being replaced by PHB based Polymer nanocomposites, because its properties are comparable with the properties of traditional composites. The resultant nanocomposites shows an improvement in the surface morphology, it has been analysed that the increase in porosity of the nanostructures, increases its end use applications in the field of medicines particularly for cell proliferation and differentiation. The properties such as tensile

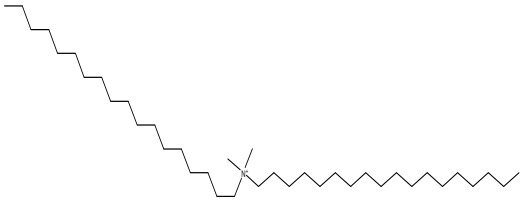
modulus and strength, heat resistance, outstanding barrier properties are also improved by the incorporation of nanofillers in the polymeric matrix (Bockstaller *et al.,* 2005; Iyer *et al.,* 2010; Iyer *et al.,* 2008, Lagashetty and Venkataraman, 2005). Organo-modified montmorillonite nanoclays are utilized for the development of PHB based nanocomposite by blending process. These nanocomposites have improved physical and mechanical properties i.e. glass transition state, stress-strain, tensile strength etc.

1.13: Nanoclays and Surface modified clays

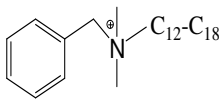
Clays, such as montmorillonite (MMT), belong to the structural family of 2:1 phyllosilicates. They have a unit layer which includes octahedral sheet of aluminium oxide or magnesium oxide sandwiched between two tetrahedral sheets of silicate oxide (Marras *et al.,* 2007; Swartzen and Matijevi, 1974).

Silica and aluminum atoms present on the adjacent sheet of MMT are bonded together by sharing of oxygen atoms. The surface of inorganic minerals is hydrophilic in nature due to the presence of hydrated cations and is not a suitable solvent for organic compounds. the adsorption of organic cations altering the properties of inorganic minerals are used to synthesized the organo-modified clays (Burgentzle *et al.,* 2004).

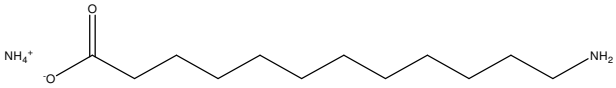
There is no formal interaction occurs between the anionic charge defect in the octahedral layer with the cations present on the top of silica layer (Gronski *et al.,* 2000). These cations can have the ability to exchange with the other cations of similar charge defects. The below figure shows the sodium cation can replace the the ions with alkyl ammonium, phosphonium, imidazolium and other +1 cation to yield orano-modified clays (Ogawa and Kuroda, 1997; Blumstein, 1965). The montomorrillonite should be organically treated, otherwise its dispersion in polymeric matrix would never be possible and preparation of successful polymeric nanocomposite will never be achieved. The virgin MMT is micro-sized in nature, serving as traditional filler and in order to make it nano sized, it should be organically treated. The most commonly used organic treatment is an alkyl ammonium with variety of chain lengths or functionality present, and can be a quaternized primary, secondary, or tertiary amine. The one common feature to a successful clay organic treatment is the presence of at least one long (12 carbons or more) alkyl chain, as without this microcomposites are typically obtained (Zilg *et al.,* 2000).



Dimethyl di(octadecyl) ammonium



Benzyl dimethyl alkyl ammonium



12-aminododecanoic acid ammonium salt

Figure 1.5: Typical alkyl ammonium organic treatments

Expansion of interlayer gallery and lowering of clay energy surface is caused by the presence of surfactants providing the compatibility of clay with the organic liquids (Burgentzle *et al.,* 2004) or polymers (Giannelis, 1996).

These properties of Surfactant-treated layered silicates have attracted significant attention of various researchers and scientists.

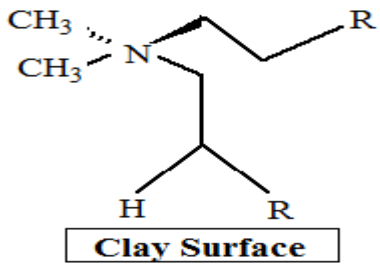


Figure 1.6: The structure of organo-modified clay with alkyl ammonium

1.14: Clay-Nanocomposite preparation

Diffusion of organo-modified clay into the polymeric matrix surface at nano-level leads to a substantial improvement in the physical properties of polymeric matrix (Marras *et al.,* 2007). For the preparation process, dispersibility of clay into the polymer matrix is of much importance. Proper dispersion of silicate layers nanoclays is of fundamental importance in the

designing of biomaterials with desired properties, which can be utilized for number of applications (LeBaron *et al.,* 1999). Various factors are responsible for the preparation of a successful nanocomposite (molecular weight, presence of reactive groups and hydrophobicity of polymers and the type of solvent system used for the blending, i.e., polar, or non-polar organic liquids and clay mineral type) (Scott *et al.,* 2004). The above factors are responsible for the different interaction mechanism (dropping of pressure into the nano-silicate layers, homogenized mixture of polymer and clay, presence of hydrogen, electrostatic and coordination bonding etc) and in the preparation of successful polymer-clay nanocomposite. Polymer layer silicate nanocomposites are prepared by plethora of methods. The major techniques are: in situ intercalative polymerization, melt intercalation process and exfoliated adsorption method (Marras *et al.,* 2007).

1.14.1. In situ intercalative polymerization

The in-situ polymerization involves the liquid monomer, which leads to the swelling of layered silicate (i.e. MMT) and further polymerization will be carried out by heat or radiation. Suitable organic initiators are an another option for the polymerization process (Usuki *et al.,* 1993; Alexandre and Dubois, 2000; Rehab and Salahuddin, 2005; Beron, 1999; Halvatty and Oya, 1994; Hussain *et al.,* 2002). For example: Nylon-montmorrillonite was successfully made by the approach (Kawasumi *et al.,* 1997). Various thermoset-clay nanocomposites are prepared from this method (Lan *et al*., 1995). The advantage of this method is its tethering effect. In-situ polymerization method enables the organic chemical i.e. 12 aminododeconoic acid (ADA) present on the clay surface, acts as an intermediate to link the nylon 6 polymer chains during polymerization. Thus helps in the preparation of a successful nanocomposite.

1.14.2: Exfoliation-Adsorption

The process is based on the solvent system in which the polymer is completely soluble and the silicate layers of the nanoclays are swellable. Dispersion of layered silicates in adequate solvents such as water, acetone and chloroform can be easily possible due to the present of weak interaction forces between the clay stacks. The adsorption of polymer occurs onto the delaminated sheets and reassembling of sheets occurs when the solvent is completely evaporated. The polymeric material then sandwiched between the stacked layers. Epoxy-clay nanocomposite are successfully synthesized by this strategy (Lee and Jang, 1998). The main disadvantage of this approach is that large amount of solvent is utilized and removal of

solvent is a critical issue (Kornmann, 2001). Due to the dispersion of layered silicates in aqueous solution, emulsion polymerization is carried out (Rehab and Salahuddin, 2005). Polyimide nanocomposites are produced by this method implemented by the Toyota Research Group (Alexandre and Dubois, 2000; Yano and Usuki, 1993).

1.14.3. Melt Intercalation: This technique does not require solvent system for the preparation of nanocomposite (Burnside and Giannelis, 1995; Rehaband Salahuddin, 2005; Vaia *et al.,* 1996; Dennis *et al.,* 2001). This method involves the mixing of silicate within the polymeric matrix in the molten state. Conventional methods such as extrusion and injection moulding are used for the mechanical mixing of thermoplastic polymer (Kornmann, 2001). Nanocomposite are formed by the intercalation or exfoliation of polymer chains. This technique is mainly utilized for those thermoplastics, that are not utilized in situ polymerization and adsorption strategies (Ray and Okamoto, 2003).

1.15: Characterization of Nanocomposites

Florescent Emission Scanning electron microscopy (FESEM), Differential Scanning Calorimetry (DSC), Thermo-gravimetric analysis (TGA), Instron tensile testing are the major techniques which are utilized for the elucidation of physical, mechanical and nano-level properties of the prepared nanocomposites.

1.15.1: Differntial Scanning Calorimetry (DSC)

For the measurement of various phenomena occurring during the thermal heating of polymer nanocomposite, Differential Scanning Calorimetry has been widely used. The various parameters such as upper working temperature, melting temperature, enthalpy of melting (ΔH_M), Crystallization temperature on heating [$T_{hc}(^oC)$], Enthalpy of crystallization temperature on heating [ΔH_{hc} (J/g)] and glass transition temperature (T_g) are analysed by sophisticated DSC technique (Corcione and Frigione, 2012). The information of melting and crystallization transition, along with the extent of crystallization covered under the area of melting transition (melt enthalpy) are analysed by measuring the peaks generated by the DSC technique. The effect of nanoclays on the crystallization behaviour of virgin polymeric matrix for the preparation of a new improved material can be easily analysed. The organic fillers are being modified by chemical synthesis in order to obtain the surface modified organo-clays. The DSC technique has been used for the characterization phase transitions and dynamics of

the modified surface of organo-clays (Mittal, 2009). Many phenomenon that occurs during the thermal scanning of Polymer/clay nanocomposites are easily analysed by Differential scanning calorimeter. The dispersion of nanoclay filler brings peculiar changes in the properties of nanocomposites, when compared with virgin polymer.

1.15.2: FESEM

The particle size, its distribution and surface porosity are determined by Florescent Emission Scanning electron microscopy (FESEM). The SEM used for this study is capable of differentiating particle detail as small as 1 nm depending on elemental contrast and other parameters. Due to the non-conductive nature of most of the polymers including PHB, instabilities, false x-ray signals are some of the problems faced during the FESEM analysis. Charging is a condition during which charge gets accumulated on the polymeric surface. Poor quality of images due to excessive brightness is produced during the charging of non-conductive surface. Fine gold layer coating is required to avoid the issues and to analyse the polymeric surface with good quality images (Lu *et al.,* 2005).

The basic measurement of nanocomposites quality as a final material includes various parameters such as interfacial de-bonding, aggregation of particles, presence of cracks, porosity and voids formation are analysed by Scanning electron microscope. The particle dispersion, its orientation and exfoliations are the important parameters required for the preparation of successful nanocomposite are characterized using scanning electron microscopy (SEM) (Sheiky *et al.,* 2010).

1.15.3: Thermal Gravimetric Analysis

The change in the decomposition temperature and the effect on the weight loss of nanocomposite by the addition of nanoparticles are determined by thermo-gravimetric analysis. Function of temperature and time plays a very important role in measuring the weight of sample by TGA. The change in the wt% of sample due to the process decomposition on heating is corresponding at specific temperature (Torre *et al.,* 2006). The attachment of nanoparticles onto the surface of polymeric material corresponds to the amount of weight loss experience by the nanocomposite during decomposition experiment.

Thermal decomposition behaviour of nanocomposite at different loading rates is measured by TGA. Thermal stability of the nanocomposite is also affected by the presence of inorganic clays at different concentrations (Corcione and Frigione, 2012). Density and interphase bonding are also confirmed by this sophisticated technique (Ciprari *et al.,* 2006).

1.16: Current Work

Current work involves development of strategies for the production of PHB in economical way. Various media modification protocols, mutagenic induction protocols, optimization protocols will be utilized for the strategies on efficient and economical production of PHB from various PHB producing isolates and already established strains acquired from strain banks. Further work will lay emphasis on preparation of blends and nanocomposites of PHB with organo-modified nanoclays for improvement of its physical properties and enhancing its properties at nano-levels.

REVIEW OF LITERATURE

2.1: Plastic and its Environmental Impact

Plastic, made up of chemicals, is very hazardous for the environment and additional energy is also required for its extraction and processing. These xenobiotic plastics are now posing a great threat to humanity and other life forms by contaminating the land, water, soil and overall environment (Luengo *et al*., 2003). It is almost non-biodegradable and has recalcitrant in tons every year due to all types of anthropogenic activities. Synthetic polymer i.e. plastics are utilized in every manufacturing industries i.e. printing, coating, food packaging, building construction products, toys, electric wires, and medical devices i.e. gloves, synthetic catheters etc. The structure of these polymers can be easily modified chemically and morphologically to different shapes and of varying strength, as a fibre or as thin sheets to suit array needs. This unique property of modification increases the widespread application of synthetic plastics in different fields. It has high durability and is resistant to various chemicals (Atlas, 1993). In 1862, the man made plastic named as "Parkesine" was announced by Alexander Parks at International Exhibition in London. This synthetic plastic was chemically made up of cellulose nitrate. In 1950's, many products were introduced and the major growth of plastic manufacturing based industries affected the lives of people, by introducing improved packaging material to chemically based textile goods (Flechter, 1990). With course of duration more development has taken place in plastic goods manufacturing industries both in terms of technology and quantity of production. This upsurge in technological development has led to the production of halogenated plastics which are technically totally indestructible even under higher temperature and variety of conditions. It is very tedious task to dispose these types of plastic wastes after their shelf life is completed. Due to the increased demand year by year and time needed for its degradation which is unknown, it's become an issue of global concern.

The third largest manufacturing industry is the plastic industry reported by Society of Plastics Industry, Washington D.C. In 1980, the average growth rate of plastic is 3.4%.

Kurupaalil in 2010 investigated that around 1.1 million workers were employed in plastic industries and provides nearly $379 billion in shipments every year. Evan's in 2010 also observed that the recycling of common synthetic plastic i.e. polyethylene, polystyrene require more cost as compared to the production of new plastics

The statistical analysis of Environmental Protection Agency (EPA) concluded that majority of waste are originated from plastic industries especially the food packaging industries reported by Municipal solid waste in United States. In 2007, approximately 30.7 million tons of plastic waere generated, which accounts for almost 12.7% of total Municipal Solid waste (MSW). It was also reported that less than 7% of the plastic waste was recovered i.e. 28.6 million tons (Municipal solid waste in the United States, 2007). Average usage of plastic per person was 100 kg/year in European countries investigated by Mulder in 1998. The worldwide production of synthetic plastic in the 20th century is approximately reaches 130 million t/year reported by Fomin in 2001.

The man made plastic being a xenobiotic, becomes highly resistant to microbial degradation (Atlas, 1993). Therefore, much more efforts are required for the development of natural or biodegradable polymers and to provide a sustainable benefit to both the environment and living beings (Willett and Shogren, 1995).

2.2: Green Plastic

Naturally derived renewable resources including Green plastics are the topic of interest for contemporary scientists as it is a substitute of traditional chemical based plastics. The green plastic must have following properties i.e. it should be derived from renewable source, biodegradable in nature and must be eco-friendly (Stevens, 2003). Biopolymers are the plastics that are biodegradable and are produced from bio-based source (i.e. they are produced from renewable sources. Biodegradability of the plastic depends on their chemical structure; some plastics are bio-based but are non-biodegradable. There is a strong carbon-carbon single bond existence in the structure of certain polymers i.e. polyethylene are derived from renewable masses such as sugarcane. The strong bonding of carbon in their structure makes it non-degradable, yet it is recyclable and is produced from bio-based source (Phillips, 2008). It was reported in literature that the share of biopolymers in the market is less than 1%. In year 2015 the expected growth rate of the biopolymers is almost just over 1%. The polymers whose physical and chemical properties undergo deterioration and are completely degraded by microbial species are termed as biodegradable polymers. The biodegradable nature of

polymers is due to the inherent oxygen or nitrogen atoms in their backbone chain. It was investigated by American Society for Testing and Materials (ATSM) that biodegradable polymers could be classified as compostable material only, if it should yield carbon dioxide, inorganic compounds and water at the rate paralleled with the other known compostable materials (Stevens, 2002). For number of years, the development of bio-based innovative polymers has been in progress and a topic of interest by various nanotechnologists, material designers and advance material developers. The data analysis shows that the shipments from Canadian bioplastic industry enhanced by 10.6% from 1995 levels to $9.1 billion in year 1996 (Charron, 1999). Leaversuch in 2002 reported that the demand for biodegradable plastic per year was increased by 30%.

Economic concerns for the development of biopolymers must be taken in concern as the cost competitiveness and the ability of the society to pay for it is the major factors on which the future of the product depends. Sustainability is also a major factor connected directly with development of the product. Biodegradable biopolymers are the source materials that can solve problems, both related to environmental concerns and can also be significant in numerous other fields. They can replace plastic either alone or by blending with materials which can enhance their important physical properties. Polyhydroxyalkanoate is a family of such type of biodegradable biopolymers (Luengo *et al*., 2003).

There is burgeoning need of biodegradable eco-friendly materials and solid waste management to overcome the current menace.

These materials are easily processible, resistant to water and retain their individuality during normal conditions and degrade completely in biologically rich medium i.e. in human body fluids. A biodegradable polymer is one, which is completely degraded by living organism and converted to carbon dioxide, water and humid material (Vroman and Tighzert, 2009)

Biopolymers came as a solution to the problem and is now emerging a new research field. These polymers can reduce the use of plastic to an extent. Biologically based polymers studies are being encouraged by many governments by introducing many programmes and workshops on the designing and development of bio-based materials/green plastic. These areas of biodegradable polymers are being supported by the government of Europe and North America along with the German government giving more interest (Grigat *et al.,* 1998).

The bio-polymer development has been accelerated worldwide so as to find the alternative of fossil based synthetic plastic. In recent years, there is an increase in the number of publication citations on the subject of green plastic or polymer reported by ISI. Web of Sciences and Thomas Innovations (Chen and Martin, 2012).

The contribution of bio-based polymers helps in reducing the dependency on petroleum based fossils and also helps in the development of positive environmental impacts such as mitigation in the emission of carbon dioxide due to the burning of synthetic plastics.

The bacterial fermentation helps in the production of bio-based plastics by synthesizing the different monomers derived from renewable resources including fatty acids, lignocellulosic biomass and organic waste material. The properties of biodegradable plastics are similar to the conventional plastics and have its applicability in every field.

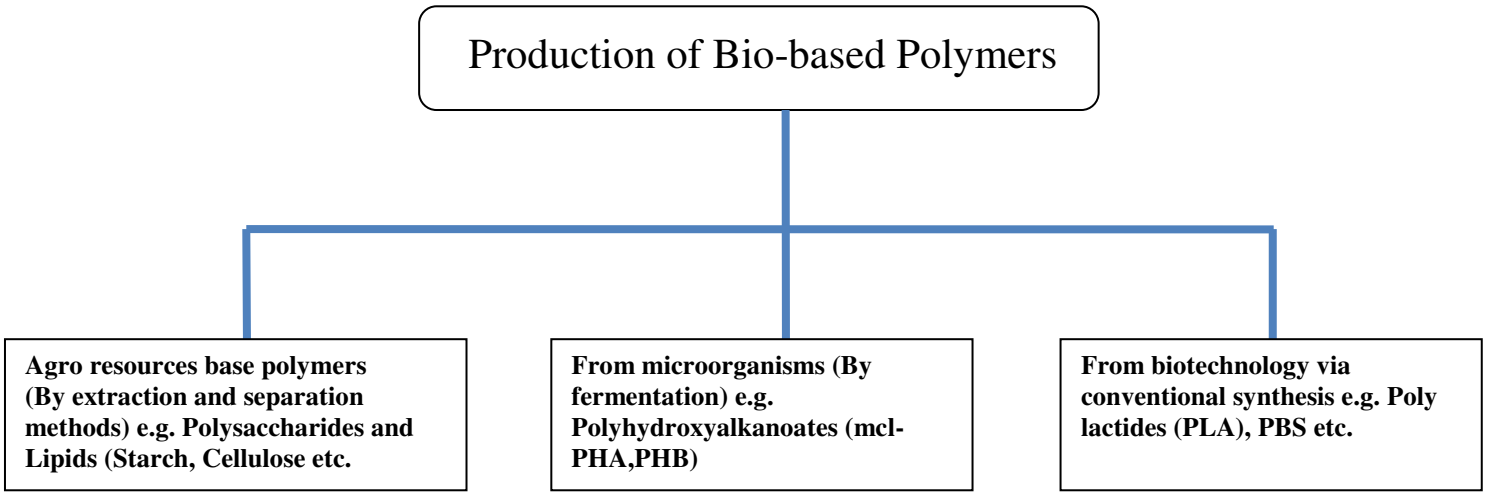


Figure 2.1: Different categories of bio-based polymers production from various processing methods (Luc and Eric, 2012)

Natural bio-based polymers are produced from natural renewable resources such as polysaccharides (chitosin, collagen), proteins and nucleic acids. The unique properties of these bio-based polymers attracts the attention of researchers in terms of their commercial applications. Modern Green plastic i.e. bio-based plastics are categorized into three groups based on the use of different renewable resources:

1. Natural bio-based polymers extracted directly from plants and animal source with or without modification. For example: modified polysaccharide polymers, cellulose derived polymers, natural rubber from rubber tree, agar from red algae, chitin and chitosin.
2. Conventional methods of polymerization or fermentation are used for the preparation of bio-based monomers by naturally occurring raw-material. For example: naturally occurring lactic acid monomers are processed into polylactic acid (PLA).
3. Large scale fermentation helps in the production of polymer by bacterial fermentation such as Poly hydroxyl/butyrate (PHB), co-polymers of PHB i.e. Polyhydroxybutyrate-co-valerate (PHBV).

2.3: Natural bio-based polymers

2.3.1: Starch

The starch is a bio-based polymer consisting of linear polysaccharide amylose and highly branched polysaccharide amylopectin. It occurs in nature as discrete granules and is present as an end-product of photosynthesis process in plants. The major sources of starch are corn, rice, potato and wheat, which are rich in carbohydrate and are present abundantly in nature. The industries are giving more interest in the utilization of thermo-plastic based starch. 15 weight% of plasticizer addition in the starch greatly influences the thermal and mechanical properties (Sanyang *et al.,* 2015). The glass transition temperature of starch is between -50°C and 110°C whereas the young modulus was compared with polyolefins as observed by Jane in 1995. The ductile nature of the starch is due to the complex molecular structure and it is partially non-linear polymer.

Retrogration is a stage in which the high crystalline nature of polymer over time leads to an increase in the brittleness. This phenomenon of retrogration is mostly an issue while working with the bio-based polymer i.e. starch and starch based thermoplastic. The issue could be resolved by adding an appropriate plasticizer, which enhances its mechanical properties. Starch blends with plasticizers and composites provide a new opportunity for the development of an improved thermoplastic with better mechanical strength, flexibility and wide applications in the field of food packaging industries by adding water barrier properties

(Maurizio *et al.,* 2005). The leading most company in the processing and production of starch based thermoplastic is Novamont as reported by Li *et al.,* 2009. The company engaged in manufacturing starch based shopping bags, packaging materials, food containers, sanitary products etc, with wide range of other applications. The group of scientists had investigated in Europe 2002 that approximately 7.9 million tons of starch and starch derivatives were consumed. Out of which 46% was utilized for non-food applications and 54% for food applications studied by Frost & Sullivan report in 2009.

2.3.2: Cellulose

The main element present in the cell wall of plants is cellulose. Complex polysaccharide with crystalline morphology is the main characteristic feature that exists in cellulose. The structure of cellulose is quite different from starch, consisting of glucose units linked by β-1,4-glycosidic bonds whereas the linkage in starch structure are α-1,4 linkages. Cotton, fibres and wood are the main raw material sources required for the production of cellulose. An orange brown solution i.e. viscose is obtained by dissolving the plant fibre in sodium hydroxide and carbon bisulphite solution, and then treated with sulphuric acid and sodium bisulphite to reconvert the viscose to cellulose in cellophane. This methodology is the basis of manufacturing rayon fibres and transparent cellulose films. To separate the cellulose from other wood constituents, two main methods are currently used reported by Yan *et al.,* 2009.

The first approach utilizes sulphite and pre-hydrolysis kraft pulping along with high pressure in order to separate the cellulose from lignin and hemicelluloses. The cellulose obtained by this method is more than 97% pure. For industrial purpose, the main derivatives of cellulose are cellulose esters used for molding and extrusion, cellulose acetate etc. The Cellulose has high tensile strength of 62-500 MPa and elongation at break is 4%. Bisanda and Ansell, 1992; Eichhorn *et al.,* 2001 observed that the fibres of cellulose were very hard on touch.

Eastman chemical is the major producer of cellulose based polymer. Certain types of microbial species have the ability to pure cellulose. The purity and high strength are the characteristic features of bacterial derived cellulose. Cellulose based materials are very costly, due to which its applications are only restricted to the field of food and biomedical designing. Thus, the high cost production and low yield of cellulose derived from bacteria acts as a barrier to the large scale industrial applications as reported by Prashant *et al.,* 2009.

2.3.3: Chitin and chitosan

Natural amino polysaccharide found abundant in the shells of prawns and crabs. Chitin and chitosan is a valuable bio-based polymer and have number of biomedical based applications. Roberts in 1997 investigated that chemical extraction methods were used for the commercial production of chitin and chitosan derived from the wastes of shrimp, crab and prawn wastes. Various aggressive processes are involved for the extraction of chitosan with chemicals. The steps include demineralization by acids, deproteination by alkali followed by the process of deacetylation.

Another approach for the synthesis of chitin and chitosan involves various enzymatic activities by the process of hydrolysis, but was not economically feasible on industrial level as reported by Win and Stevens, 2001. Ravi Kumar in 2000 studied the worldwide industrial scale pilot plants for the production of chitin and chitosan which were majorly located in Scandinavia, USA, some parts of Asia and Canada. The unique features of chitosan based polymers include biocompatibily, good mechanical strength, unique property of forming films, cost effective and are chemically inert investigated by Virginia *et al.,* 2011; Marguerite, 2006; Liu *et al.,* 2012. The potentiality of chitosan increased multi-folds of its applicability in various fields ranging from pharmaceutical, cosmetic products to waste treatment and plant protection.

The change in the degree of acetylation and molecular weight affects the different properties of chitosan in each application. Ravi Kumar in 2000, concluded that Chitosan has high level of applicability and is used in the field of cosmetic based products (shampoos, rinses, and permanent hair-colouring agents) because it was highly compatible with many biologically active components.

Ramya *et al.,* 2012; Bae and Moo-Moo, 2010 documented that the properties of chitosan enhances its applicability in various fields i.e. for the preparation of various medical devices (scaffolds, sutures and various implants) and pharmaceutical products.

The skin care industries were also used derivatives of chitosan for the manufacturing of various products. Valerie and Vinod, 1998; Bansal *et al.,* 2011; Hafdani and Sadeghinia, 2011 observed that the cost effectiveness feature of chitosan improved its applicability in the

preparation of skin moisturizer as its properties are comparable with that of hyaluronic acid. The vast applications and the production of bio-based polymeric material of chitin and chitosan make it an important material in the field of biomaterial and nanotechnology.

2.3.4: Pullulan

Pullulan has high solubility in water and a linear molecule, mainly composed of maltotriose units connected by α-1, 6 glycosidic units. In 1983, it was first reported by Bauer during fermentation experiment. Experiment involved fermentation in broth culture by *Aureobasidium pullulans* (black yeast like fungus). The simple experiment consisted of simple sugars as a feedstock, which were then converted into Pullulan as reported by Bernier, 1958; Sena *et al.,* 2006; Catley, 1971. Chemical modification or the introduction of reactive groups in Pullulan produces novel polymers which are hydrophobic in nature (less or not soluble in water). The presence of glycosidic linkage imparts unique features to the Pullulan.

The parental properties of Pullulan i.e. high water solubility and low viscosity increases its scope in various commercial applications i.e. an additive in food, substitute to blood plasma and as an adhesive were investigated by Zajic and LeDuy, 1973; Cheng *et al.,* 2011; Singh *et al.,* 2008; Leather in 2003 concluded that the strength, toughness and transparency of Pullulan were comparable with polystyrene (PS) as it has the ability to be moulded into any form or shape. Pullulan is tasteless as well as odourless and a slow digesting macromolecule. The application of Pullulan as a low calorie food additive improves the bulk and texture of the food products. Conca and Yang in 1993 reported that Pullulan was an excellent food preservative used in the preparation of various food products, it possesses good moisture retention and oxygen barrier properties, and also it inhibites the growth of fungus. The biomedical applications of Pullulan have been studied in various aspects and researchers found that it could be used in targeted drug delivery, healing of wounds, sutures and tissue engineering applications as was concluded by Rekha and Chandra, 2007. Other emerging markets for pullulan include oral hygienic products as reported by Barkalow *et al.,* 2002 and formulations of capsules for dietary supplements and pharmaceuticals as reported by Leathers in 2003, leading to increased demand for this unique biopolymer.

2.3.5: Collagen and gelatin

The extracellular matrix and connective tissue of animals are composed of collagen fibres. These fibres are insoluble in water and organic acids and partially soluble in alkaline solution

(10%). The fibres are rich in protein component. The collagen is flexible in nature and its main application is in the preparation of scaffolds that helps tissues to withstand stretching. 27 different types of collagens are discovered in the recent years. The animals are the main source of collagen which includes pork, skin of pigs and bones of cattle. The source of industrial collagen is mainly obtained from non-mammalian species as was reported by Gomez-Guille *et al.,* 2011. The process of hydrolysis helps in the production of collagen. Johnston-Banks in 1990 observed that the parameters responsible for the degree of conversion of collagen into gelatine are function of temperature, extraction time and pH. The unique properties of collagen i.e. its biodegradable and biocompatible nature, weak antigenicity, increases its applicability in the biomedical sciences as it has all the features required for the preparation of a good and unique biomaterial as was investigated by Maeda *et al.,* 1999. Rubin *et al.,* 1973 reported that it has an important application in the area of ophthalmology where drug delivery systems are prepared for slow and controlled release of drugs into the targeted body cells. Lee *et al.,* 2001 also investigated its applications in the field of tissue engineering i.e. for the skin replacement, as a bone substitute and development of artificial blood vessels and valves.

2.3.6: Polylactic acid

The scientist's discoveries and researches on bio-based plastics bring a tremendous change in the worldwide markets. The world population are attracted towards the unique properties exhibited by polymers derived from renewable resources and is termed as Green Plastic. The Polylactic acid (PLA) is one among them. It has been known since 1845 but until early 1990, it has not been commercialized. Aliphatic polyester is the family to which PLA belongs and consists of basic constitutional unit i.e. lactic acid.

Corn (starch) or sugar derived from renewable resources undergoes bacterial fermentation to produce monomeric units of lactic acid. The monomer of lactic acid consists of hydroxyl carboxylic acid groups. Polycondensation reactions of lactide monomers from lactic acids could be an approach for the synthesis of PLA. As compared with polyethylene terephthalate (PET), PLA has some similar properties and is a polymer of commercial interest with wide range of applications. The unique characteristics of PLA include high rigidity, glossy appearance, resistible to different processing conditions and transparent in appearance.

The wide applications from packaging to electronics and automotive in which the conventional plastic (Poly ethylene, Polystyrene, and Polycaprolactone) performed a major role are being replaced by the thermoplastic polyester (PLA) having similar and comparable properties (Majid *et al.,* 2010).

The thermal properties of PLA are not attractive due to low glass transition temperature of 60°C. Blending with other polymers could be one of the strategies to improve the mechanical properties of PLA. Another approach including changing the stereochemistry of the polymer i.e. crystallinity of the polymer is influenced by changing the ratio of L and D isomer.

PLA based material has been widely used in food packaging which includes spoons, knifes, food trays and plates. PLA with carbon and kenaf fibres with enhanced flame retardency and thermal properties was manufactured by a renowned company NEC Corporation situated in Japan. An improved PLA based blend was prepared by the Fujistu Company in Japan to manufacture computer hosing. The applications of PLA and its blends are acceptable world-wide because of its unique and enhanced properties in the field of automotive and chemical industry (Babu *et al.,* 2013).

2.3.7: Polyhydroxyalkanoates

The hydro-carbon based plastics (Synthetic plastic) are being replaced by the polyesters belonging to the family polyhydroxyalkanoates (PHA's) having paralleled properties and a promising material to replace the petroleum based polymers. These polyesters can be produced by bacterial fermentation reactions in large scale fermentor through downstream processing. A number of microbial species are capable of producing PHA's under starvation conditions. Recombinant engineered microorganisms can have the ability to tailor the properties of PHA's by adding the co-polymers in their chains inside the cells (Chee *et al.,* 2010).

The simplest PHAs i.e. Polyhydroxybutyrate is a biodegradable and biocompatible polymer and has become a topic of interest by various researchers. The unique characterization of pathway and its production at industrial scale has enhanced its application in the field of bioengineering technology as reported by Savenkova *et al.,* 2000.

Reis *et al.,* 2008 investigated that the shortcoming of PHB i.e. narrow thermal processing window, crystalline at room temperature and tendency to creep, restricts its utility in many industrial applications. The blending of PHB with copolymers of PHA are widely used for improving its properties both at physical and mechanical levels i.e. 80-95% (R)-3-hydroxybutyric acid monomer and 5% to 20% of a second monomer (valerate), hence develops a new and improved material i.e. PHBV (Polyhydroxybutyrate-co-valerate).

2.4: Comparison of PHAs and PLAs

Biopolymers, under development for industrial purpose include polylactides (PLAs), polyglycolic acids, polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and their co-polymers and blends. After the rise of petroleum prices in USA in mid-2008, on large scale, PLA (Poly lactic acid) and PHA (Polyhydroxyalkaonates) came into existence. Both polymers have their own pros and cons. Production cost of PHA is twice as that of a PLA, while PHA has comparatively more brittleness and elasticity. PLA is chemically synthesized while PHA is synthesized biologically as an intracellular product. PHA is produced by at least 10 companies all over the world using microbial fermentation, on the other hand only one company Nature works produces PLA because polymerization of LA to form PLA is difficult. The properties of PHA and PLA vary on the basis of their structural units. Due to the availability of PLA in large quantity, it is researched widely for its applications. The energy crisis in the present time leads to the research focus on PHA. For the reduction of carbon dioxide emission PHA is now considered as a useful bio-plastic. Efforts are required to reduce its production cost (Chen, 2009).

Various chains of PHA are also useful in medical field such as in Bone Tissue engineering, as drug carriers. These can also be used as raw materials in many packaging companies. Most researched PHA is PHB (Polyhydroxybutyrate).

Table 2.1: Comparison of Poly lactic acid (PLA) and Polyhydroxyalkanoate (PHA) (Chen, 2009)

PLA vs. PHA	**Poly lactic acid (PLA)**	**Polyhydroxyalkanoate (PHA)**
Structures of monomeric units	Only D- and L- lactic acid (LA)	At least 150 monomers
Methods for Production	Bio-production of LA and chemical synthesis of PLA	Intracellular polymer, which are biosynthesized
Cost of Production	Comparable with conventional plastic like PET	Atleast twice that of PLA
Properties of material	Poor in nature, adjusted by regulating D- and L- LA ratios	It can be changed to brittle, flexible nad elastc and are fully controllable
Advancement in the technology	LA production well established, yet LA polymerization toPLA is complicated. Only one company, Nature work, produce PLAon a large scale.	At least 10 companies, worldwide produced or are producing PHA upto 2000 t per year scale via mictrobial fermentation.
Cost effectiveness	Large scale capital investment: Nature Work has invested 1 billion US$ over the past several years to run a 1,40,000 ton PLA plant. It is not a cost effective approach	Small investment: existing aerobic microbial fermentation plants with process modification can be used for PHA production
Intellectual properties	Cover almost all areas of production and application	Still a lot of space for exploitation
Applicability in different areas	Food packaging, medical implants, 3 D imprints ,yet limited by Tg of 65-75^{o}C for cheaper P (L-LA)	Almost all areas of conventional plastic industry, limited only by current higher cost.

2.5: Synthetic Plastics versus biodegradable plastic PHB

Polyhydroxyalkanoates (biopolymers; PHA) are an important class of polymers found in bacteria. Earlier, since polyhydro-xybutyrate (PHB) was discovered by Lemogine in *Bacillus megaterium* in 1926, an extensive research started towards industrial PHB production for substituting synthetic polymers (Anderson and Dawes, 1990). Ojumu *et al.,* 2004 studied about the alternative source, which could take the place of thermo-synthetic plastics produced from petroleum based products. He investigated that bio-based plastics produced from renewable resources are completely synthesized by microorganisms or produced from plant based products i.e. starch, cellulose, chitin etc.

The properties of biodegradable plastics i.e. PHA's are very much comparable with synthetic based plastics. High cost of PHAs required for its production has restricted its application. The main focus of the studies right now is to develop a new green technology which can act for commercial low cost production of PHB.

Rivard *et al.,* 1995 analyzed that synthetic plastics have numerous application in every field, not only due to the favorable mechanical and thermal properties, but also but mainly due to their stability and durability. Discarded plastic have deleterious impact on the environment, including negative effects on wildlife and on the aesthetic qualities of cities and forest. The increased cost of solid waste disposal as well as the potential hazards from waste incineration such as dioxin emission from PVC (Polyvinyl chloride) makes synthetic plastic a waste management problem.

Muller *et al.,* 2001; Amass *et al.,* 1998 studied that from the past two decades, there have been a growing public and scientific interest regarding the use and development of biopolymer (biodegradable polymers) materials as an ecologically useful alternative to plastics, which must retain the desired physical and chemical properties of conventional synthetic plastics, hence offering a solution towards the existing grave problem of plastic waste.

A number of biodegradable plastic materials mostly biodegradable polyesters, namely aliphatic polyesters, polyhydroxyalkanoates (PHAs), polysaccharides, polylactides, and copolymer, blends of these, have come up over the past few years and extensive research is

going on to fulfill the demands in various areas of industries. Lee, 1996; Steinbuchel and Fuchtenbusch, 1998 have studied that these biomaterials provide solution to managing of waste and in certain cases, can act as good substitute for conventional plastic where mechanical properties are met.

Steinbuchel, 1991 observed that a number of soil inhabiting bacterial species accumulated PHA as intracellular granules, which can be utilized as carbon and energy storage materials under starvation conditions or nutrient limited conditions. Byrom, 1994 studied that the stored PHA gets degraded by intracellular depolymerases and further metabolizes as energy source. Different microorganisms with different growth medium produces PHA's of molecular weight ranging from 2×10^5 to 3×10^6 daltons.

Table 2.2: Comparison between Synthetic Plastic and biodegradable plastic

Synthetic plastic	**Biodegradable plastic**
Synthetic plastics are man-made plastic prepared by various non-renewable petroleum derived products by chemical synthesis	Biodegradable plastics are prepared from renewable resources i.e. starch, resins, wood, bacterial fermentation etc.
They are non-degradable under natural conditions	They can be degraded by various natural factors i.e. ultra-violet radiation, oxygen and nitric acid and various bacterial sp.
Synthetic plastics are not cost–effective, as they are produced from expensive petro-chemical based resources	Biodegradable plastics are cost-effectives, as they are produced from cheaper resources
The degradation time for synthetic plastics is very long or negligible and creates the problem of solid waste management	Long or short period of time is required for its complete degradation and resolves the problem of solid waste management
eg:- polyethylene (PE), polycaprolactone (pcl) and poly vinyl chloride (PVC)	eg:- Poly-lactic acid (PLA), Polyhydrobutyrate (PHB) and its related chains

2.6: PHA (Polyhydroxyalkoanates) and its subclasses as most favorable material for replacement of synthetic Plastic

PHA is a family of completely biodegradable polymers and can be used for medical applications due to its non toxic nature. PHA are the polymers of HA (hydroxyalkanoates) containing hydroxyacyl as its monomeric unit. PHA granules accumulate in the cytoplasm of the bacterial cell. They are degraded into oligomers and monomers and then further to carbon dioxide and water. They are classified according to their monomeric units in short chain length (scl) and medium chain length polymers (mcl). Scl have C3-C5 carbon atom as its monomer while mcl have C6-C16 carbon atoms as monomeric unit. Also there are PHA copolymers with monomers of both short and medium chain length (Tripathi *et al*., 2012).

PHA properties which make them useful for future use are a) PHAs are thermoplastic in nature, b) These are not soluble easily in water, c) They generally consist of R-stereoisomerism, d) Less toxic in nature, e) Biodegradable and Biocompatible, f) P3HB & PHBV has piezoelectric properties, g) Renewable sources are used to obtain various PHAs, h) They are hydrolyzed by PHA depolymerases enzymes and provide energy and carbon sources to microbes (Bonartsev *et al.,* 2007).

PHA family includes PHB, PHBV, P4HB, P3HB4HB, P3HO, PHBHHx. PHA can be used as biodegradable plastics and fibre materials, as medical implants such as orthopedic pins, adhesion barriers, tendon repair devices, cardiovascular patches, sutures, stents etc. and as drug delivery carrier. PHA monomers and oligomers can be used as nutritional supplements. Commercially available PHA are PHB (polyhydroxybutyrate), PHBV (poly-3-hydroxybutyrate valerate), P3HB4HB (poly-3-hydroxybutyrate-co-4-hydroxybutyrate), PHBHHx (poly- 3- hydroxybutyrateco - 3- polyhydroxyhexanoate), P4HB (Poly-4-hydroxybutyrate). PHB is one of the PHA subclass, studied mostly of all. Its degradation temperature and melting temperature is very close and also has similar physical properties (Chen, 2009).

As compared to PHB its copolymers such as PHBV have better thermal and mechanical properties but its production in bacteria is very less as compared to PHB. It has been studied that when PHB is mixed with PHV then the polymer obtains high flexibility and strength (Otari and Ghosh, 2009). The property of PHBV changes according to the proportion of the HV monomers added to it. As per the carbon source used in the medium, the PHBV structure

can be modified (Wang *et al*., 2013). Copolymer PHBV is less brittle and less crystalline than PHB and can be used in bone tissue engineering and in making of biodegradable drug carriers (Chen and Wu, 2005). Major disadvantage of PHBV is its low productivity which is in fractions as compared to PHB.

2.7: Structural Details and Storage of PHB

Doi *et al.,* 1992 provided structural details about the majority of PHAs, which are composed of R-(3)-hydroxyalkanoic acid monomers having C3 to C14 carbon atoms with varying saturated or unsaturated and straight or branched chains containing aliphatic or aromatic side groups.

Discrete granules of PHB were observed inside the bacterial cells when analyzed by transmission electron microscopy (TEM), their size and number depends on the type of bacterial species.

Winfred and Robards, 1973 provided the information that Poly hydroxy butyrate (PHB) is a biodegradable thermo-polyester synthesized by bacterial sp. Lipid inclusions as PHB was observed in the cellular structure of *Bacillus* sp. In *Alcaligenes eutrophus*, 8-13 granules were observed per cell with a diameter of 0.2 to 0.5µm reported by Byrom in 1994.

Ostle and Holt, 1982 analyzed that granules appear as highly refractive inclusion when observed under electron microscope. Accumulated PHB granules can be easily identified by screening with Sudan black B staining. Steinbuchel, 1991 worked on about 250 different bacterial isolates, including gram-negative and gram-positive species, and reported them to accumulate various PHAs. Ojumu *et al.,* 2004 analyzed that PHA synthetases of *A. eutrophus* can only polymerize 3HAs of Short Chain Length (SCL) size, while that of *Pseudomonas oleovorans* can only polymerize 3HAs of Medium Chain Length (MCL) size. In Short Chain PHAs the monomer units are oxidized at any positions except the third carbons while for Medium Chain PHAs monomers units are oxidized at the third position except in few cases. According to Anderson and Dawes, 1990, there is flexibility in PHA biosynthesis which makes possible to design and produce related biopolymers having useful physical properties ranging from stiff and brittle plastic to rubbery polymers. Atlic *et al.,* 2011 concluded that *Cupriavidus necator* is the most extensively studied strain for PHA production on industrial

scale due to its versatile behavior for accumulation of poly [(*R*)-3-hydroxybutyrate] (PHB) and its copolymers.

2.8: Production of PHB and Subclasses

2.8.1: Role of naturally occurring microbes

Bacillus megaterium NCIM 2475 has been used for the production of PHV with PHB. The growth of cells and polymer production studied from the 8th hour to 100th hour provided best results in 96th hour. In the presence of sucrose PHB-co-PHV was obtained. (Otari and Ghosh, 2009).

Ralstonia eutropha has been widely researched for the production of PHAs. Wang *et al.,* (2013) studied the production of PHBV in presence of levulinic acid (LA) concentration as a co-carbon source. The structure of LA is analogous to pentanoic acid and it is an easily available cheap carbon source as well. Highest yield obtained with LA is 12.61 g/litre, which is upto 81.2% of cell weight. NH_4Cl was used as the nitrogen source and casein peptone produced 70% more PHBV as compared to urea as a nitogen source initially. The study revealed that HV monomer concentration can be modified by using stress resistance condition and appropriate C:N ratio.

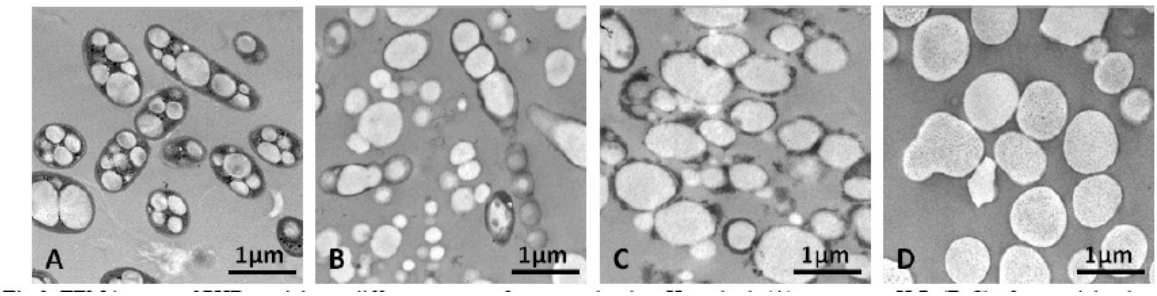


Figure 2.2: TEM images of PHB particles at different stages of recovery by the pH method: (A) *in vivo* at pH 7; (B-C) after partial polymer recovery; (D) after complete polymer recovery (Porter *et al.,* 2011)

Ramsay *et al.,* (1990) studied six microbes for the production of PHBV. These were *Bacillus cereus, Alcaligenes eutrophus, Pseudomonas pseudoflava, Alcaligenes latus, Micrococcus halodenitrificans and Pseudomonas cepacia.* Under the nitrogen limiting conditions, and glucose, propionic acid in media (sucrose for *Alcaligenes latus*) 17g/l PHBV was produced from *Alcaligenes eutrophus* and 43% (w/w) PHBV was produced from *Alcaligenes latus*. The

concentration of propionic acid was used to regulate the HV monomer concentration in the PHBV. 3.4 mole % HV monomers were produced by *Alcaligenes latus* while 50 mol % HV monomer by *Bacillus cereus*, were produced under same conditions. *Alcaligenes sp.* A-04 produced a terpolymer P(3H-co-3HV-co-4HB) which also contains valerate and butyrate in it, and it is reported to have better physical and chemical properties over other PHA. 68% (w/w) terpolymer was obtained at 60h of shake flask cultivation. At the cultivation time of 96h, 93 mol% of 3HB unit was obtained (Prateep and Kulpreecha, 2006). PHA of 6 to 11 carbon atoms are also produced from *Pseudomonas oleovorans.* The carbon varied due to the variation in substrates. When hexaonate was used as a sole carbon source high yield was obtained (Brandl *et al*., 1996).

Rhodobacter spharoides U7 produced PHB and PHBV in dark conditions, with oxygen supply to it. The culture was grown at 200rpm at the temperature of 37^0C. Propionate and valerate were used as carbon sources at different concentrations (Kemavongse *et al*., 2007).

2.8.2: Role of metabolically engineered microbes

Science is growing at a very fast pace and technologies such as genome sequencing, recombinant DNA technology, engineering of metabolic pathways have provided various breakthroughs in plants. These technologies are now researched for making recombinants and to produce PHA and PHB in efficient manner and in large amount. *E.coli.* is metabolically engineered in various ways to produce PHA. One of the strains of recombinant *E.coli* produced by introducing genes coding for enzyme *PhaA, PhaB, ptb* that lead to 35g/l PHB production in the presence of glucose (Steinbuchel and Lutke-Eversloh, 2003).

In another strain, PHA synthesizing genes from *Alcaligenes latus* were transferred to *E.coli.* and this recombinant produced 42.5 wt% PHBV and also PHB at high concentration in fed batch culture where glucose concentration was made upto 20g/l and propionic acid upto 20 mM (Choi and Lee, 1999).

Carbon sources are also manipulated to get high polymer amount. Polythioesus are added to *Ralstonia eutropha* (wild type) which produced PHB. Different monomers of varying chain length were produced when acrylate was added to *Ralstonia eutropha,* as an inhibitor of β-oxidation pathway. Properties and yield of the polymers are also manipulated by the process of mutagenesis as well (Aldor and Keasling, 2003). Enzyme PHA synthase is mutated in

some cases to increase PHA quality. CO_2, oil of plants, waste material is used as cheap carbon source, which are renewable and therefore, reduce the cost of production (Tsuge, 2009).

Engineering was done in the strain of *Pseudomonas putida* KT2442 to produce PHB and PHHx by deleting β-oxidation cycle in it. PHB produced was 58 mol% while PHHx produced was 42 mol%. The blend of PHB and PHHx came up with better structural and mechanical properties (Tripathi *et al*., 2012).

Li *et al*., 2011 also used the same strain and transferred $phaPCJ_A$ (a PHA synthesizing gene obtained from *Aeromonas caviae*). 70 mol% of PHB and 30 mol% of PHBV was produced from it. The properties such as tensile strength and Young's modulus were found to be improved as well.

2.8.3: PHB production from environment Soil

Wei *et al.,* 2011 worked on the isolation and screening of PHB producing strains originated naturally from soil sources i.e. *Cupriavidus taiwanensis* strains. 7 bacterial isolates of *Cupriavidus taiwanensis* i.e. 184, 185, 186, 187, 204, 208, 209 were screened for PHB production along with *Pseudomona oleovorans* ATCC 29347 taken as control. An optimized pH of 7.0, temperature at 30^oC and agitation rate of 200rpm were used for the production of PHB. It was observed that the PHB yield was 10% and PHB production was 0.14g/l. The carbon and nitrogen sources were also optimized and it was analyzed that for *C. taiwanensis* 184, the best optimized carbon source was gluconic acid and best nitrogen was ammonium chloride. Optimized carbon/nitrogen ratio was also determined for high PHB production. It was observed that after optimization studies, PHB yield was 58.81% and PHB production was 2.44g/l. The best optimized carbon/nitrogen ratio was taken as 8/1 for the high PHB production on the selected strain i.e. *C. taiwanensis* 184.

PHB produced from microbial strains from environmental samples are being tested by gas chromatography-mass spectrometry (GC/MS) reported by Elhottova *et al.,* 2000.

Nurbas and Kutsal, 2004 worked on the Infrared (IR) and Gas Chromatography (GC) for PHB analysis. PHB content and its production was significantly increased by two stage fermentation strategy investigated by Hartmann, 2010.

C. taiwanensis 184, when undergoes two stage fermentation technology with optimized condition of temperature and pH, enhanced the PHB yield upto 72% with PHB content of 7g/l. The potency of indigenous strain *Cupriavidus taiwanensis* 184 for PHB production was screened by Sudan Black B staining reported by Burdon, 1946.

Ceyhan and Ozdemir, 2011 worked on the isolation and identification of PHB producing strains i.e. *Enterobacter aerogenes* (designated *E. aerogenes* 12Bi). Biochemical and phylogenetic characterization were used for the identification of strains.

Scientists reported first time that domestic wastewater medium (DWW) could be utilized as an inexpensive or cheap source for high production of PHB. DWW medium was utilized as sole carbon source and it was observed that the PHB yield was enhanced upto 90% cdw, by utilizing DWW as a substrate. Chien *et al.,* 2007 observed a high number of intracellular lipid granules in the bacterial strain, which were analyzed by Transmission electron microscopy (TEM). The scientists reported that PHB production was determined by Hypochlorite method and PHB yield was increased from 16.66 to 96.25% (w/w).

Rodriguez-Contreras *et al.,* 2013a reported that the conventional media were utilized by a novel bacterium sp. capable of producing industrial biopolymers of different chain lengths. The biopolymer was then characterized and identified. Among the isolates, it was investigated that strain S29 through fed-batch fermentation in shaker flask could generate bio-based polymer. The identification result shows that the biopolymer is a homo-polymer of Polyhydroxybutyrate. The molecular characterization shows that the strain S29 was a novel strain of *Bacillus megaterium.* The unique properties of the strain attracts researcher and scientist, not only to work on its culture growth and PHB accumulation but also on the thermal stability of the produced polymer i.e. PHB.

2.8.4: PHB production from Saline environment and Halophilic bacteria

Wei *et al.,* 2011; Chien *et al.,* 2007 worked on the PHB production from the *Vibrio* sp. BM1 isolated from Saline Marine environment. The study analyzed production of PHB by *Vibrio* sp. BM-1, isolated from marine environment through improvement of medium constituents and implementing an appropriate fermentation strategy. The studies developed a glycerol-yeast extract-tryptone (GYT) medium that facilitates the growth of *Vibrio* sp. BM-1 leads to

the production of 1.4 g/L PHB at 20 h cultivation. This study proved that GYT medium was the most effective medium for cultivating *Vibrio* sp. BM-1. Mineral salts such as Na_2HPO_4, KH_2PO_4, $(NH_4)2HPO_4$ and $MgSO_4 \cdot 7H_2O$ significantly affected PHB production. The findings of this study acts as a reference for further work into the use of PHB for manufacturing biodegradable polymers using marine microorganisms.

Roohi *et al.,* 2012 worked on the isolation and characterization of Halotolerant and Halophilic bacteria from salt mines of Karak, Pakistan. The workers investigated the isolation of twenty one halotolerant bacterial strains from salt mines. The salt concentration in media was in the range of 5-40% concentrations. These strains can grow in media with 5-40% NaCl concentrations. The growth conditions i.e. temperature, pH and aeration rate were optimized in order to study the morphological, physiological and biochemical characteristics. On the basis of sodium chloride concentration, halophiles were divided into 3 groups i.e. slightly halotolereant, second; moderately halophilic and third, extreme halophilic bacteria. Various factors responsible for the growth of halophiles were temperature, Ph, NaCl and medium composition. Lichfield and Gillevet, 2002 reported that ecological, taxonomical and phylogenetically studies have been conducted along with their biotechnological applications.
The results show that slightly and moderately halophilic bacteria were present more abundant than the extremely halophilic bacteria. Quesada *et al.,* also observed these types of results in 1982.

The analysis also shows that saline environment favors the growth of moderately halotolerant bacterium sp. as compared to extremely halophilic bacteria reported by Rodriguez-Valera, 1988. The physiology of extremely halophilic bacteria requires high concentration of sodium chloride and most of them also require magnesium ions for their growth. The slightly and moderate halophiles, do not require magnesium ions reported by Grant *et al.,* 2001. Optimized conditions were taken for the growth of halophiles. It was investigated that a 28–37ºC temperature and 7.0–8.0 pH, supplemented with 5-20% of sodium chloride in medium culture was utilized for the growth of halophiles.

In the study by Hongyu *et al.,* 2009 halophilic bacteria was isolated from salt ponds of China and growth of these microorganisms was observed at the temperature of 35-40ºC and at pH 7.0–8.0 with 20% (w/v) of NaCl.

Tekin *et al.,* 2011 investigated the work on the extreme halophilic archaeon *Heloferx* sp. MA10 isolated from Camalti Saltern, Izmir. Izmir Camaltı Saltern is the biggest seawater-based saltern lake in Turkey. In this study, the scientists Tekin *et al.,* isolated 14 extremely halophilic archaea, and screened for PHB production. One strain was then selected as best PHB producer and was further cultivated in different PHB media. PHB was extracted from cells and measured with spectrophotometer, and further the amount of PHB was measured through comparison with standard PHB. The detected PHB yield was 6.53% of the dry cell weight in the PHB medium supplemented with acetate. Partial 16S rRNA gene sequence analysis showed 99% similarity to *Haloferax alexandrinus* strain TMT; therefore, the strain was named *Haloferax* sp. MA10. *Haloferax* sp. MA10 and *Haloferax alexandrinus* TMT have some differences in phenotypic and biochemical properties. With this study, the discovery of PHB-producing extreme halophilic archaeon *Haloferax* sp. MA10 at İzmir's Camalti Saltern was reported for the first time.

Upasani, 2008 reported in his work on the Sambhar Lake, the largest inland saline and alkaline lake of India is situated in the State of Rajasthan. He reported that isolation and characterization of extremely Haloalkaliphilic microorganisms especially archaebacteria from Sambhar Lake. Isolation of moderately halophilic bacteria (*Haloferax volacanii*) was also achieved. Scientist in this work isolated and characterized several strains of red, extremely haloalkiphilic archae from the salt samples.

2.9: Enhanced PHB Production through Optimization strategies

Elsayed *et al.,* 2013 screened PHB producing strains *Azomonas macrocytogenes* isolate P173 by utilizing various screening methods. It was analyzed by the studies that the strain produced 24% PHB per cell dry weight after 48 h. Elsayed *et al.,* 2013 carried out further studies with several experiments for optimization of the composition of the culture medium and environmental factor for maximum PHB production. For optimum PHB production, various parameters were optimized and the results shows that temperature of 37^{o}C, aeration rate of 60% and pH of 7.5 were required for high PHB production. Culture medium were also modified and designed to increase the PHB yield by adding 0.7% glucose and 100 mg/L potassium nitrate as carbon and nitrogen sources. The combination of optimized parameters along with modified medium were utilized and it was reported by Elsayed *et al.,* 2013 that the PHB yield was increased 24 to 42% in a time incubation of 24 hrs. Second experiment

included the mutagenesis studies on the variant 173A2 by Acraflavin as mutagenic agent, which increased the PHB Yield to 47% in a time incubation of 24 hrs using same optimized condition combination with modified medium.

Poly hydroxyl butyrate was produced by *Rhodobacter sphaerodies* as a major component with a yield of 97%. Small amount of poly hydroxyl valerate (PHV) was also produced by the bacterial sp. i.e. 3% under anaerobic conditions reported by Brandl *et al.,* 1991.

Mahuya *et al.,* 2005 investigated the effect of nutrient limitation on accumulation of PHB by *Rps. palustris* SP5212. Khatipov *et al.,* 1998 analyzed combinations of various ratios of carbon and nitrogen substrates to study poly-β- hydroxybutyrate accumulation and H_2 evolution by *Rhodobacter sphaeroides* RV.

Studies were investigated on tannery effluents of Warangal district of South India, for the isolation and screening of purple non-sulphur bacterial strains (Merugu *et al.,* 2010).Nine bacterial species, were isolated which included *Rhodospirillum rubrum, R. acdiophila*, *Rhodo pseudomonas palustris*, *Rsp. Photometricum R. rutila*, *Rhodopila globiformis*, *Rhodobacter s phaeroides*, *Rb. capsulatus*, *Rhodobacter sp.* and *Rhodocyclus gelatinosus*. Among them *Rhodopseudomonas palustris* KU003 was the best producer of Polyhydroxybutyrate (PHB). Further media was optimized and nitrogen limitation was observed on the production of PHB. PHB accumulation was found more when nitrogen limitation of 78 mg/L of ammonium chloride was maintained. The maximum PHB produced was 180 mg/L of BP (Biebl and Pfennig's) medium containing glucose as carbon source.

Morgesin and Schinner, 2001 investigated that halotolerant photosynthetic bacteria have the advantage over other microorganisms for their ability to adjust themselves to both presence and absence of light as well as able to survive in saline condition which offers a multitude of applications in various fields of biotechnology.

The research was carried out on Egyptian soil to isolate the highly efficient PHB producing bacterial isolates. 30 different isolates of *Acetobacter* were studied and screened for PHB production. It was investigated that optimized carbon and nitrogen sources at different temperature and aeration, effects the accumulation of PHB in the cell. The maximum yield of

PHB was observed as 0.88gm/mg dry cells. The efficient bacterial isolate was molecular characterised by 16S rRNA and named as *Azotobacter vinelandii* (El-Shanshoury *et al.,* 2013).

The scope of the study was to develop a fermentation process which includes high PHB producing bacterial strains along with optimized conditions, which favors high PHB production. The effect of nutrients and environmental conditions on the PHB accumulation was studied on the selected strain *R. sphaeroides* N20. The scientists concluded from their work that the highest PHB production was estimated as 8.02 ± 0.10 g/l and 88% PHB yield. Cheap substrates were utilized for high PHB production.

2.10: Enhanced PHB production through Mutagenesis strategies

Adwitiya *et al.,* 2008 isolated gram-positive bacterium that accumulated PHB from local garden soil in Bangalore. Based on its morphological and physiological properties, and nucleotide sequence (about 1.5 kb) of its 16S rDNA it was identified as *Bacillus thuringiensis* IAM 12077. Scientists observed PHB-producing capacity of putative mutant strains of *Bacillus thuringiensis* IAM 12077. The strain was capable of producing PHB 10%-15% dry cell weight (DCW). PHB producing strains were grown in nutrient broth medium for 48 hrs and was subjected to random mutagenesis by physical (UV) and chemical (acridine orange) method. The scientists investigated that among the UV-mutants screened, 19 putative mutants produced more PHB than the parental, while 2 strains produced less. Of these mutants, B8 exhibited promising PHB accumulation (24.68%; 1.54-fold) with more PHB production (1.3 g/l; 5.4-fold) than the parental strain. Chemical mutagenesis yielded putative mutants, of which 6 had a decrease, 3 had no change, and 2 had an increase (B3, C2) in PHB production. While the increase in accumulation (19.69%, 1.43-fold and 22.22%, 1.62-fold, respectively, for B3 and C2) was comparable to the increase shown by the UV mutant (B8), the yields did not concomitantly increase (0.43g/l, 1.13- fold and 0.6g/l, 1.57-fold, respectively).

Sreeju *et al.,* 2011 studied about the strain improvement, which can be carried out by physical agents such as gamma-rays, x-rays and ultraviolet irradiation, and chemical, agents such as ethyl methane sulphonate (EMS) and nitroso-methyl guanidine (NTG). The best mutagen and conditions for its use are not identical for all organisms and can only be found by trial and error. The efficiency of alkylating mutagen treated bacterial isolates showed effective

reactions depending on the duration of exposure. EMS mainly added the ethyl group to numerous positions on bases of DNA.

On the basis of the results obtained, all the four cultures namely, *P. putida*, *B. megterium*, *E. coli* and *R. eutropha* have higher activity on duration of exposure to UV light. When the period of exposure is for longer duration, less will be the survival of the specific cells and all the mutant forms which have the ability to survive alone will remain forever. Similar results were found by the Random Amplified Polymorphic DNA (RAPD), a PCR based method which could be used to distinguish strains within a species. Genetic analysis for identifying mutation using the primer A9 revealed a moderate genetic variability proving mutation.

Sangkharak and Prasertsan, 2007 worked on chemical and physical mutagenesis effect on three halotolerant bacterial strains; *Rhodobacter sphaeroides* ES16 (the wild type) and two mutant strains of *R. sphaeroides* ES16, namely N20 and U7. Chemical mutagen was N-methyl- N'-nitro-N-nitrosoguanidine (NTG) and physical mutagen was ultraviolet (UV) radiations at 254nm respectively. Glutamate-malate (GM) medium was used as culture medium. Further, screening was carried out for PHB production. The parental strain produced a PHB of 19.5% cdw, whereas the mutant strain produced PHB of 53.9% and 42.0% of dcw.

Katircioglu *et al.,* 2003 investigated mutant strains for high PHB production i.e. *B. subtilis* K8, *Bacillus megaterium* Y6, and *B. firmus* G2 for their poly-hydroxybutyrate (PHB). Experiments were carried out to induce mutations by using Ultra-violet light, acriflavin and 5-bromouracil. SDS-PAGE was used for the comparison of proteins extracted from 59 bacterial strains. The range of PHB yield was 1.46-63.45%, depending on the type of each strain. Eight mutant strains have high level of PHB production as compared to wild-type strain. Mutant strain of *B. sphaericus* X3 was analysed and it was observed that no increase in PHB was observed in mutant strain as compared with parental ones. SDS-PAGE revealed the protein profiling and it was observed that the protein profile of parental and mutant strain was entirely different.

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2.11: PHB and its applications

Saharan *et al.,* 2012 focused his work for production of bioplastic towards sustainable development. The current work focused on the production, fermentation, types, challenges, applications, sustainability, process development and use of cheap substrates for bio-based plastic production. Synthetic plastics in many applications are being replaced by bio-based plastics, as they are derived from renewable resources and are full degrade in natural biological systems. Commercial development of bio-based plastics is very expensive as they required large scale fermentation costs along with proper sterilized conditions. Engineered microbial strains, mixed culture approached, two-stage fermentation technology and use of cheaper substrates can be used as inexpensive methods for PHA's production. Broad area of applications and the presence of unique characteristics of bio-based materials i.e. PHA's show an extremely promising future.

Chen, 2009 worked on the microbial polyhydroxyalkanoates and concluded from his studies about the industrial importance of PHA.

Production of bio-based polymers i.e. Polyhydroxybutyrate by different microbial sp. have been investigated by various academic researchers, polymer expertise and medical researchers. PHA production was established extensively in research and development departments (R$D) of many companies.

For the improvement of industrial microbial robustness, PHA synthesis from microbial strains is very useful in this aspect. Specific drug targeting applications were achieved from the proteins encoded by certain enzymes present in the biosynthetic mechanisms of PHB production i.e. *PhaP*, *PhaZ* or *PhaC*. The production and applicability of PHAs require an interdisciplinary research, which includes various fields of sciences and technology. Some of the fields are chemistry, physical sciences, material sciences, polymer sciences, biology and biomedical areas reported by the scientists.

Orts *et al.,* 2008; Chen and Wu, 2005 have strongly promoted the PHA field to become an industrial value chain ranging from packaging, plastics, agriculture, fermentation, biofuels, medicine to nutrition and fine chemicals. Sufficient amount of PHA's availability attracts the

other specialized companies to get involved and increases its applicability as a substitute of synthetic thermo-polyesters.

The widespread application of PHA's are covered in critical review by Chen *et al.,* 2009 ranging from its utility in bioplastic to medicines.

Table 2.3: Various application of PHA class material (Chen *et al.,* 2009)

Applications	Description	Example
Packaging industry	All packaging materials that are used for a short period of time, including food utensils, films, daily consumables, electronic appliances example:	PHBV, PHB
Other bulk chemicals	Heat sensitive adhesives, latex, smart gels. PHA nonwoven matrices can be used to remove facial oils.	PHB
Fine chemical industry	PHA monomers are all chiral R-forms, and can be used as chiral starting materials for the synthesis of antibiotics and other fine chemicals.	PHB and PHBV
Healthy food Additives	PHA oligomers can be used as food supplements for obtaining ketone bodies.	PHB
Medical implant Biomaterials	PHA have biodegradability and biocompatibility,and can be developed into medical implant materials. PHA can also be turned into drug controlled release matrices	PHB, PHBV, P3HB4HB, P4HB, P3HO (poly-(R)-3-hydroxyoctanoate) and PHBHHx
Medical	PHA monomers, have therapeutic effects on especially R3HB Alzheimer's and Parkinson's	PHB

	diseases, osteoporosis and even memory improvement.	
Biofuels or fuel additives	PHA can be hydrolyzed to form hydroxyalkanoates methyl esters that are combustible.	PHB
Specific drug delivery	Coexpression of PhaP and specific ligands can help achieve specific targeting to diseased tissues	PHB

2.12: Application of PHB and related classes in bone tissue engineering

As per studies done on PHB and related classes, these polymers comes up with many applications in different fields. As the composition of PHB changes under particular physiological conditions, its mechanical and biocompatible property also changes. This feature leads to use of PHB, PHBV, P4HB etc. and their blends in making medical implants such as bone marrow scaffolds, sutures, nerve guides, repair patches (Chen and Wu, 2005). Immunological response during organ transplantation is one of the major problems across the medical field. Transplantation of whole organ from human as a donor or donor from another species like pig has led to rejections due to immunological barriers. Concept of tissue engineering has evolved with the idea of growing cells on the base called as scaffold, for the development of organ before implantation. A biochemical base is provided by scaffold till the time cells produce their own extracellular matrix. As the cells grow, deposit and organize the scaffold starts degrading, leaving an organ/tissue at its place (Stock and Vacanti, 2001).

Poly 4-hydroxybutyrate and copolymers of 3-hydroxybutyrate and 4-hydroxybutyrate including P3HB-co-3HV (3-hydroxyvalerate) P3HB-co-3HD (3-hydroxydecanoate) and P3HB-co-3HH (3-hydroxyhexanoate) are the polymers among the PHA family with very high elasticity and 400-1,100% of elongation. In the cardiovascular tissue engineering these polymers are showing much progress. In the bone marrow and cartilage engineering, the application of these elastic and soft PHAs is still under investigation, as they have potential there, more exploration is required (Chen *et al*., 2012).

PHAs are biocompatible, support growth of cells; allow in growth of tissues, lead to degradation of non-toxic products, organization of cells, which make them useful for tissue

engineering (Williams *et al.,* 1999). PHA family can be exploited for the heart valves and blood vessel's tissue engineering as they are flexible and produce less inflammatory responses. PGA (Polyglycolic acid), PHA and P4HB were studied for the making of scaffolds and it was concluded that PHA and P4HB provide cell attachment and collagen formation, also their molding into trileaflet valve is easy because of thermoplastic nature. Scaffolds made from the composite of PGA and P4HB and trileaflet valve from porous PHA, provided increase in collagen content and laminated fibrous tissue formation observed in histology (Fuchus *et al*., 2001).

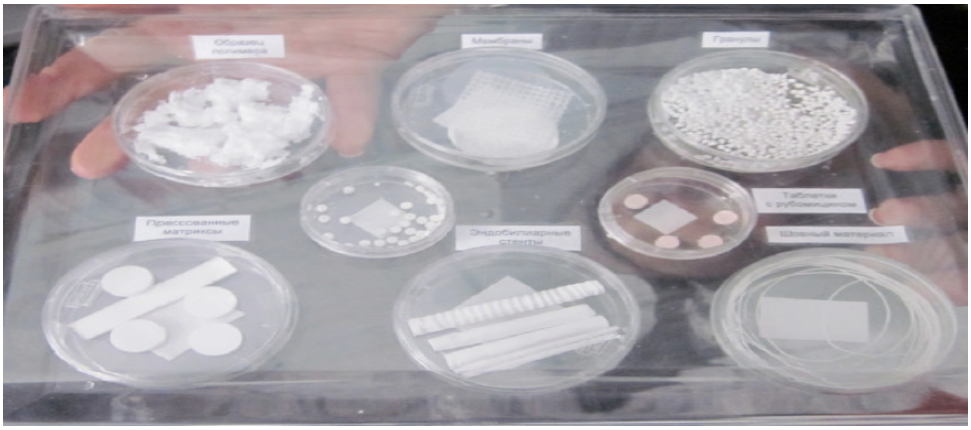

Figure 2.3: Schematic structures of representative polyhydroxyalkanoates. The representatives shown here are biocompatible and have been examined in different medical applications (Brigham and Anthony, 2012)

In the field of bone tissue application, role of P3HB is well researched and identified. After 12 months of transplantation no chronic inflammatory response was diagnosed, a satisfactory bone tissue adaptation was obtained. During the transplantation done in vivo, no sign of structural breakdown was obtained. In the rabbit bone marrow and chondrocytes, P3HB-3HH (3-hydroxyhexanoate) has shown better attachment, proliferation and differentiation capacity (Doyle *et al*., 1991). PHBHHx being immunotolerant and with low toxicity, is exploited for the growth of human mesenchymal stem cells (hMSCs), embryonic stem cells (hESCs) and spontaneously differentiated human embryonic stem cells (SDhESCs). Results obtained after 20 days and it was observed that culture shows 50% cell viability for SDhESCs and 90% for hMSCs in PHBHHx collagen hybrid scaffold. Adipogenic, chondrogenic and osteogenic differentiation on hybrid scaffold was observed for RUNX2, SOX9, PPARγ genes respectively (Lomas *et al.,* 2013).

2.13: Blending of PHB with other polymers for tissue engineering

Bone tissue engineering consists of making of scaffolds for the growth of tissues on them. It was studied that mechanical strength and bioactivity of scaffold can be improved by a blend of PAM (polyacrlamide)-PHBV-Bioactive glass. The process used was photo-initiated polymerization. In this new scaffold, hydrophilicity of PHBV is increased by PAM, strength and bioactivity is incremented by bioactive glass. This scaffold is efficient enough to be used for bone repair mechanism (Wang *et al*., 2004). PHB and PHBV were mixed in the ratio of 91:9 to make foam, inoculated with oesteoblasts taken from rat periosteum and studied for bone regeneration (Orban and Marra, 2002). Composite of P3HB and Bioglass with Vitamin E in it, increased the rate of cell proliferation of MG-63 human osteoblasts for bone regeneration. It may be due to increase in protein adsorption and hydrophilicity. Albumin was reported as the most adsorbed protein (Misra *et al.,* 2009). As vitamin E, used for surface modification, ammonia was also used for PHB thin films using plasma treatment at low pressure. This treatment changed the hydrophobic group to hydrophilic, making surface better for cell attachment.

RGD peptide with PhaP (a PHA granule binding protein) coated on a PHA scaffold, provided more adhesion of the cells on surface, equal distribution of cells all over, better differentiation and proliferation as compared to uncoated scaffold, after three weeks (You *et al.,* 2011). Porous patches of P4HB led to organized tissue formation when seeded with ovine venous cells (Martin *et al.,* 2003). Abdominal aorta of lambs was replaced by copolymer of PGA and PHA up to the length of 3 to 4 cm (Fuchus *et al*., 2001). Chen *et al.,* 2009 determined P3HB4HB (poly-3-hydroxybutyrate-co-4-hydroxybutyrate) with 4% 4HB monomer in it, was the best for growing RaSMCs (Rabbit blood vessel smooth muscle cell). The formation and accumulation of elastin was improved by 4HB component of P3HB4HB, which can be further researched for the making of artificial blood vessels.

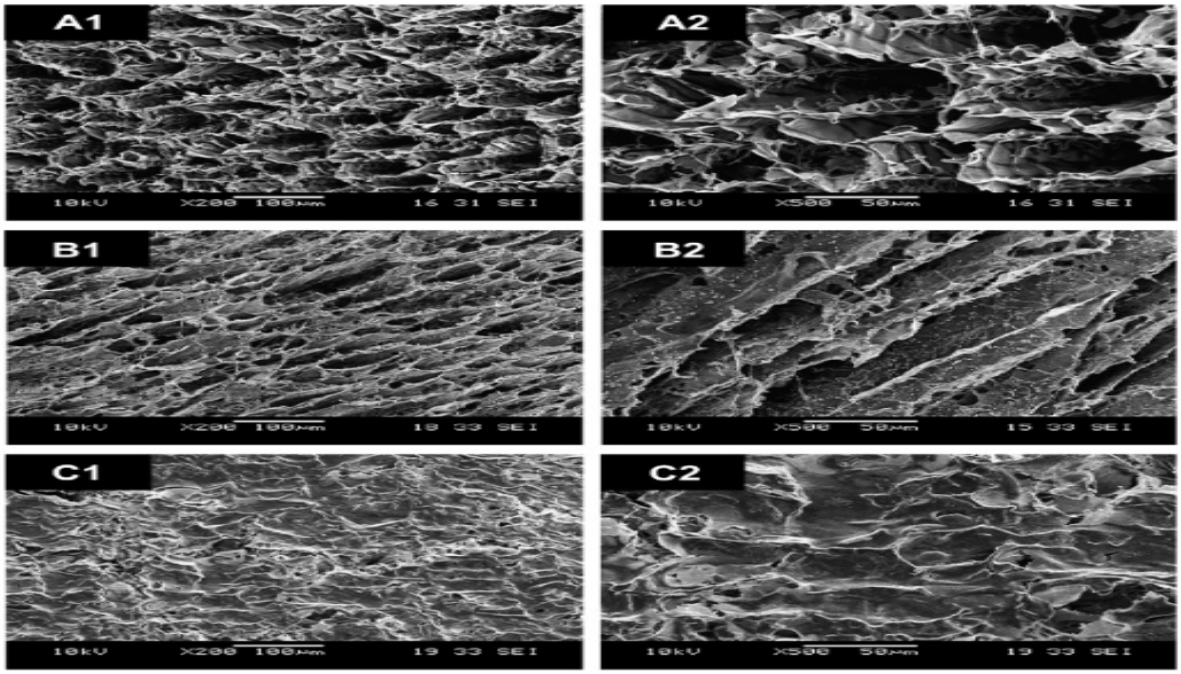

Figure 2.4: Scanning Electron Microscopy (SEM) study of PHBHHx scaffolds with or without cell growth. (A) Uncoated scaffolds sections; (B) PhaP-RGD coated scaffolds; (C) Chondrogenic differentiation studies by inoculating PhaP-RGD coated scaffolds with hBMSC cells for an incubation period of 21 days (You *et al.,* 2011)

The research was carried out on Poly(3-hydroxybutyrate) applicability in tissue engineering. Solvent casting and particulate leaching techniques by employing commercially available sugar cubes as porogen were utilized for the preparation of Poly(3-hydroxybutyrate) [P(3HB)] foams exhibiting highly interconnected porosity (85% porosity). In the scaffold microstructures, bioactive grade (BG) particles of 45S5 Bioglass⁻ grade were incorporated both in micrometer ((m-BG), <5 mm) and nanometer ((n-BG), 30 nm) sizes. Simulated body fluid (SBF) assay was done to confirm the in vitro bioactivity of the P(3HB)/BG foams. It was observed that the foams shows high level of protein absorption after immersion in SBF solution for 10 days. The blend matrix has interconnected porous microstructure, which proved to be suitable for MG-63 osteoblast cell attachment and proliferation. The analysis shows that the foams implanted in rats as subcutaneous implants and observed that implant was non- toxic and do not produce any immunogenic responses after 1 week of implantation. The bioactivity and biocompatibility of the implant were proved by analysis its bactericidal properties. *Staphylococcus aureus* as a pathogenic bacteria was tested against the P(3HB)/BG composite foams. Selected concentration of Vitamin E was also incorporated in addition to BG, in order to develop multifunctional scaffolds. The whole work concluded that the P(3HB)

scaffolds with multifunctionalities viz. bioactive, bactericidal, antioxidative behaviour, electrically conductivepaves the way for the development of an improved material with enhanced properties, which could be utilized in the advanced scaffolds for bone tissue engineering (Misra *et al.,* 2010).

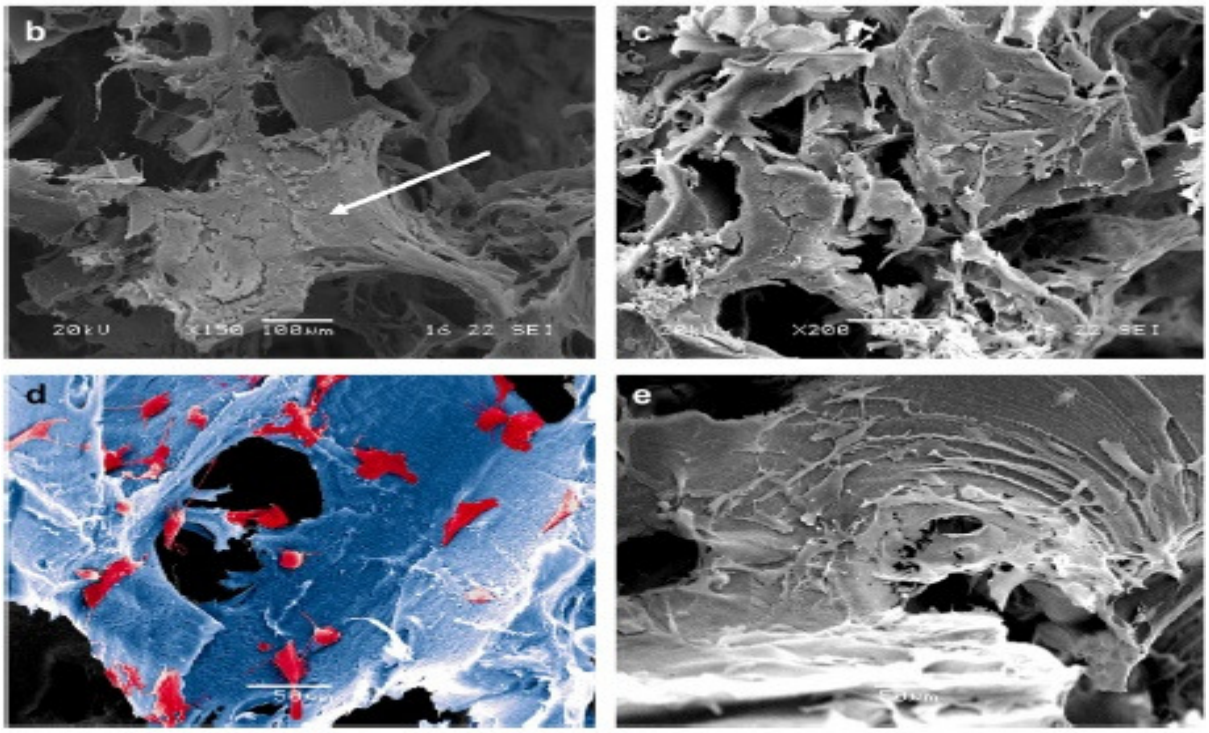

Figure 2.5: (b) Poly-3-hydroxybutyrate foams consisting of MG-63 cell layer on its surface, (c) Preparation of Poly-3-hydroxybutyrate /Bioactive Glass foams, (d) P(3HB) foams highlighting the spread of the cells and bridging the pores and, (e) P(3HB)/m-BG foams demonstrating the ability of the cells to bridge the pores and also to take up the contours of the substrate (Misra *et al.,* 2010)

2.14: Application of PHB in drug delivery system

Yilgor *et al.,* 2009 studied the use in drug delivery system. They made a 3-D construct to deliver growth factors. PHBV was used for making nanocapsules carrying BMP-7 which helped in long term release of it. In invitro release of drug various parameters such as drug loading, additional coating of the implant surface and type of active reagent used are very important.

In the therapy of chronic osteomyelitis, the implantable rods were made of PHB, PHBV, P (3HB-4HB) for the local antibiotic i.e. ducoid and sulparazone administration. It was observed that drug release was more dependent on dissolution of drug than on diffusion or degradation of polymer. The polymer coated rod released antibiotic at constant rate for two weeks while the uncoated rod released them in less than one week. The release from ducoid rods was more as compared to that of rods containing sulperazone (Chen and Wu, 2005).

It was reported that release of drugs from PHBV and PHB matrices is obtained by formation of pore and water penetration. Also, the mass of these matrices is reduced on a slow rate as compared to PGLA (polylactideglyotide) system. The blending of these polymers can be done to control erosion rate, porosity and therefore release rate of drug.

2.15: Application of PHB in therapeutics

PHB is not only confined to bacterial species. The complex PHB (c-PHB) is found in various organisms of different phyla where it performs many physiological functions. The c-PHB structure has altered physiochemical properties which are very helpful in maintaining its stable form, in cytoplasm, intracellular fluids, membranes and lipoprotein as well. C-PHB is an important part of metabolic cycles as it dissolves salts and transfers them through hydrophobic barriers (Reusch, 1995).

The therapeutic use of PHA such as oligomer P4HB and monomer 4HB is stated for various diseases. They are circulatory collapse, withdrawal of drug addiction, narcolepsy, atypical psychoses, neurosis, cancer and chronic schizophrenia. Further, they can be used for the production of growth hormones, anesthesia induction, euphoria, and production of muscle mass and heightened sexual desire (Williams and Martin, 2000). According to the investigation done on PHB, PHV, PHHX, it was observed that these are administered as a nutritional or food supplement or injected intravenously, the level of blood ketone rises which is very useful for treatment of diabetes, metabolic disease control, epilepsy, parenteral nutrition, increasing cardiac efficiency and effects in neurodegenerative disorders (Martin *et al.,*1999).

2.16: Degradation of PHB in Biological System

Degradation of the scaffolds is also required, when they are implanted in the body. If the degradation is slow, it can be recognized as a foreign material and can led to inflammatory responses. R-3-hydroxybutyric acid is a component of blood and eukaryotic cell envelope, which makes PHB as a biocompatible material. Also, the degradation rate of PHBHHx can be controlled by manipulating the molar ratio of the monomer, 3-hydroxyhexanoate. In-vitro and in-vivo biocompatibility is good in case of P(HB-HV) (Yang *et al*., 2004). The study shows that the crystalline nature and melting temperature of PHB are reduced when it is mixed with TCP (Tricalciumphosphate). As less crystallinity leads to higher rate of degradation, PHB/TCP composites can be used for tissue regeneration (Wang *et al*., 2001).

Hydrolytic enzymes degrade biological polymers very fast, in the biological medium. But the chemically produced synthetic organic polymers degrade comparatively slow (Hasirci *et al.,* 2001). The mechanical properties of polymers, their service life and their response towards biological system are influenced by rate and mode by which they are degraded. It was studied that amorphous PLLA takes only 1 year to get degraded in biological system than to polymeric PLLA, that takes about 5.6 years. However, PHB and PHBV take long time than PLLA and PLGA (Hasirci *et al.,* 2001).

Holland *et al.,* in 1990 reported that at the pH of 7.4 and 37^{o}C temperature, PHBV took 400 days to get degraded, which can be reduced by treatments such as blending. Amylose, sodium alginate, talcum powder and dextrin can be used to blend with PHBV to increase rate of degradation. Matrixes made up of Poly (anhydride-co-imide) are capable of adhering osteoblasts, and polygonal cell morphology as was observed within 24 hours. In 14 days, interconnecting layers were formed. The rapid proliferation can be due to presence of an essential amino acid, alanine, in Poly (anhydride-co-imide) matrix.

In the study of osteoblasts attachment on PHBV matrix, it was inferred that, pH change in degradation is insignificant to be taken as degradation indicator, while changes in density and weight can serve the cause. Degradation of PHBV foam with time without affecting structural integrity is required as it implies that structure do not provide any restriction to the growing cell.

2.17: Introduction to Nanocomposites

Nanotechnology is the science of 21st century promising greater technological development at nano level. In material science research, development of polymer nanocomposites could enhance the polymer applications in different fields of science and technology. Wide varieties of "nanomaterials" are being developed with controlled size and composition for the aim of improving optical, mechanical, magnetic, electrical and other related properties. Polymer based nanocomposites are considered as the next industrial revolution materials. The current focus area is identification of nanocomposite material which is light weight, eco-friendly, bio-degradable, cost-effective, performance-oriented and also suits diverse applicatory needs. Some above functionalities can be achieved by using organic and natural materials as filler material in different polymer blends. Polymer nanocomposites provide a new alternative as compared to conventionally filled polymers with significant commercial application. Clay minerals are abundant natural material found on earth surface. Clay material have size less than 2 μm size possessing specific properties of plasticity, shapeability, rheology, slip formation, ion-exchangeable property, etc. within nanosized layer. Montmorillonite clay acts as an inorganic ion exchange material which is composed of alluminosilicate layers which are stacked one over the other, so are 2:1 layer type silicates. A high percentage of MMT is present in Bentonite clay. Modification done at chemical and structural level for raw bentonite clay makes possible the preparation of new nanostructural material which can be suitable for varied applications. Combined properties and synergetic effect of comprising components of polymer nanocomposites material would yield advance morphologies and improved properties which could not be obtained from the individual material. There are plethora of methods available for production of polymer nanocomposites such as simple mixing of required inorganic materials with polymers (Lagashetty and Venkataraman, 2005), in-situ polymerization of monomers inside the galleries of the inorganic host (Okamoto *et al.,* 2000), melt intercalation of polymers (Ramos *et al.,* 2000; Wu *et al.,* 2006) etc. Polymer nanocomposites fibres are prepared by the technique of electrospinning, which has attracted great interest among researchers and industrial scientists. Manufacturing of polymeric blends availability in current market is estimated to be more than 700,000 metric tons/year. The average growth rate is estimated to be 6-7%.

The polymer blending offers possibility of adjusting the cost-performance balance and tailoring the technology to make products for specific end use applications i.e. biocompatible

thin coatings, optics, drug delivery system electrical devices, and photoconductors, biodegradable scaffolds and filter systems (Zhu *et al.,* 2000; Berkland *et al.,* 2001; Ma *et al.,* 1999; Messersmith and Giannelis, 1993; Mu and Feng, 2001; Oriakhi and Lerner, 1995; Rosca *et al.,* 2004), enhancing resin's performance, improving specific properties, viz. impact strength, resistant to solvent system etc., and provide means for the recycling of industrial and consumers wastes.

Polymer nanocomposites become a unique type of engineered material where the polymeric system properties could be improved by loading the concentration of nanoclays in the polymeric system. Loading of nanoclays improves the thermal, mechanical and optical properties of the polymeric matrix system. Nanoparticles, used in the processing of nanocomposites are hazardous to human health and environment due to its size and high reactive nature. The drawback could be resolved by preparation of nanocomposites using plant derived nanoparticles or sourced from naturally derived products i.e. naturally occurring polyhydroxybutyrate (PHB) extracted from microorganisms. The resulting nanocomposite may have desirable properties and lesser environmental impact.

Poly hydroxybutyrate, a hydrophobic molecule with unique properties of biocompatibility and degradability, also have higher melting temperature and crystallinity. For its end use, many properties i.e. its strength, thermal stability should be improved. The properties could be enhanced by blending strategy approaches, which utilized mixing of PHB polymeric system with other biodegradable materials i.e. nanoclays and resins.

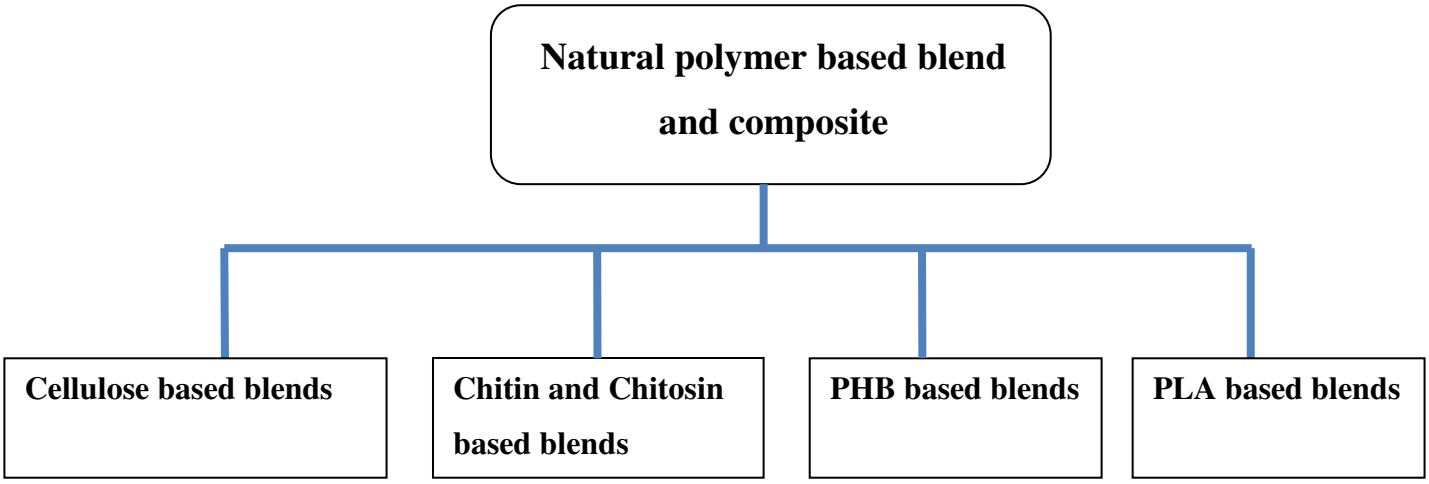

Figure 2.6: Classification of natural polymers and its blend

2.17.1: Cellulose based blends

The vogue for material applications has become more by the utilization of renewable resources. A natural raw material in this respect is cellulose, major component of wood and most plants. In the field of nanomaterial, the cellulose composite at nanoscale has started to steal the limelight as a strengthen materials because of their versatile and unique properties i.e. their low cost of production, availability at higher rates, renewable in nature, dimension at nanoscale level, high surface area, uniqueness in morphology, ease of modification in chemical structure, low density, and resistant to stress and strain. Various experiments have been involved for the extraction of cellulose nanofibres from wheat straw by chemical treatment, resulting to purify cellulose (Alemdar and Sain, 2008a). Chemically treated fibre further undergoes mechanical treatment (cryocrushing, disintegration, and defibrillation steps) to individualize the nanofibres from the cell walls. Collectively, the technique was known to be chemo-mechanical technique (Alemdar and Sain, 2008b). The analysis shows the physical characterization of the nanofibres in the range of 10-80 nm (diameter) and a few thousand nanometers (length).

There have been many methods that have been described for preparation of cellulose based nanocomposites. The method i.e. one-pot synthetic reaction has been used for the preparation of waterborne polyurethane (WPU)/ cellulose nanofibres (CN) composites (Eichhorn *et al.,* 2010). The nanocomposite were formed in this case by the presence of exposed hydroxyl functional group present on the surface of CNs and isocyanate on the ends of the WPU prepolymer, added during the preparation of nanocomposite.

The properties of cellulose nanofibres are analyzed by surface morphology and by observing other thermal and physical properties (Kalia *et al.,* 2011).

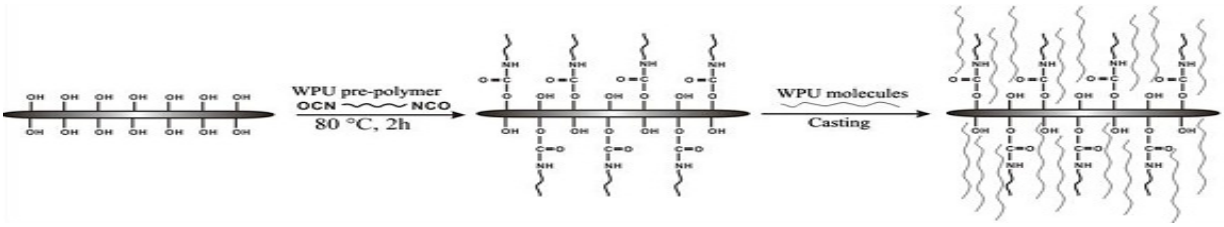

Figure 2.7: This work represented that cellulose nano-crystal blends with water borne polyurethane (WPU) in order to improve its interfacial adhesion (Cao *et al.,* 2009)

The incorporation of grafted WPU as filler in the cellulose nanocrystals enhanced the crystallization behaviour of WPU polymeric chains. Utilization of nanocrystals as a template for the preparation of an improved nanomaterial having enhanced properties was a very useful and paradigmatic approach. The resulted nanocomposite prepared showed a good dispersion and strong interfacial adhesions as compared with the pristine polymer. The blending of CNs WPU, leading to the development of cellulose nanocrysal grafted waterborne polyurethane. Many other examples were involved in the preparation of improved nanomaterial from the plants derived cellulose i.e. Eucalyptus. The derived cellulose was firstly dissolved in N-methyl morpholine oxide (N-MMO) at 120^{o}C and diluted with dimethyl sulfoxide (DMSO). The resulted solution as a sheath solution was used in an external concentric capillary needle. In the internal concentric capillary needle, sulphuric acid hydrolysis of sisal bleached and cotton fibres resulted in the production of cellulose nanocrystal suspension at the end of the reaction. These nanocrystal fibres as compared with pure cellulose fibres are superior in their mechanical properties. This technology of reactive extrusion to develop starch/cellulose acetate blends leading to the development of improved and superior nanomaterial both in their physical and mechanical properties.

Reactive processing technology has been employed for the development of thermoplastic polysaccharides i.e. cellulose- 2, 5 acetate. Utilization of grafting technique on cyclic lactones, simultaneously onto polysaccharides, hydroxy-functionalized plasticizer, and optionally also onto hydroxy-functional fillers has been employed (Warth *et al.,* 1997).

Casting methods were utilized for the preparation of nanocomposites in which cellulose nano-crystals suspension from flax fibres were undergone acid hydrolysis and then reinforced in plasticized starch with a low loading rate ranges from 5 to 30wt%. Better adhesion properties in interfacial areas were shown by cellulose nano-crystals as filler in the plasticized flax cellulose matrix with uniform dispersivity as analyzed by Scanning Electron Microscopy (SEM). In the glycerol rich phase (*Tg*1), no changes were observed at low temperature, when fillers were loaded from 0 to 30 wt% reported by the scientists. In the starch rich phase, it was observed that flax cellulose nano-crystals restricts the mobility of starch chains by forming strong intermolecular interactions between the starch chains and stiff fillers, due to which the temperature ranges shifted from 43.3 to 48.8^{o}C.

The FTIR spectra of the neat PS and other loading rates of FCNs i.e. PS/ FCNs-10, PS/FCNs-20 and PS/FCNs-30 films had been analzsed to study the presence of various functional groups, which depicts its molecular structure. Chen *et al.,* 2008; Zhang and Han, 2006 reported the peaks at 3280 cm^{-1} corresponds to the –OH group (stretching vibration of the hydrogen bonding) and 993 cm^{-1} corresponds to the stretching vibration in starch of carbonyl bonding. Due to the chemical similarity of cellulose and starch molecular structure, the nanocomposite FTIR analysis depicted the same FTIR spectra for cellulose nano-crystals as fillers in plasticized starch polymeric matrix system.

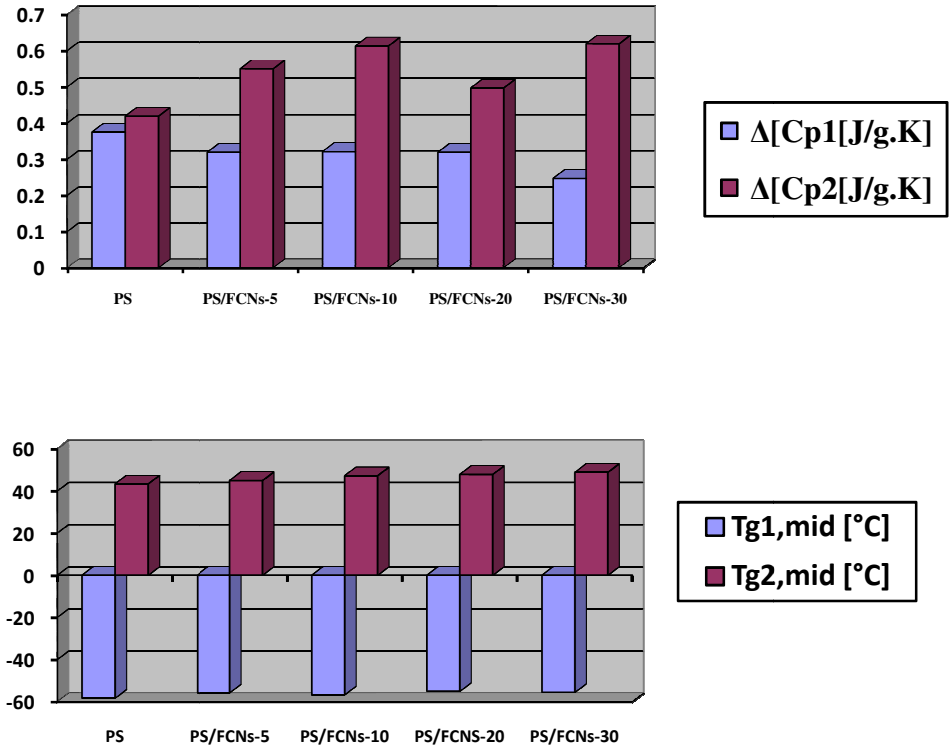

Figure 2.8: The Differential Scanning Calorimetry data of PS and the PS/FCNs nanocomposites (*ΔCp* is a characteristic constant number for a given amorphous polymer (Cao *et al.,* 2008)

*Tg*1: glycerol rich phase of temperature range –80 to –50°C

*Tg*2: starch rich phase of temperature range 30 to 60°C

2.17.2: Chitin and chitosin based blends

Chitosin, a naturally derived polyesters produced from the deacetylation of chitin fibres, which is a major components of crustaceans shell (Moraes *et al.,* 2010).

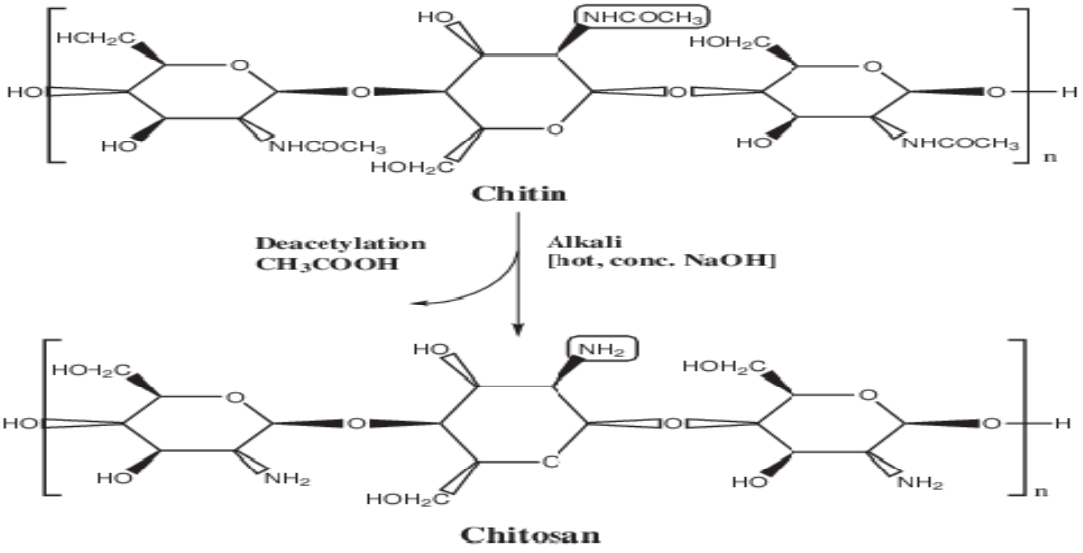

Figure 2.9: Production of chitosan from chitin by deacetylation and its chemical structural details (Ramya *et al.,* 2012)

Patel *et al.,* 1996 reported that the chitosan in pharmaceutical industries attracts various pharmaceutical chemists due to its many interesting properties. Chitosan could be used as a biomaterial, if the fragile nature and membrane permeability of the material should be improved. The unique functional properties of the chitosan are useful in the development of various blends with other polymeric systems investigated by Japanese Chitin and Chitosan Society, 1994). Studies have been carried out for the preparation of chitosan with various polymers to improve its properties both at thermal and mechanical levels observed by Kim *et al.,* 1992; Hasegawa *et al.,* 1992; Yao *et al.,* 1996; Guan *et al.,* 1998; Xiao *et al.,* 2000.

There have been important studies carried out by various workers. One of the studies by Moraes *et al.,* 2010 has the goal to prepare and characterize Silk Fibroin/chitosan blend films. The aim was to prepare insoluble film, with more stable crystalline structures. Blending of natural derived polymers is a challenging task because they do not posses regular molecular structures. The presence of wide range of molecular masses along with the influence of processing factors results in the production of different products. The factor which was necessary for the effective interaction of SF/CHI composite was pH. The pH 5.5 should be

adjusted prior to mixing in order to avoid phase separation and for the successful preparation of SF/CHI nanocomposite.

The optimized pH conditions, favors the Chitosan (CHI) to expose their amino groups in protonated form and allows its interaction with the carbonyl groups of Silk fibroin. Chemical and thermal properties of the blended CHI/SF films were analyzed by sophisticated instruments. The functional groups of chitosan can be modified by blending it with other polymers increases is utility in many useful applications. Materials with unique properties were prepared by the development of polymeric blends especially derived from natural materials (Moraes *et al.,* 2010).

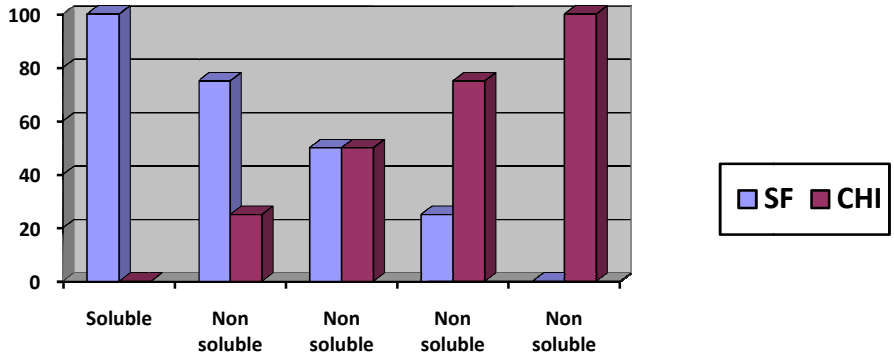

Figure 2.10: Water solubility test of the Silk Fibroin/Chitosan blends (Moraes *et al.,* 2010)

The physical integrity of the SFI/Chitosan was evaluated by observing the water solubility test of blended films. The physical structure of the blends were maintained after immersing in water for a period of time, shows that the blends were insoluble in water as compared to silk fibroin, which shows its solubility in water. The stability of SF/CHI composites paves a way for the development of more stable and improved materials.

The scientists concluded that in XRD analysis by adding CHI into the blends, some crystalline halos at $2\Theta = 14°$ and 17° could be observed. Tao *et al.,* 2007 reported that increase in CHI content is directly proportion to the intensity of halos. CHI diffractograms characterized the CHI by observing the halos at $2\Theta = 20°$. Difference in CHI content depicted the difference in halos intensities as observed by Kweon *et al.,* 2001a. Strong inter and intra molecular hydrogen bonds were formed by the presence of –OH and $–NH_2$ groups in the chitosan structure. Duan and his co-workers investigated with his studies and also proved that

the presence of regularity in the chitosan structures promoted the formation of crystalline regions, which were depicted by the intensity of halos.

The FTIR analysis shows that the spectra at 3454 cm^{-1} corresponds to the –OH stretching vibration for pristine chitosan and migrated to a lower peak at 3434 cm^{-1} as observed in 1:1 Chitosan/Silk Fibroin blend. Terin and Elvan in 2009 investigated from their studies that intermolecular interactions were formed between the SF/CHI blends, which were depicted by the disappearance of C=O amide group observed at spectral peak at 1628 cm^{-1} as compared to neat chitosan.

Kweon *et al.,* 2001b; Park *et al.,* 1999 studied that the silk fibroin was more stable in the presence of chitosan with 25wt%, which shows that maximum CHI content should be incorporated in the silk fibroin solution to induce modification in structural conformation of silk fibroin and to make it more stable and crystalline.

DCS analysis shows that the thermograms of the blend films, presented a mixture of components. It observed that, as compared with to pure chitosan, the blended composite do not show peaks at 204.6 and 224°C. Kweon *et al.,* 2001a observed similar results in *A. pernyi* SF/CHI blends, in which crystallization exothermic peak of SF was not presented in the composite material. The silk fibroin chains undergo pre-crystallization, which was due to the diffusion of acetic acid in the blended films. The acetic acid was used to dissolve the chitosan fibres. Water evaporations leads to decrease in the initial weight, which was below 100^{o}C. The silk fibroin provides thermal stability to the blended film with each increase in the loading wt%. As the content of SF increases, thermal degradation temperature of the blend also increased. It was analyzed that 50:50 was the weight residues and thermal weight residues of the blended film. Similar behavior was observed in all the blended films with a weight loss peak of below 100^{o}C and thermal degradation peak near to 300^{o}C.

The pure chitosan % decomposition was low as compared to blend chitosan/silk fibroin (1:1) analyzed by thermo-gravimetric analysis thermogram (TGA).

Degradation percentage was increased from 14 to 50%, when compared with neat chitosan. Blended films was more stable than parental ones (Paul *et al.,* 1989 Sandford *et al.,* 1990) but

sometimes unusual behavior was due to the presence of crystalline structures and variation at morphological levels in the blended films reported by Viciosa and Dionisio in 2004.

The molecules of silk fibroin show interaction in the system reported by Kweon *et al.,* 2001b; Park *et al.,* 1999. Silk fibroin is a natural derived protein fibres with excellent mechanical properties. It has good biocompatibity and is permeable to oxygen and water molecules. Its applicability could be broaden in the area of medical sciences particularly in wound dressing. The silk fibroin is very brittle when dried during casting, which limits its practical use. Reinforced structure preparation was done by blending of Silk Fibroin with chitosan broaden its applications in biomedical field (Bansal, 2010).

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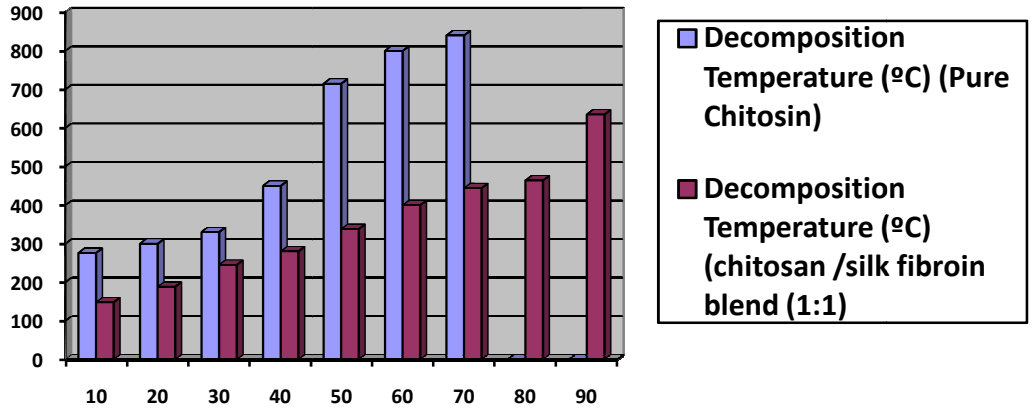


Figure 2.11: Comparative Analysis of Decomposition Temperature of Pure Chitosin and Chitosan/ Silk fibroin blend (1:1) (Ramya *et al.,* 2012)

Binary blended Silk Fibroin/Chitosan films have smooth surfaces and could be used as antibacterial and antioxidant compound in wound healing. Chitosan in the blended film acts as a barrier for microorganisms and removes excess fluids from the wound sites. The unique properties of the film with plant extracts find a potential application as a wound dressing contact layer. The properties include high area to volume surface ratio, diameter of fibre in micro-scale, light weight and large pore size.

2.17.3: PLA based blends

The biodegradability, ease of processing, transparency and price makes bio-based biodegradable polymers, one of the most attractive material that posses facile commercial availability. Although, scientists are increasing their interest in using PLA for disposable degradable plastic articles; however, there are properties such as high flexibility, gas retardency, high melt viscosity and melt strength/elasticity that are often not good enough for some end-use applications, such as blow molding (Singh *et al.*, 2003; Di *et al.,* 2005). Different investigations were carried out, to improve the physical properties of PLA, especially in terms of thermo-mechanical stability by the addition of different fillers (nanoparticles) in PLA was explored (Sinha and Okamoto, 2003; Pollet *et al.,* 2003; Utracki, 2004; Pluta *et al.,* 2002). Various melt blending techniques were used to prepare PLA based nanocomposite by the addition of 5 % wt of an organically modified montmorillonite (CLO) [CLOISITE 20A], unmodified sepiolite (SEP) [PANGEL S9] and organically modified zirconium phosphonate (ZrP) [ZrP 102]. At room temperature, a completely amorphous structure for the polymer matrix was obtained. The results revealed that during the film cooling process (*ca*. 10°C/min), the PLA matrix was not able to crystallize. For the PLA/CLO and PLA/ZrP nanocomposites, the WAXS analysis indicated a similar broad halo with maximum at. 2θ = 17º and shows an amorphous structure of the polymer matrix in these specimens. So, depending on the type and functionalization of nanoparticles, different dispersion levels were analyzed by Scanning electron microscopy (SEM) and Wide angle X-ray scattering (WAXS) (Fukushima *et al.,* 2012).

Further, Differential Scanning Calorimetric analysis shows that pristine PLA on heating was fully crystalline, whereas the addition of ZrP could promote extent of PLA crystallization, but the addition of CLO and SEP to PLA did not significantly affect the crystallization on heating and melting behavior of PLA matrix. There was remarkable increase of elastic properties (E') of PLA nanocomposites by the addition of all the nanoparticles shown by the Dynamic Mechanical Thermoanalysis (DMTA). The presence of CLO and SEP attributed to the formation of a PLA/nanoparticle interconnected structure within the polymer matrix and analysis shows that there was a significant enhancement in the melt viscosity and dynamic shear moduli (G!,G") of PLA nanocomposites. The addition of SEP and ZrP nanoparticles to the PLA polymer matrix did not significantly effects the oxygen permeability of PLA. The results also revealed that only the CLO has the positive impact on the permeability of PLA.

The analysis shows that the addition of CLO leads to about 30% decrease as compared to PLA permeability, due to the good clay dispersion and clay platelet-like morphology. Based on these achievements, a high potential of these PLA nanocomposites in sustainable packaging applications could be envisaged (Weber, 2011).

2.17.3.1: PLA/Layered Silicate Nanocomposites

Organo-modified nanoclays are easily dispersed in the hydrophobic polymeric systems for the preparation of successful polymer/nanoclay composite. Organo-clays are prepared by utilizing various organo-modifiers of different chain lengths and are examined in MMT, bentonite, smectite and mica. Comparative studies were carried out on nanoclays by incorporating organo-modifiers and it was analyzed that the gallery spacing was increased in case of mica, when treated with organo-modifiers. The smallest galleries were observed in case of smectite layers. Clay of larger size restricted the conformation of modifier by physical jamming. The mica has the property of ion exchange, thus utilizing these properties in formation of organo-modified clays (Okamoto *et al.,* 2002).

The PLA nanocomposite with smectite shows an increment in the young's modulus as compared to the nanocomposites formed by MMT or mica. The same clay loading in smectite system has better dispersion than other clay systems. Improvement in the gas barrier properties were observed in smectite PLA composite as compared with MMT or mica systems. The large galleries of MMT or mica are stacked in nature, within their nanocomposites. PLA i.e. Poly lactic acids are produced from the fermentation processes, which includes naturally derived or plant based substrates such as potato, corn and other waste products. The green technology behind the production of PLA makes it a potential packaging material. PLA applicability in biomedical devices includes sutures with bioabsorbent, medical implants, culturing of tissues (Kohn *et al.,* 1994) and controlled drug delivery carrier (Park *et al.,* 1994).

For the end use applications, thermal and mechanical properties should be enhanced, which could only possible by the development of improved nanocomposites. The properties are thermal degradation, melting temperature, crystalline temperature, enthalpy of melting, young's modulus, enlongation at break, stress and strain etc. Pristine PHB blending with two dimensional alumino-silicate layer galleries is one of the successful way to enhance the

material performance (Giannelis, 1996; Garces *et al.,* 1835; LeBaron *et al.,* 1999; Vaia *et al.,* 1995; Usuki *et al.,* 1998; Lan *et al.,* 1996; Alexandre and Dubois, 2000; Maiti *et al.,* 2002).

Table 2.4: Characteristics of the clay used (natural, organically modified and in nanocomposites) (Ray and okamoto, 2003)

Properties	**Smectite**	**MMT**	**Mica**
Size[a] (nm)	50-60	100-130	200-300
CEC (mequiv/100g)	87	113	120
D(001)[b](clay) Na^+ (nm)	1.2	1.2	1.25
D(001)[b](organoclay with C16 clay (nm)	1.87	2.13	2.44
D(001)[b] in nanocompositeswith C16 clay (=3wt%) (nm)	2.27	2.55	2.69
Crystalline size[c] of organoclay with C_{16} salt (nm)	7	12	20
Crystalline size[c] in nanocomposites (=3wt%) (nm)	3.5	10	22

[a]size of organically modified clays x

[b]calculated from wide-angle X-ray diffraction data

[c]Calculated from Scherrer equation $D_{hkj} = (k\lambda)/(\beta \cos\theta)$

where k is a constant, β is the half-width, and θ is the peak angle.

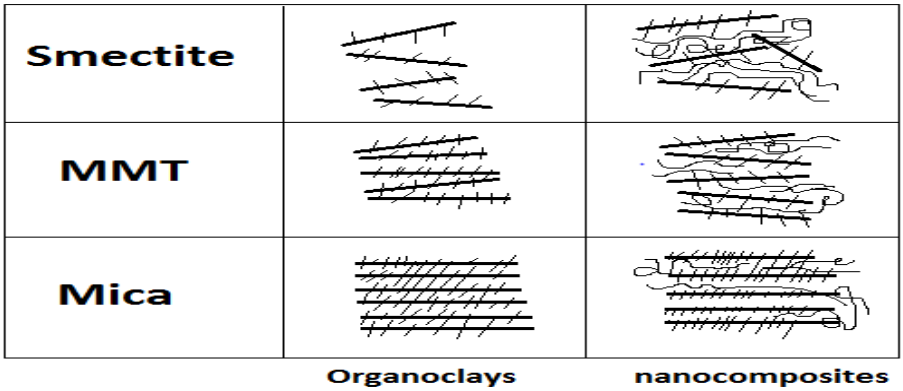


Figure 2.12: Organoclays and its nanocomposites

Poly (lactic acid) (PLA) based nanocomposites prepared by adding 5wt% filler content of organically modified montmorillonite (CLO) and unmodified sepiolite (SEP) were obtained by melt blending procedure. WAXS and SEM analysis shows a good dispersion of CLO and SEP into the polymer matrix (Boo *et al.,* 2007; Wu *et al.,* 2009; Alberti *et al.,* 1991). PLA containing ZrP shows a limited dispersion on PLA matrix as compared to CLO, likely due to its significant lower interlayer thickness, leading to formation of a polymer microcomposite. DSC analysis shows that neat PLA and composites presented no tendency to crystallize on cooling. Nevertheless, it was observed that PLA was able to crystallize on heating, and that the addition of ZrP can promote the extent of crystallization of the PLA on heating, which can be explained by the regular distribution of ZrP micronic particles able to act as efficient nucleating agents for PLA crystallization. The dynamo-mechanical results showed improvements in storage modulus (E!) with the addition of all particles, resulting in a remarkable increase of elastic properties for PLA composites, especially at temperatures close to the glass transition temperature. These (E!) increases were associated to possible interactions between the particles and PLA molecules originated from hydrogen bonding between the carbonyl group of PLA and the hydroxyl groups belonging to the structure of CLO, SEP and ZrP. Interestingly, similar increases of E! was obtained upon the addition of ZrP into PLA as compared to CLO and SEP, despite its low dispersion level into the PLA matrix, possibly explained by the high crystallinity of ZrP particles as well as the very regular distribution of ZrP micronic particles along all the polymer matrix. The melt viscosity and dynamic shear moduli of nanocomposites were also enhanced significantly by the presence of CLO and SEP (Fukushima *et al.,* 2012).

The oxygen permeability of PLA did not significantly vary upon addition of SEP and ZrP particles (Ray and Okamoto, 2003). Only addition of CLO leaded to an important decrease of the PLA oxygen permeability. Optical transparency measurements show the transparency of PLA in the visible region with the presence of particles, thus preserving the good optical properties typical of PLA. The improvements in the viscosity of PLA composites as well as the similar transparency and improved gas barrier properties could provide to these composites an opportunity in the field of packaging. Even more, it is reasonable to expect that by controlling the nanoparticle content and processing conditions, PLA based nanocomposite films with improved properties could be obtained for various industrial applications.

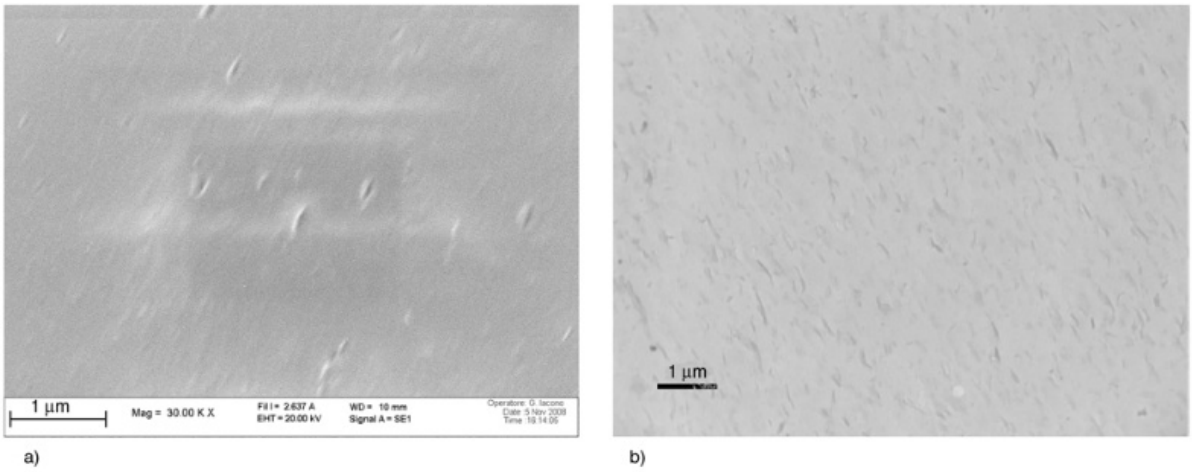


Figure 2.13: Scanning electron micrograph (a) and transmission electron micrograph (b) for PLA + 5% CLO (Fukushima *et al.,* 2012)

2.17.3.2: PLA with PHB along with Maleic Acid (MA) as compatibilizer

Melt mixing method was utilized for the preparation of partial miscible blend of PLA and PHB (Ohkosh *et al.,* 2000). Maleic anhydride was used as a compatibilizer which creates interpenetration network structure between the two matrices by providing additional interactions. The compatibilizer also increased the misciblity of two matrices, which was further characterized by FT-IR and SEM analysis. Hydrogen bonding with intermolecular flexibility was uniformaly distributed within the matrix of polymer blend. As compared to virgin PLA matrix, it was analysed that the blend enlongation at break and Izod impact strength has been analyzed at a maximum of 540.17% and 99%, respectively (Hongzhi and Jinwen, 2011). The tensile strength of the blend matrix was improved by the incorporation of layered silicates into the blend and has been modified to 49% without compromising the flexible characteristics. To promote the thermal stability of the blend, the insulation property of intercalated/exfoliated layered silicates works very efficiently. Natural montmorillonite (NaMMT) and commercially modified montmorillonite Cloisite 30B [(C30B)] were utilised for the comparative study, to analyse the effect of each layered silicates on the physical properties of the mixed blend composed of PLA/PHB/MA (Jandas *et al.,* 2014).

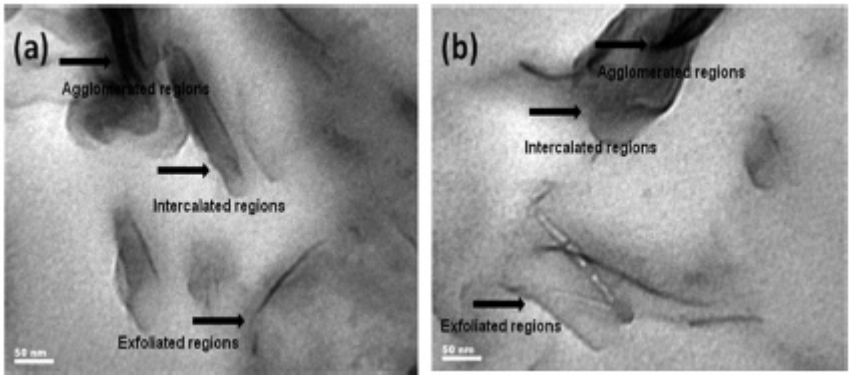


Figure 2.14: Transmission Electron Microscopy images of the blend composites prepared from (a) Organo-modified MMT and (b) Cloisite 30B (Jandas *et al.,* 2014)

2.17.4: PHB blends and its superiority for biomedical applications

Poly(hydroxybutyrate)-PHB (-[CH(CH3)CH2COO]n-) has attracted much attention in recent years. It is eco-friendly in nature and is fully bio-based thermo-polyester, which is produced by the fermentation of bacterial strains. Polyhydroxybutyrate produced by microbial fermentation as intracellular lipids inclusion within the cytoplasm of many microorganisms. Griffin in 1993 reported that the properties of PHB are very similar to the synthetic derived polymers i.e. polypropylene, which increases its applications in industries. The properties include high crystalline nature (50-70%) and excellent insulator for gas (permeable to water vapor 560 g.μm/m^2/day) etc. The drawbacks of PHB include high fragile nature indicate 3-5% elongation at break and are not physically stable above its melting point with degradation starts from 200°C. These drawbacks hindered the technology of thermo-polyester processing. Three factors are responsible for the brittle nature of PHB i.e.

1. Glass transition temperature (Tg ^{o}C) is close to room temperature.
2. Storage at room temperature leads to secondary crystallization
3. Low nucleation density.

All these drawbacks are derived its high purity nature and regularity in stereo-chemical structures. Enhancement in thermal and mechanical properties of PHB are achieved by blending technology i.e. with other biodegradable polymers, resins etc. Misra *et al.,* 2006; Doyle *et al.,* 1991; Knowles *et al.,* 1992; Luklinska and Bonfield, 1997 reported that the composite of PHB/hydroxyapatite (HA) have been utilized in the preparation of scaffolds to treat the fractured bones.

Shum-Tim *et al.,* 1999 investigated from their studies that a blend of polyglycolic acid (PGA) and PHB were used in the preparation of pulmonary valve leaflets and arteries scaffolds in animal model i.e. sheep. Sodian *et al.,* in 1999 designed PHB based scaffolds used in pulmonary valves and inserted again in the sheep animal model. Biopolymers pave the way for the development of successful scaffolds, which could enhance its application s in tissue engineering. Novikov *et al.,* 2002 reported that after several experiments and investigations, PHB has been successfully used as a graft matrix for neuronal generation after spinal cord injury in rats. PHB films were also found to provide scaffolding to patch a large bowel defect in rats and were shown to degrade more readily in vivo (Freier *et al.,* 2002).

The exceptional properties of polymer/nanocomposites based PHB gained huge popularity among researchers and industrial scientists, because its properties are superior as compared to virgin polymer. Homogeneous dispersion of nanoclays by blending technology is carried out in polymeric matrix. These nanoclays are inorganic in nature with atleast one dimension measures in nanometer scale. The interaction of nanoclays with polymeric matrix by intermolecular hydrogen bonding substantially improves the physical and mechanical properties. Ray and Bousmina, 2005 investigated that Poly (-3-hydroxybutyrate) (PHB) is rapidly gaining recognition as one of the most promising biopolymers.

Many of the researchers have worked on blending and modification of PHB characters. The major concluding output come reveals that much work is needed for preparation of appropriate blends of PHB, which can prove appropriate for various industrial applications.

Low molecular weight additives incorporation in PHB polymeric matrix lowers the glass transition temperature which deteriorates the tensile properties of polyhydroxybutyrate. Decrease in the extension ratio at break was due to the necking and cold drawing at room temperature and was observed at elevated testing temperature in blends of 40 and 50% PHB polymer.

The PHB content of less than 60% observed to have a crystallinity of less than 0.4%. The amorphous component (extension ratio $\lambda R > 3$) should have a molar mass of $Mn > 30{,}000$ g/mol when blended with the polymeric matrix in order to obtain good mechanical properties.

Various blends of PHB have been prepared taking into consideration the shortcomings of pristine PHB. Strategies have been followed to increase the mechanical and physical strength of this important polymeric material.

2.17.4.1: PHB/nanoclays

Blending of PHB with certain nanoclays or resins improves the material performance including biodegradation. Addition of inorganic nanoclays to form nanocomposites (Garces *et al.,* 2000; LeBaron *et al.,* 1999; Vaia *et al.,* 1995; Usuki *et al.,* 1995), enhance the material performance both at thermal and mechanical levels.

The flexibility and optical clarity of the neat polymer was achieved by improving the barrier properties, which could only be possible by clay nanocomposites investigated by Yano *et al.,* 1997; Xu *et al.,* 2001. The charged nature of inorganic clays is hydrophilic in nature, which make its incompatible with most of the hydrophobic natural polymeric material systems is one of the major consequence. Alteration at the polarity of inorganic clays helps in the successful formation of polymer-clay nanocomposites. The ion exchange reactions occur in the hydrophilic clays by cationic exchange such as alkylammonium ion reported by Maiti *et al.,* 2007. 12-aminododecanoic acid (ADA) was added in MMT as an organic modifier, in which sodium ion was being replaced by amino acid *via*. cationic exchange by ion-exchange reactions (Ling *et al.,* 2002).

$$Na^{+}\text{-CLAY} + HO_2C\text{-R-}NH_3^{+}Cl^{-} \longrightarrow HO_2C\text{-R-}NH_3^{+}\text{-CLAY} + NaCl$$

2.17.4.2: PHB blend with MMT modified by 12-aminolauric acid

Montmorillonite (MMT) nanoclay was modified with 12-aminolauric acid and mixed with PHB solution. PHB/clay nanocomposites with 1 wt% loading shows a great effect on the crystallization behavior of PHB (Cheng *et al.,* 2014; Galego *et al.,* 2002) . The mechanical properties of PHB/MMT, including tensile strength, Young's modulus, elongation at break and toughness, were characterized by Instron (Sun *et al.,* 1999). An exfoliated morphology with almost complete dispersion of the silicate layers was observed. The exfoliated nanocomposite exhibits higher crystallization temperature and faster crystallization rate resulted from the heterogeneous nucleating effect due to the nano-scale dispersed silicate layers. The effect became less evident when the MMT loading was increased. Due to the formation of nanocomposites, small amounts of clay acts as effective reinforcing filler to

enhance the mechanical properties of PHB, such as tensile strength and Young's modulus. The novel effect of addition of weight load % of nanoclay for the formation of nanocomposite will benefit to the processing and commercial applications of microbial poly(3-hydroxyalkanoate) (Cheng *et al.,* 2014).

Blending of PHB is carried out for improving its physical properties. Cheng and his co-workers, worked on the physical and mechanical properties of polyhydroxybutyrate/clay nanocomposite membranes. They investigated that montmorrillonite (MMT) type of nanoclay organically modified with 12-aminolauric acid and further blended with PHB for the preparation of nanocomposites. The scientists observed that PHB/clay nanocomposites with 1 wt% loading show a great effect on the crystallization behavior of PHB.

2.17.4.3: PHB blend with MMT modified by octadecylamine (C_{18} MMT)

Nanocomposites were prepared by various loading wt% of organically modified MMT with octadecylamine (C_{18}MMT), which were uniformly dispersed in polymeric system i.e. PHB by melt intercalation method. Achieving improved thermal properties was the main aim of the current studies. Morphological characterization of the composites was conducted by x-ray diffraction analysis (XRD). The effect of clay content on the thermal properties of the material was studied by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) (Zuburtikudis *et al.,* 2008).

The structure of the nanocomposite materials was investigated by XRD using a Rich. Seifert 3003 TT diffractometer and Ni-filtered CuKα radiation (λ=0.154 nm) (Marras *et al.,* 2007). The scanning range was varied from $2\theta=2^{o}$ to 10^{o}. Basal spacing (natural polymer)=1.2 nm, Basal spacing (C_{18}MMT)=2 nm. Thermal Gravimetric Analysis measures the melting behavior of pure and nanocomposite PHB. TGA data for pure PHB and its hybrids, was taken in a N2 atmosphere and in the temperature range of 150^{o}C, (where the samples are considered to be free of humidity), to 750^{o}C. With a heating rate of 10^{o}C/min, PHB volatilizes completely in a single step beginning at about 220^{o}C (Zanetti *et al.,* 2001). The introduction of organophilic inorganic material significantly improves the thermal stability of the polymer raising the temperature of decomposition initiation. The degradation properties of the polymer clay nanocomposites were also investigated under oxidative atmosphere and isothermal conditions (190^{o}C). The organoclay addition significantly delays the polymer mass loss. The

incorporation of 10wt% of OMMT into the polymer system, enhanced its weight loss time % five times more as compared to neat polymer film as observed by Zuburtikudis *et al.,* 2008.

The nanocomposites displayed improved thermal stability compared to pristine polymer. The dispersed inorganic platelets assist the formation of a protective layer on the surface of the polymer, which acts as an insulator and a mass transport barrier retarding the decomposition rate of the polymer matrix.

Marras *et al.,* 2007 demonstrated the modification of sodium montmorillonite (NaMMT) through the insertion of amphiphilic hexadecylammonium cations into the clay's interlayer galleries. The researchers concluded that the important aspects for incorporation of the organomodified MMT particles into different media for various applications such as polymer nanocomposite preparation. From these studies scientists confirmed about the implications of the alkylammonium surfactant concentration in the modification process of NaMMT which influences significantly preparation of polymer nanocomposites.

2.17.4.4. PHB blend with PCL/ stearate Mg-Al LDH

Liau *et al.,* 2014 observed that XRD shows the increasing of basal spacing from 8.66 to 32.97^{o}A in modified stearate Mg-Al LDH, and TEM results revealed that the stearate Mg-Al LDH layers were homogeneously distributed in the PHB/PCL polymer blends matrix. Scanning electron microscopy (SEM) proved that clay improves compatibility between polymer matrix and the best ratio 80PHB/20PCL/1stearate. Mg-Al LDH surface was well dispersed and stretched before it breaks gradually which increases the flexibility and compatibility of nanocomposites. The presence of 1wt% of stearate Mg-Al LDH content shows higher interlayer spacing and production of intercalated nanocomposites by addition of high filler content. The Mechanical analysis show that an 80PHB/20PCL/1stearate Mg-Al LDH nanocomposite was drastically enhanced by about 66% and 300% for its tensile strength and elongation at break (Liau *et al.,* 2014).

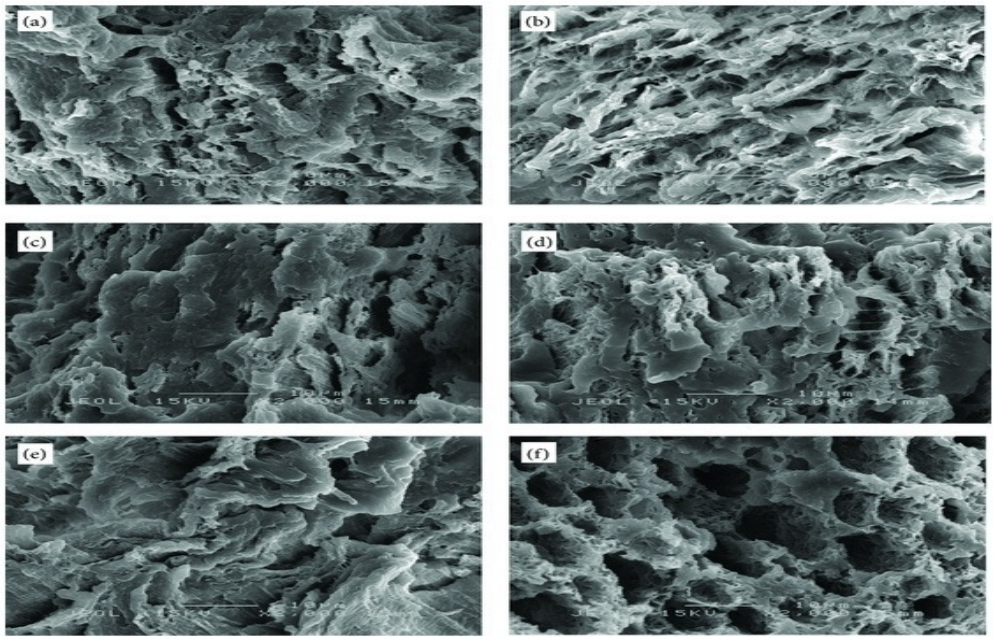


Figure 2.15: Scanning electron micrographs (SEM) of (a) 80: 20 Polyhydroxybutyrate: Polycaprolactone blend and Polyhydroxybutyrate: Polycaprolactone: stearate Mg-Al LDH nanocomposites with 0.25%, 0.5%, 1%, 1.5% and 2 wt% stearate Mg-Al LDH content (Liau *et al.,* 2014)

2.17.4.5: PHB/Carbon nanotubes

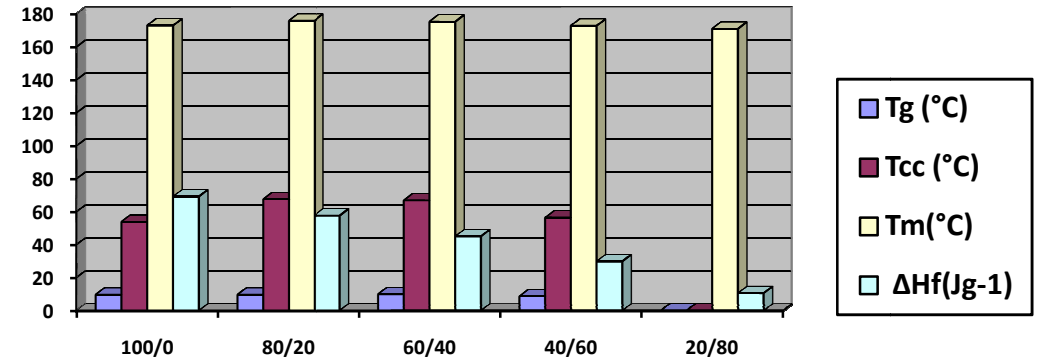
The most effective way for improving the properties of PHB has been developed by the addition of carbon nanotubes (CNT). Addition of carbon nanotubes even at low loading rate wt% significantly improves the thermal and mechanical properties of the PHB reported by Maiti *et al.,* 2007; Xu and Qiu, 2009. The work by Xu and Qiu, 2009 was focused on the analysis of the dynamic crystallization and melting behaviour of PHB nanocomposites containing small amounts of environmentally friendly IF (fullerene) like nanoparticles including layered metal dichalcogenides such as WS2 and MoS2 with unique physical and chemical properties. In DSC analysis, neat PHB shows a Tcc (Crystallization temperature) around 80ºC; however, with blending Tcc shift to around 94, 105 and 104ºC for PHB/IF-WS2 (0.1 wt%), PHB/IF-WS2 (0.5 wt%) and PHB/IF-WS2 (1 wt%), respectively. The results indicate that IF-WS2 act as highly nucleating agents for PHB crystallization. Complementary information on the nucleating effect of IF-WS2 was also investigated through the melting behavior of the PHB/IF-WS2 nanocomposites, for samples previously crystallized at various cooling rates. Two melting endothermic peaks (Tm1 and Tm2) are observed for both neat PHB and the PHB/IF-WS2 (1 wt%) nanocomposites by Xu and Qiu, 2009. Variation in the

shape of two melting endotherms was found due to increase in cooling rate. The magnitude of Tm_2 in area relative to that of Tm_1 became larger with increasing cooling rate, indicating that the re-crystallization of PHB was enhanced with increasing cooling rate. Under the same condition, Tm2 does not appear so obvious in the nanocomposite than in neat PHB, indicating that the presence of IF-WS2 may hinder the re-crystallization of PHB in the nanocomposites compared with that of the neat PHB. The restricted re-crystallization of PHB in the nanocomposites may be explained by the strong heterogeneous nucleation role that IF-WS2 plays in enhancing the crystallization rate of PHB. As compared to neat PHB, crystals of nanocomposites were more perfect and stable as observed by Naffakh *et al.,* 2011.

2.17.4.6: PHB/ starch blend

Investigations on the thermal behavior and structural morphology of PHB with starch acetate (SA) blends were carried out by Zhang *et al.,* 1997. Derivatives of cellulose and starch were naturally derived biodegradable polymers. The thermal behavior and surface morphology of PHB/starch acetate blends were studied by various sophisticated techniques have been already discussed in many part of literature. The sodium acetate component does not affect the glass transition temperature of the blended composite and remain same at 9^oC, similar to that of virgin PHB. Small depression areas were seen when the content of sodium acetate increased in the PHB blend, which shows a little increase in the value of melting temperature, when compared with pure PHB.

The FTIR spectra shows peak at 3470 cm^{-1} corresponds to the hydroxyl groups of sodium acetate and another peak at 1724 cm^{-1} correspond to the carbonyl groups were observed in the blended films. The addition of sodium acetate affects the crystallization nature of PHB both from melting to cooling and from glassy state on heating. The pure PHB has higher temperature and enthalpy of non-isothermal crystallization, when compared with blended nanocomposites. The peaks of cold crystallization of polyhydroxybutyrate in the blends were shifted to higher temperature as compared to virgin PHB.



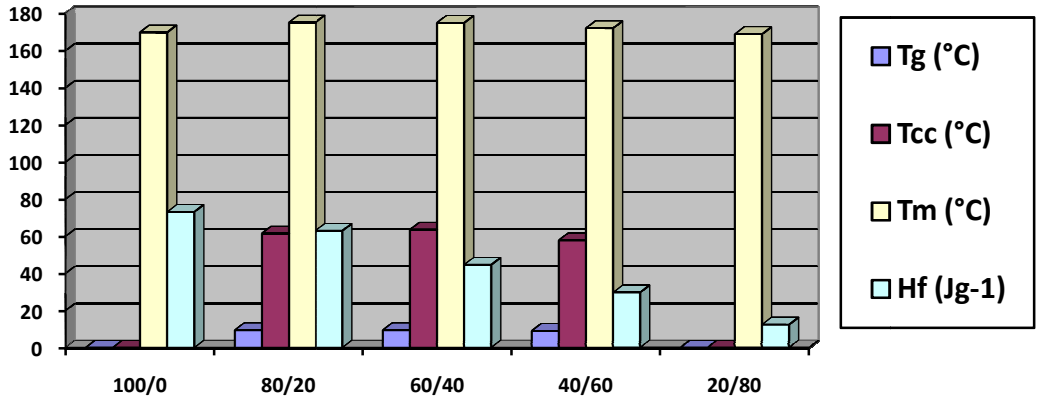


Figure 2.16: Differential Scanning Calorimetry results on heating run after rapid cooling (Run I) and (Run III) (Zhang *et al.,* 1997)

T_g = glass transition temperature, T_{cc} = temperatures of cold crystallization, T_m = Melting temperature, ΔH_f = apparent melting enthalpy

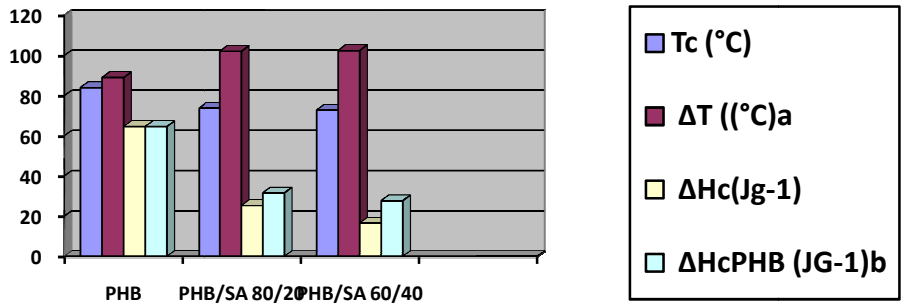


Figure 2.17: (T_c): Temperature and enthalpy of non-isothermal crystallization and ΔHc, from the melt at a rate of 20^0C min^{-1} (Zhang *et al.,* 1997)

T_c: Temperature of crystallization; ΔH_c PHB(Jg^{-1})

ΔH_c: enthalpy of crystallization; ΔT: supercooling temperature (Zhang *et al.,* 1997)

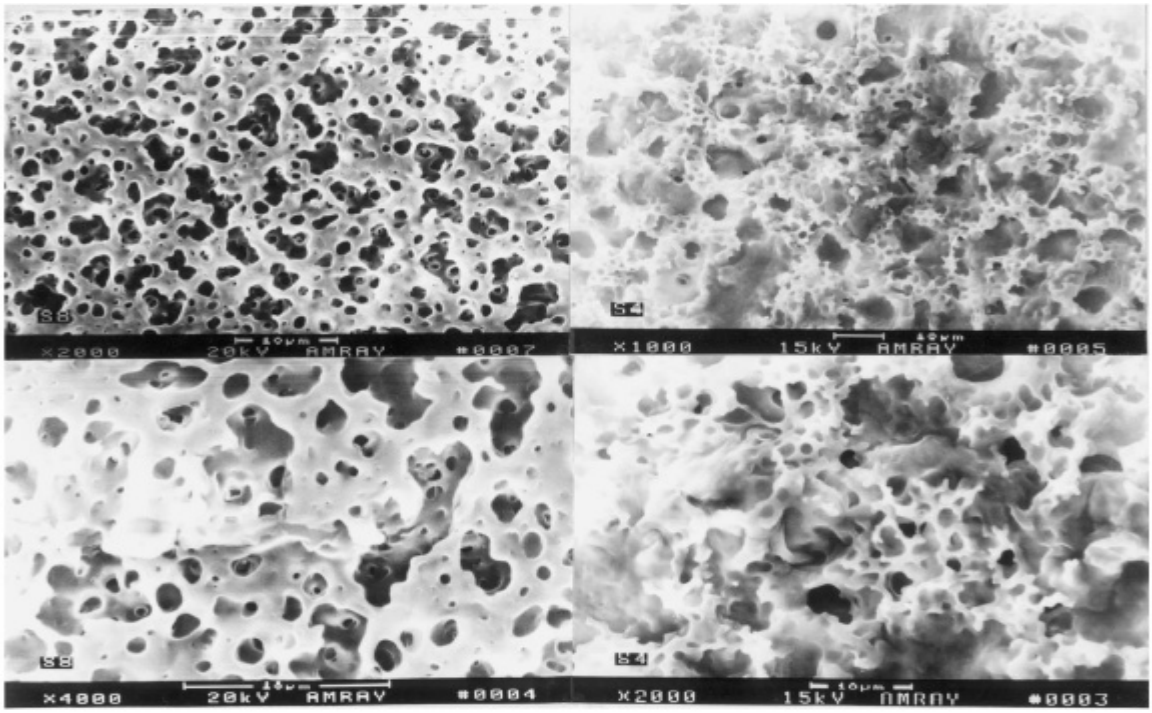


Figure 2.18: Scanning electron micrographs of the blend (a) PHB: SA 80:20; (b) PHB:SA 40: 60 (Zhang *et al.,* 1997)

2.17.4.7: Blends of poly[(R)-3-hydroxybutyrate] with poly(g-benzyl-L-glutamate)

Recently work have begun on exploration of a new blend family of PHB with poly (amino-acid)s or their derivatives because of their attractive potentials as biomaterials. The combination of tough PBLG with high-crystalline PHB may produce PBLG-based blends with good mechanical properties. In the present work, our investigation mainly focused on the miscibility, thermal behaviour, morphology and mechanical properties of PHB/PBLG blends. The mechanical properties of the blends have been well correlated, in particular with regard to blend morphology (Deng *et al.,* 2001). The DSC curves of the melt-quenched samples (run IV), except for pure PBLG, shows a single distinct glass transition and a cold-crystallization peak during the heating run from -30^{o}C to 100^{o}C. No glass transition is observed for pure PBLG under the present experimental conditions.

Deng *et al.,* 2001 investigated work on the miscibility, thermal behavior, morphology and mechanical properties of poly [(R)-3-hydroxybutyrate] (PHB) with poly(g-benzyl-L-glutamate) (PBLG) by means of differential scanning colorimetry (DSC), scanning electron microscopy (SEM) and tensile tests.

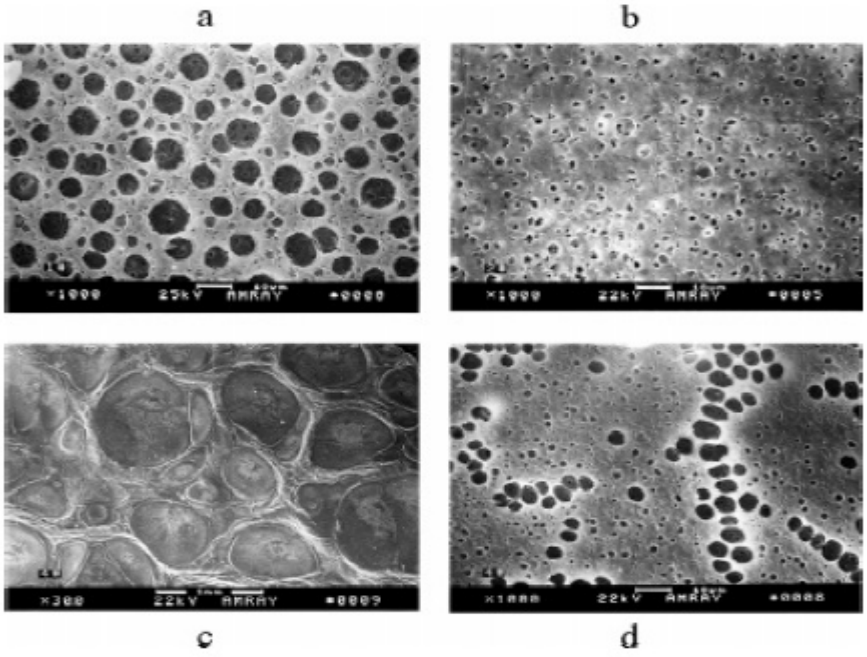


Figure 2.19: Scanning electron micrographs (SEM) of blend films after tetrahydrofuran etching: (a) PHB: PBLG (80:20); (b) PHB:PBLG (60:40); (c), (d) PHB:PBLG (40:60). (Deng *et al.,* 2001)

The mechanical properties varied considerably with blend composition. Compared with pure components, the PHB/PBLG (20/80) blend shows a certain improvement in mechanical properties. The composition-independent Tg definitely showed the immiscibility of PHB/PBLG blends in the molten state over their whole composition range. Such immiscibility also exists in the amorphous state due to a constant T_m together with a clear two phase separated structure revealed by SEM observation. The current blending patterns produced show improved properties over pure PHB. Naffakh *et al.,* 2011 investigated their work on the PHB nanocomposite and analyzed the composite, crystallization and melting behaviour by time resolved synchrotron X-ray diffraction.

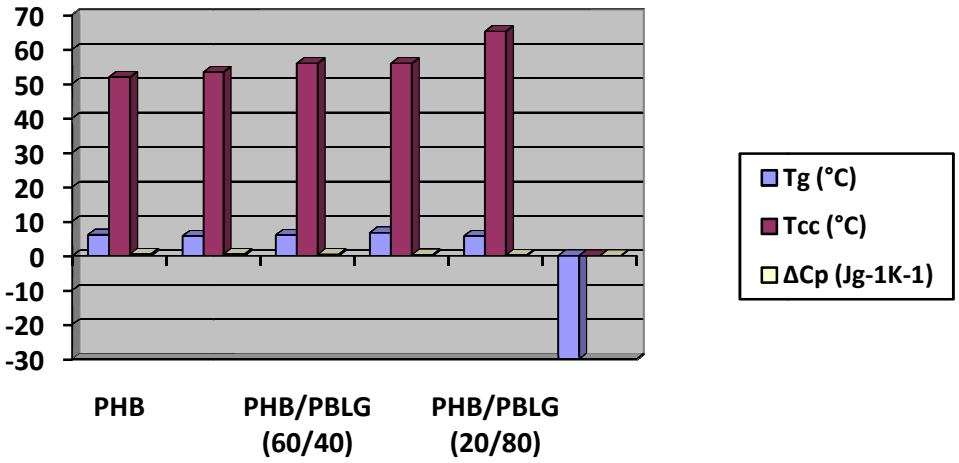


Figure 2.20: Data from heating curves of PHB/PBLG melt-quenched samples

T_g= Glass transition temperature; T_{cc}= Cold-crystallization temperature ; ΔC_p= Specific heat increment (Deng *et al.,* 2001).

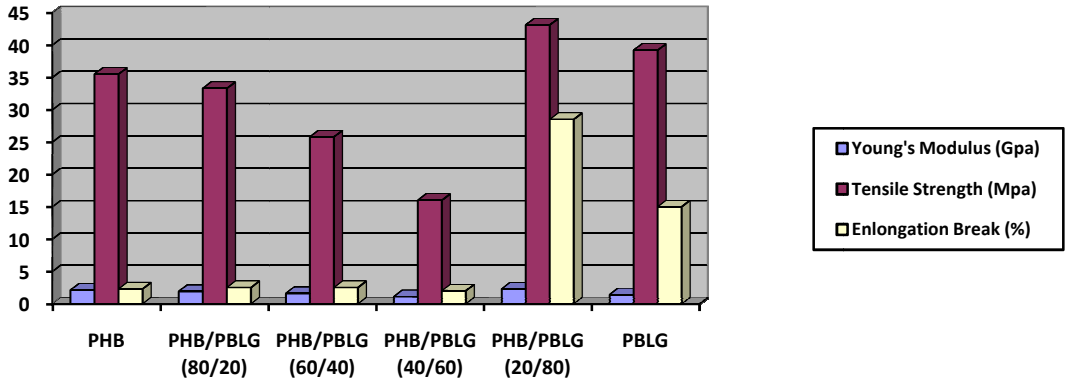


Figure 2.21: Mechanical Properties of PHB/PBLG blends (Deng *et al.,* 2001).

The mechanical properties of the blends depend markedly on composition. The PHB-based blends show poor mechanical properties. However, for the PHB/PBLG (20/80) blend, where PBLG replaces PHB as the matrix phase, improved mechanical properties were obtained compared to either of the individual components.

SEM analysis shows the morphology of the PHB/PBLG blend and concludes that PHB/PBLG (80/20) and PBLG (60/40) blends possess a microphase separated structure, in which PBLG acts as the dispersed phase of the diameter below 0.01mm. However, in the case of the PHB/PBLG (40/60) blend, the dispersed phase was PHB owing to the occurrence of phase

inversion. The size of the dispersed phase of this blend was as large as 1mm in diameter, characteristic of a macro-phase separated structure. For the PHB/PBLG (20/80) blend, the micro-phase separated structure appears again with the diameter of the dispersed phase also being below 0.01mm.

2.17.4.8: PHB blend with epoxy rubber

In this work the scientists investigated about the chemical modification of pristine montmorillonite with an alkyl-titanate complex. The hydroxyl functionality on the surface of the clay platelet was substituted by alkyl-titanate group from the titanate modifier making the surface organophilic. These titanate coupling agents form chemical bonds between inorganic and organic species via proton coordination and form an atomic layer on the surface of the clay by chemical modification (Monte, 2002; Parulekar and Mohanty, 2005). This large alkyl group also increases the inter-clay spacing and hence can facilitate intercalation and exfoliation.

Various toughening mechanisms for PHB were employed in the current work. The optimal toughening was achieved by the incorporation of various organo-modified clays and functionalized elastomeric components into the PHB matrix. The compatibilizer was also investigated to improve the interfacial adhesion between the incompatible elastomer and plastic phases. Different blends of PHB were prepared i.e. PHB+Rubber, PHB+Rubber+Comatibilizer, PHB+Rubber+Compatibilizer+Cloisite 30B, PHB+Rubber+Co mpatibilizer+Modified Clay and TPO+Clay, where TPO and PHB acted as control. Various techniques have been used to analyze the morphology and toughening of the blends (Duncan, 2003).

Izod notched impact (impact strength) properties of the materials were measured on an Izod impact tester (TMI model 43-02,TMI,NY) as per ASTM D256 for notched Izod impact testing with a 5lb-f pendulum. The studies revealed that the Semi-crystalline polymer and natural rubber form an immiscible blend, rubber used in the experiment was of high purity and hence its viscosity values were a magnitude of 50 higher than that of PHB. The blending of rubber with the PHB should be continuous, which could not be possible because of high viscous property of rubber. Thus, compatibilizer having Maleated polybutadiene with reactive maleic anhydride group should be added with the blend which improves its properties and

provides high impact strength and toughness. The toughening reduces the modulus which was regained to the permissible extent by the addition of novel nanoclays i.e. addition of ENR and MMT. Elemental analysis and contact angel measurements validated the successful modification of pristine montmorillonite clay using titanate coupling agent as calculated by X-ray diffraction analysis (XRD) (Sherwood, 1990). The modulus of the toughened PHB reduced to 0.7 GPa from 1.6 GPa of virgin PHB because of the impact modulus. The titanate-modified clay nanocomposite regained the modulus to 0.9GPa (Duncan, 2003). This improvement in modulus from the toughened PHB gives a material with the required stiffness for structural applications as well as retains the impact strength. Nanocomposite with this modified clay shows more than 400% improvement in impact properties and around 40% reduction in modulus in comparison with virgin PHB. The TEM micrograph of the titanate-modified clay shows morphology of the clay platelets. Clusters of platelets have been observed that suggest incomplete exfoliation. Further processing and material optimization studies are in progress to find more improved performance of these developed toughened PHB based nanocomposites (Duncan, 2003).

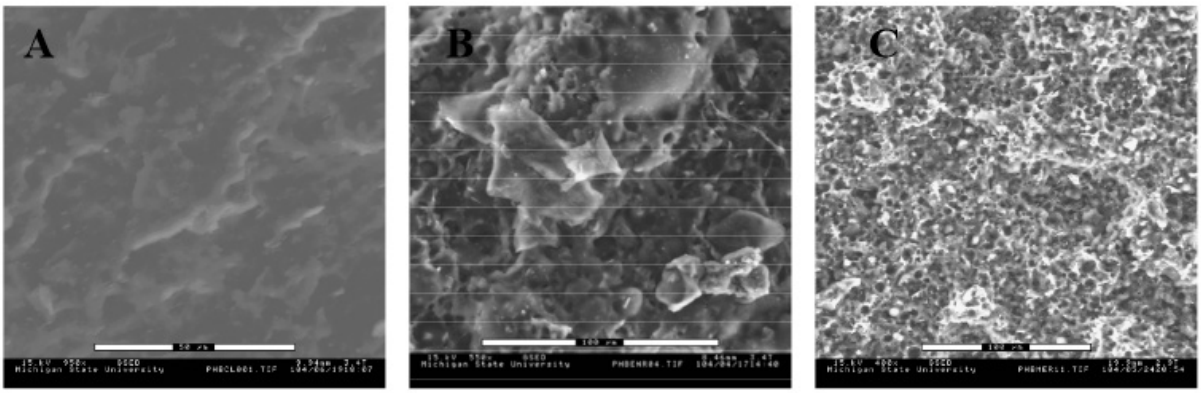


Figure 2.22: ESEM micrographs of fracture surfaces of pure PHB and its toughened blends. A)

Pure PHB (scale bar: 50μm), B) PHB:40% ENR (scale bar: 100μm), C) PHB:10% MR: 30% ENR (scale bar: 100μm) (Parulekar and Mohanty, 2005)

2.17.4.9: PHB/Hydroxyapatite blends

Fabrication technology was used to prepare the composites of porous hydroxyapatite and polyhydroxybutyrate (HA-PHB). In this method, centrifugation was done to infilterate PHB micro-/nano-particles inside rigid HA scaffolds which were followed by heating at 175°C so

that the PHB melts into appropriate sized scaffolds. Pielichowska and Blazewicz, 2010; Porter *et al.,* 2011 investigated that hydroxyapatite scaffolds were obtained by heating trabecular bovine femur bone at 1350°C to remove the organic content and sinter the HA.

Microbial fermentation technology was utilized for the recovery of PHB granules. Investigations were carried out on mechanical properties, HA:PHB ratios, surface adhesion and porosity were compared with neat hydroxyapatite scaffolds. The porosity percentage was observed to be ~54% in case of filled composites and ~67% in case coated composites. Addition of PHB slightly increases the strength of all the hydroxyapatite and polyhydroxybutyrate blends. The incorporation of PHB in filled composites does not show any stiffness, whereas the coated composites show an increased in the stiffness as compared to HA scaffolds. It increased from the range of ~35 MPa to ~105 MPa. Strong intermolecular interactions between hydroxyapaptite and polyhydroxybutyrate lead to an improvement in the stiffness of coated composites. Shishatskaya *et al.,* in 2006 observed that filled composites shows very little inter-constituent adhesion

In this paper, Medvecky in 2012 studied the preparation, microstructure, and properties of the polyhydroxybutyrate-chitosan-hydroxyapatite composite scaffolds using the polymer precipitation by mutual polymer solution mixing. This preparation process allows the fabrication of above composite scaffolds without applying the toxic fluorinated co-solvent, whereas the propylenecarbonate and acetic acid were used as polyhydroxybutyrate (PHB) and chitosan (CHT) biopolymer solvents. The researchers aim was to prepare relatively soft biocomposite material, which could be simply tamable to required shape.

The composite with PHB : CHT = 3 : 1 had more compact microstructure, where a very small fraction of large 100 μm pores and the high amount of irregular pores with dimension <10 μm can be observed. Despite increased amount of the large spherical-shaped pores with chitosan content in composites, the high amount of pores with average size of 10-20 μm were found in microstructures. From the analysis of micrographs result it could be concluded that the microstructures of all samples are very porous and the micropores with dimensions less than 1 μm are clearly visible too. The thermal decomposition of both biopolymers in composites was strongly affected by the addition of nanohydroxyapatite. The PHB thermal decomposition in PHB-HAP composite was shifted about 70°C to lower temperature in comparison with

pure PHB. Chen and Wang, 2002 shows approximately 20°C depression in the degradation temperature after 30 wt% addition of HAP to polyhydroxybutyrate-co-valerate biopolymer (composite was prepared by compression moulding).

The results of the current experimental work can be summarized in following points.

1. Observation of high porous microstructure in composite scaffolds and the macroporosity of scaffolds rose with the chitosan content in composites.
2. Rise in amount of the amorphous PHB component was found after chitosan addition to the PHB-HAP mixture.
3. Reductions in both the melting and thermal degradation temperatures of PHB and chitosan biopolymers in composites were noted, which confirms mutual interaction between polymers and the decrease of PHB lamellar thickness.
4. Biopolymers were homogeneously distributed in composite scaffold microstructures.
5. Compressive strengths of macroporous PHB-CHT-HAP scaffolds were approximately 2.5 MPa.
6. High nanohydroxyapatite loading of biopolymer matrix was achieved; which preserves the appropriate in-vitro apatite-ability forming of composites.
7. Composite scaffolds were prepared without applying of the high toxic fluorinated co-solvents.

With the analysis and review of all data available it can be concluded well that PHB is an important biopolymer and can be used in numerous types of applications. The hinges stopping the widespread use of this polymer are its cost and improvement required in the physical and mechanical properties. The economical production of PHB can be carried out by utilizing various strategies of optimization and production enhancement through mutagenesis approaches. On the contrary the enhancement in physical and mechanical properties can be carried out by blending approaches. Nanoclay based nanoparticles can prove to be of immense importance in enhancement of the physical and mechanical properties of the material. Use of PHB polymeric material in tissue engineering and scaffold preparation owing to its high level of biocompatibility makes it the most sought after material in the advance field of material and polymer science.

2.18: The Gap associated with the present proposal

1. PHB analysis from individual isolated bacteria has been done earlier. But no comparative analysis has been performed for the PHB production.
2. PHB production from halophilic bacteria of Sambhar Lake Rajasthan has never been explored. The proposed work involves the investigation of PHB production from isolates of Sambhar Lake.
3. Mutagenic investigations have been sparingly carried out for PHB production. The current work involves novel approach for carrying out mutagenic experiments on the isolated strains for PHB production and modifying their capability. The investigations will also involve mutagenesis of known PHB producers (procured from microbial cell banks) and comparative analysis between mutated and non mutated strains.
4. Investigations regarding improvement in physical properties of PHB will be carried out through blending. Blending with nanoclays, can improve the property of PHB at enhanced levels. Blending experiments will be carried out with compounds that have never been used by other workers earlier. The main objective of the approach is to develop an improved biomaterial which will be a product of green technology, a biodegradable material which will be a substitute of the commercially used synthetic plastics.

HYPOTHESIS OF STUDY

Synthetic manufactured plastics mainly polyethylene and other related classes are utilized as industry material, in day to day commodity uses etc. There anthropogenic use is increasing every passing day and after the passage of their life cycle, they end up as large garbage material. In countries like India where the management of waste material is already quite tedious with large population outputs, plastic waste material management becomes a nightmare. This causes accumulation of plastic waste material as large dump yards at most of the places; choking water bodies, rivers, canals, lakes, ponds etc. The degradation time counted for this kind of polluting material is more than 1000 years which worsens the condition more. This condition is causing the focus of peoples on demand for material which is having properties almost similar to plastic but having self biodegradation capability. The closest materials that appear with all such parameters are the bioplastics. Among all bioplastics, Poly-hydroxybutyrate and its related classes are the strongest contenders; they are supposed to be very innovative molecules that could be used for the purpose and in place of chemically manufactured plastic. These PHB molecules can be isolated from variety of bacteria and halophiles. They can be used as such or can be modified for the enhancement of their properties. The modification process involves blending of PHB material with variety of resins and different clay material to control their rigidity, tensile strength and degradation time for suiting across the needs of various sectors. With all these modification this future material can very well be used in place of Polyethylene based plastic with their degradation time already defined. With degradation time defined and inherent basic biocompatible nature of PHB material, it could also be used for exclusive biomedical applications such as bone tissue engineering, manufacture of scaffold material for production of artificial organs, sutures etc. The basic difficulty in achieving this task is expensive production of PHB material. There could be certain strategies employed for economical production of PHB material, such as through increased production by mutagenesis and with well formulated strategy for optimization of production of PHB with variation in basic ingredients of growth such as carbon and nitrogen sources and their concentration.

The current strategy has been followed in this thesis work and proves that definitely the production level of PHB in bacteria can be enhanced by these strategies. The mixing of PHB with various nanoclay materials has also been worked out here. Organically modified nanoclay material provides better filling and mixing with PHB matrix material. Mixing with nanoclay material increases the mechanical and physical property of the PHB material. The output of the blending experiments with nanoclay material justifies the hypothesis. These materials have enhanced medical application as carrier molecules for drug delivery, scaffold development for tissue engineering and numerous other fields.

OBJECTIVES

The objectives undertaken to accomplish the proposed work were:

- Isolation and screening of PHB producing strains from agriculture and saline environment.
- Strain improvement protocols for isolated strains and standard strains of PHB production through mutagenesis.
- Optimization of media and large scale fermenter production of bacterial extracted PHB from improved strains and its characterization for quality.
- Exploration of pure form of PHB for production of nanocomposite biomaterial through mixing methods and its characterization through various nanomaterial characterization techniques.

MATERIAL AND METHODS

5.1: Chemicals

All the chemicals and microbiological media were procured from Loba Chem, Moly Chem and Himedia. The nanoclays utilized for work were procured from Sigma Aldrich, USA. The PHB utilized in nanocomposites preparation were procured from Good fellow UK.

5.2: Isolation and screening of PHB producing strains from agriculture and saline environmental sites was the first objective of the work and was accomplished through following experimental procedures

Soil samples were chosen as the basic environmental entities for screening and isolation of PHB producing microorganisms. Soil of natural environment are subjected to stress due to varying climatic conditions hence have higher chances of inducing stress and causing variation in physiology of bacteria for production of PHB. Induction of PHB production occurs under stressed conditions.

Dry agricultural sites and saline lake soil sites were chosen for sample collection as they pose considerable physiological stress to bacteria thriving at these sites. Hence chances for presence of PHB producing bacteria are quite high at these sites. Details of geographical sites of sample collection are given under.

5.3: Collection of Soil samples from different geographical areas

Soil samples were collected for screening and isolation of PHB producing bacterial strains from two types of environment sites.

(i) Agriculture soil sites: Various agriculture sites were selected for isolation, screening, of PHB producing microorganisms.

Sites selected were from different geographical regions of Punjab

(a) Faridkot District Agriculture sites (Malwa Region of Punjab) 30.6°N 74.7°E

(b) Amritsar District Agriculture sites (Majha Region of Punjab) 31.58°N 74.98°E

(c) Kapurthala District Agriculture sites (Doaba Region of Punjab) 31.37°N 75.38°E

(ii) Saline soil sites: Sites surrounding various Saline lakes were considered for isolation, screening of PHB producing Halophilic microorganisms.

(a) Sambhar lake (96 km south west of the city of Jaipur, Rajasthan) 26.96°N 75.08°E

(b) Pichola lake (Udaipur, Rajasthan) 24.57°N 73.67°E

5.4: Collection Method

Standard Sample collection protocols were followed (APHA, 2008). Soil Samples were collected under sterile conditions and transferred to laboratory in ice at 4^{o}C (Kalaivani and Sukumaran, 2013; Sankara and Babu, 2013). The soil samples were collected in new sterile polybags, sterile new gloves and fresh sterile labwares were used in each sample collection site. Each sample was minimum of 100 gm. Surface soils (0-5 cm) were taken from representative areas of various sites. Recently soiled surfaces and areas were not considered. Extraneous matters such as rock particles, plant parts roots and other such debris etc were not collected with the samples. The collected samples were properly sealed in the polybags, and transferred to laboratory on ice.

5.4.1: Processing of soil samples

Soil samples collected were divided in two parts

1. Part one was used for soil sample Physio-chemical analysis.
2. Part two was used for Microbiological analysis.

5.5: Soil sample Physio-chemical analysis

Physio-chemical analysis was carried out from Punjab Agriculture University (Soil Testing Laboratory) in order to know the pH and electrical conductivity of the samples.

5.5.1: Measurement of Soil pH

The soil samples collected from different sites were filtered through the sieve (6.3mm) to remove the particles greater than 6.3 mm. Take 30 grams of soil into a glass beaker and add equal amount of distilled water to it. Proper stirring was required to obtain the slurry and was covered properly. Sample was stirred after every 10-15 minutes and allowed to stand for 1 hour, which helps in the stabilization of slurry pH and temperature. The pH meter controller was maintained at the temperature of soil slurry, prior to testing. pH meter electrode was

standardized with the standard buffer solution i.e. phosphate buffer having pH 7. Sample was stirred with a glass rod before immersing the pH electrode. The glass electrode required only 30 seconds immersion into the soil slurry before it get stabilized. The stabilization of the electrode signaled positively. The pH value was read and recorded. pH electrode was rinsed with distilled water and then dab with tissue to remove the particle or film formed on the electrode (Geotechnical Engineering Bureau, 2007).

5.5.2: Measurement of Soil Electrical Conductivity

The electrical conductivity of the soil sample was measured with the help of electrical conductivity meter. The meter was calibrated with the help of dry potassium chloride solution (dissolved 0.7456 gm of dry potassium chloride in 1 litre of distilled water at temperature 25^{o}C). The electrical conductivity of this solution is 1.41 mS/cm. The experiment was carried out by dissolving 20 gm of soil with 40 ml of distilled water in 250 ml of conical flask in a ratio 1:2. Soil slurry solution was prepared and allowed to stand for a time lag of 1 hour.

Conductivity of the supernatant was measured after calibrating the electrode with the solution of potassium chloride (APHA, 2006).

5.6: Preparation and Composition of different growth medium

Nutrient agar and DSC-97 were prepared with different salt concentration in order to optimize the culture condition for the growth of bacterial isolates (Trigui *et al.,* 2011). Nutrient Agar media was utilized mainly for culture of agricultural isolates with 1, 2, 3 M NaCl salt concentration and pH 7.2. DSC-97 is a specialized media reported for culture of halophilic bacterial isolates. DSC-97 was used with 1, 2, 3 M NaCl salt concentration and pH of 7.4 for culture of halophilic isolates. Slightly alkaline pH favours the growth of halophile bacteria. Isolation was carried out in varying salt concentration. Isolates growing better in specific molarity of salt were further grown in that molarity only. The composition of different medium is as follow:

A): Composition of Nutrient Broth (NB)

Constituents	**Quantity in g/L**
Beef Extract	10
Peptone	10
NaCl	at different molar concentration
Distilled water	1.0L
pH	7.2

B): Composition of DSC-97

Constituents	**Quantity in g/L**
Casamino acids	7.5
Yeast extract	10.0
Trisodium citrate	3.0
KCl	2.0
MgO4.4H2O	20.0
FeCl2	0.023
NaCl	at different molar concentration
Distilled Water	1.0L
pH	7.4 (± 0.2)

5.7: Isolation of Microbial cultures

Soil samples from diverse environmental habitats were utilized for the isolation of pure microbial strains. Serial dilution agar plating method or plate count through viability method is the most common method utilized for isolation and further culturing of soil microorganisms (Aneja *et al.,* 1996). Selective medium (Nutrient Agar and DSC-97 with varying salt concentration) were utilized for the isolation of sample specific isolates. Temperature was maintained at 37^{o}C.

After appearance of visual colonies, streaking was carried out for the isolation of pure colonies. Selection of isolates was based on the shape, colour and morphological distinctions (Aneja, 2003).

5.7.1: Serial dilution agar plating method or Viable plate count method

In this method, a known amount (10 gm) of the samples was suspended and mixed in known volume (90 ml) of sterile saline (0.85% NaCl in Double distilled water) so a microbial suspension of 100 ml total volume is prepared. Further Serial dilutions at continuous range of 10^{-2}, 10^{-3},…..,10^{-7} were prepared by adding volumes (usually 1 ml or 10 ml) into dilution blanks (having 99 ml or 90 ml sterile saline). Finally 1 ml solution from different dilutions (triplicate for each dilutions) were added to sterile Petri dishes (containing sterile 15ml of nutrient agar medium for agriculture isolates and selective medium DSC- 97, for halophile population). The plates were inoculated to have an even spreading and sterile glass rod spreader was used to equally spread the sample aliquot over the plate. Upon drying of the aliquot, the plates were incubated, in an inverted position for 3-7 days at 37°C (Aneja, 2003).

5.7.1(i): Requirements

Soil sample
Molten and cooled media
90 ml (7-8) sterile saline solution blanks.
Sterile petri dishes (36)
Sterile syringe with needle
Sterile 10ml pipettes and micropipettes
Colony counter
Bunsen burner /Spirit lamp
Marker pen.
Autoclave bag
500 ml conical flasks
Glass Rod
L Shaped glass spreader

5.7.1(ii): Observation

After incubation period the plates were observed carefully for the growth of bacterial colonies. As the dilution plates are replicates of each other; the average of triplicate microbial count is taken. Results were recorded carefully in the chart or data book for further use. The plates were stored for further purification of the bacterial strains.

5.8. Purification of Isolated bacterial colonies by Sub-culturing (or picking off) technique

After incubation has been completed in serial dilution or spread plate method and appearance of discrete, well separated colonies has been examined, the next step was sub-culturing. Some of the cells from one of the colonies were streaked in zig-zag manner to separate nutrient agar plates with predetermined salt concentration (1-3 M), DSC-97 plates with predetermined salt concentration (1-3 M) with a sterilized loop for further examination and use. These new individual cultures demonstrate the growth of a single species and were called a pure or stock culture (Aneja, 2003).

5.8.1: Requirements

Petri plates with bacterial cultures grown in serial dilution technique after incubation period.

Nutrient agar slants and nutrient agar plates, DSC-97 media

Inoculating loops

Permanent marking pen

Microscope

5.8.1(i): Procedure

1. The nutrient agar slants and agar plates were labeled with separate markings for identification.
2. The inoculating loop was sterilized by immersing in the hottest portion of the burner flame.
3. The loop was flamed until it is red hot and further cooled for few seconds.
4. After cooling tip of the loop was touched with the surface of a selected discrete colony on the agar culture plate.
5. The fresh petri plates with required media were inoculated by lifting the lid of the agar plate at 45°C; streaking through parallel lines in the center of the plate over the hardened agar surface by the loop with culture and finally recapping them.
6. The agar slants were inoculated by removing the plug of agar slants, grasping the plug with little finger of the left hand and passing the neck of the tube rapidly over Bunsen burner flame. The loop with culture was inserted into the slant and was drawn lightly over the hardened agar surface in a zig-zag motion and the tube was closed.
7. The inoculating loop/needle was flamed again for destroying existing organism.
8. The cultures (in slants/petri plates) were subjected to incubated at 37°C for 48-72 hours.

5.8.1(ii): Observations

After incubation, the slants or plates were observed for the appearance of pure colonies. The slants and the plates having uniform and uncontaminated growth were stored for further usage.

5.9: Culture of Pure strains

Pure strains known for high production capacity for PHB were procured from MTCC Chandigarh. These strains were maintained on nutrient agar plates. The strains collected were mainly representative of high end producers of PHB:

(a) *Bacillus megaterium* MTCC 453
(b) *Pseudomonas oleoverans* MTCC617

5.10: Screening method for PHB producing bacteria

Isolated bacterial strains were further screened by Sudan Black B staining method (Krithika *et al.,* 2011; Flora *et al.,* 2010; Juan *et al.,* 1998; Collee *et al.,* 1989). Sudan black stain was used to show microbial intracellular lipid (Collee *et al.,* 1989). In the resultant visualization the bacterial cytoplasm stains light pink, while lipid inclusion granules appear blue- black stained. The bacterial smear was prepared on the glass slide and heat fixed. The stock solution was prepared by dissolving 0.3 g of sudan black in 100 ml of 60% ethanol solution and was filtered by syringe filter. Dropper was used to pour the solution drop-wise on the bacterial smear. A time lag of 15 min was given and then washing was done by distilled water. Further, 0.5% solution of safranin dye as a counter-stain was added for 30 seconds. Slide was dried under room temperature and further observed under camera microscope at 100X magnification (Magnus Microscope) (Singh and Parmar, 2011; Murray *et al.,* 1994). The selected bacterial isolates, showing positive test for PHB production were further treated for strain improvement and optimization for large scale PHB production.

5.11: Strain improvement protocols for isolated strains and standard strains of PHB production through mutagenesis was the second objective and was achieved through following experimental procedures

5.11.1: Quantitative analysis of PHB production from strains isolated in first objective through biphasic induction of PHB production:

Standard method devised for the PHB production involves 2 steps:

The first stage consists of the task of achieving high cell biomass and cell number**.** The second stage involves limiting the nutrient condition with excess of carbon source and limiting nitrogen source, which favors the high production of poly hydroxy butyrate and storage of PHB granules in the bacterial cell. So, the exact stage of maximum PHB production was achieved by introducing biphasic growth condition with excess carbon source exactly at that point where the cell biomass was maximum and the phase was known as stationary phase (Madison and Huisman, 1999; Saharan *et al.,* 2012). Individual pure bacterial isolates were grown in 100 ml of respective medium (Nutrient broth 1 & 2 M salt concentration DSC-97; 1, 2 and 3 M salt concentration) with predetermined salt concentration in which they were isolated and show best growth at 37^{o}C. These salt concentrations were determined earlier during purification stages of bacteria. The point of maximum cell biomass was ascertained by observing the growth curve of each bacterial strain with the help of spectrophotometer at O.D. 600 nm. For agricultural soil isolates, maximum cell mass was obtained at time period of 48-72 hrs, where as the stationary phase or maximum growth in case of Salty lake isolates was achieved in a period of 96-120hrs. The induction for PHB production was accomplished utilizing biphasic growth condition. The bacterial cell biomass was centrifuged at 8000 rpm for 15 minutes and the pellet was washed with mineral salt medium. Pellet was further suspended in 100 ml of mineral salt medium and incubated at 37 ^{o}C. Mineral medium composition was KH_2PO_4, 1.5 g; Na_2HPO_4. $12H_2O$, 9 g; MgO_4. $7H_2O$, 0.2 g; $CaCl_2$. $2H_2O$, 0.01 g; citric acid, 0.1 g; and trace element solution, 1 ml pH 7.2 for ariculture isolate and 7.4 for halophiles. Trace element solution contains (per liter): $FeSO_4$. $7H_2O$, 20 g; H_3BO_4, 0.3 g; $CoCl_2$. $6H_2O$, 0.2 g; $ZnSO_4$. $7H_2O$, 0.03 g; $MnCl_2$. $4H_2O$, 0.03 g; pH 7.0) $(NH_4)_6Mo7O_{24}$. $4H_2O$,0.03 g; $NiSO_4$. $7H_2O$, 0.03 g; and $CuSO_4$. $5H_2O$, 0.01 g (Adwitiya *et al.,* 2009; Mercan *et al.,* 2002; Wang and Lee, 1997). This mineral salt medium was supplemented with 2% glucose as sole carbon source inducing excess carbon condition for high PHB production. After 30 hrs of growth the bacterial cells were harvested through

centrifugation and extraction of PHB was carried out (Amoli *et al.,* 2003; Passahna *et al.,* 2013).

5.11.2: PHB production and extraction from microbial cells through chloroform extraction protocol

PHB production for quantification from microbial strains was carried out in 100 ml of conical flasks. Extraction of PHB was performed by chloroform extraction method. After incubation of the culture centrifugation of the bacterial culture was carried out at 8000 rpm for 15 minutes. Supernatant was discarded and pellet was washed with distilled water, acetone and ethanol. These pellets were dried at 37°C and weighted which was further called as cell dry weight in g/L (cdw). Digestion of these pellets in sodium hypochlorite was carried out at 37°C for 1-2 hours. Boiling chloroform was added in the digested pellet, so that intracellular lipids get completely dissolved in the chloroform and settle down at the bottom. Chloroform solution was separated from sodium hypochlorite mixture and was allowed to evaporate overnight at room temperature to obtain crystals of PHB. (Hahn *et al.,* 1993; Hahn *et al.,* 1995). The percentage of PHB was estimated by evaluating the amount of PHB present in cell dry weight.

The percentage of intracellular PHB accumulation was estimated as the percentage composition of PHB present in the dry cell weight.

PHB accumulation (%) = Dry weight of extracted PHB (g/L) × 100 / cell dry weight (g/L)

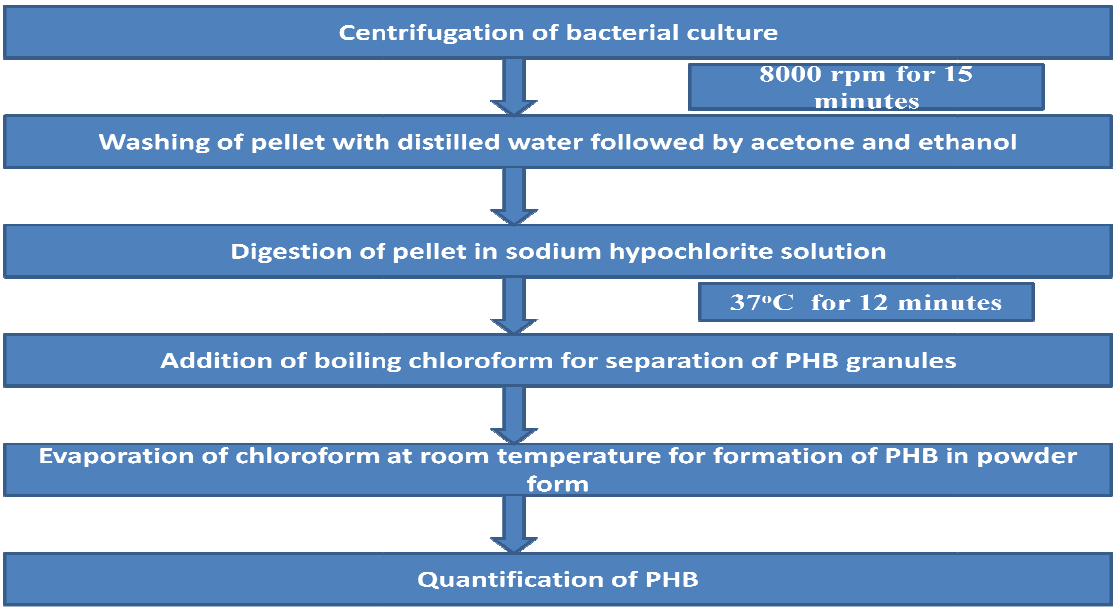


Figure 5.1: Protocol for extraction of PHB from harvested bacterial cells

5.12: Strain improvement protocols for isolated strains and standard strains of PHB production through mutagenesis

Strain improvement protocols have been utilized all over the world from long time back for improvement of production of commercially important products from microbial species. Strain improvement protocols for PHB were utilized for improvement of both isolated screened strains of PHB production and procured known PHB producer strains.

5.13: Mutagenic protocols with physical and chemical mutagens

5.13.1: Preparation of control culture solution

Overnight grown bacterial culture broth 20 ml was centrifuged at 10,000 rpm for 15 minutes at room temperature. Supernatant was discarded and pellets were taken and re-suspended in 1ml sterile saline. Sterile saline was prepared by dissolving 7.4 gm of NaCl per litre of distilled water. Pellets in sterile saline were spread on growth media plates. Incubation time and temperature was dependent on the type of bacterial strain. Total viable count (TVC) was measured (Adwitiya, 2008; Miller, 1992; Sreeju *et al.,* 2011). All the experiments were carried out in triplicates.

5.13.1.1: Physical mutagens:

5.13.1.1(i): Procedure:

UV mutagenesis was utilized for carrying out Physical mutagenesis protocol. Direct-Plate Irradiation procedure was utilized for carrying out UV mutagenesis as described by Lin and Wang 2001. Direct-plate kill experiments were performed by growing sub-cultures overnight on Nutrient broth and DSC-97 (for halophiles) (20 ml final volume) to about 1 x 10^8 cells per ml, approximately 0.7 to 0.8 OD 660 nm. When these sub-cultures reached desired optical density, they were used to make final plates for the ultraviolet irradiation by spread-plating 0.1 ml of the sub-cultures on nutrient plates & DSC-97.

After plating, the plates were kept in a black box (plastic cooler box) before and after irradiation unless otherwise noted. All UV irradiations were performed in UV chamber with glass front. Major part of the experiment was performed in the dark to avoid photo-reactivation. The only time a light source was present was during the transfer of the plates into the UV chamber. Before each irradiation, the UV lamp was warmed up for at least 30

minutes. The time set for UV exposure was 0, 1, 3, 5, 7, 10 minutes and was controlled by manually adjusting the power switch of the UV lamp. Lids of the treatment plates were removed before placing them in chamber for avoiding shielding effect by the lids. After irradiation, the lids were replaced and the plates were immediately transferred into the black box. All the plates were grown in 37°C incubator for 48-72 hrs for agriculture isolates and 96 to 120 hrs for halophiles before counting the number of colonies.

Mutant colonies were taken and total viable count was measured (Adwitiya, 2008; Sreeju *et al.,* 2011).

5.13.1.1(ii): Chemicals mutagens

(I). Inducing mutation by Acridine Orange

Procedure:

Acridine orange was used as chemical mutagen for inducing mutations for improvement of PHB production of microbial strains. 0.01 ml of bacterial culture was taken from overnight grown bacterial broth and spread on NA media plates and DSC-97 media plates (for Halophiles) without the exposure of mutagenic agent as Control. Incubation was performed at 37^{o}C. Total viable colonies were counted after 24-36 hrs. Quantity of 10 ml of nutrient broth in 3 test tubes was taken & DSC broth for halophiles. Stock solution of Acridine orange (1000 µg/ml) was separately prepared by adding 0.1 gm of Acridine orange in 10 ml distilled water. Different concentrations; 50 µg/ml, 100 µg/ml and 200 µg/ml of acridine orange were maintained in the test tubes along with inoculation by overnight grown bacterial broth. Incubation of these test tubes for 2-6 hours was carried out in dark at 37^{o}C and then further incubation was carried out in orbital shaker for 24 hours at 37^{o}C for the growth of mutated species. After incubation culture was spread over NA media, DSC-97 plates and incubation was carried out at 37^{o}C. Further total viable colony count was measured (Adwitiya *et al.,* 2008; Miller, 1992; Sreeju *et al.,* 2011).

II). Inducing mutation by EMS (Ethyl Methyl Sulphonate)

Procedure

Nutrient Agar & DSC-97 tubes were prepared. Stock solution of Ethyl methane sulphonate was prepared at a concentration of 1000 µg/ml by following standard calculation. Different concentration of EMS solution was maintained into the test tubes of growth broth from the

stock solution (50 µg/ml, 100 µg/ml and 200 µg/ml) and they were further inoculated with overnight grown bacterial strains. They were incubated at 37°C in orbital shaker. One test tube was taken as control in which no mutagenic agent was added. Incubation of these test tubes for 2-6 hours was carried out in dark at 37°C and then further incubation was carried out in orbital shaker for 24 hours at 37°C for the growth of mutated species. After incubation culture was spread over NA media, DSC-97 plates and incubation was carried out at 37°C. Further total viable colony count was measured (Adwitiya *et al.,* 2008; Miller, 1992; Sreeju *et al.,* 2011).

III). Inducing mutation by Ethidium Bromide (EtBr)

Ethidium bromide mutagenesis involves the exposure of bacterial isolates at different concentration of EtBr. Nutrient Agar & DSC-97 tubes were prepared. Stock solution of Ethidium bromide was prepared by dissolving 0.01 gram of Ethidium bromide in 10 ml of distilled water (1000 µg/ml). Different concentration of Ethidium bromide solution was maintained into the test tubes of growth broth from the stock solution (50 µg/ml, 100 µg/ml and 200 µg/ml) and they were further inoculated with overnight grown bacterial strains. They were incubated at 37ºC in orbital shaker. One test tube was taken as control in which no mutagenic agent was added. Incubation of these test tubes for 2-6 hours was carried out in dark at 37°C and then further incubation was carried out in orbital shaker for 24 hours at 37°C for the growth of mutated species. After incubation culture was spread over NA media, DSC-97 plates and incubation was carried out at 37°C. Further total viable colony count was measured (Adwitiya *et al.,* 2008; Miller, 1992; Sreeju *et al.,* 2011; Walter *et al.,* 2013).

IV). Inducing mutation by Sodium Azide

Radom Mutation was carried out by exposing the bacterial culture broth at different concentrations of sodium azide. Nutrient Agar & DSC-97 tubes were prepared. Stock solution of Sodium Azide was prepared by dissolving 0.01 gram of Sodium Azide in 10 ml of distilled water (1000 µg/ml). Different concentration of Sodium Azide solution was maintained into the test tubes of growth broth from the stock solution (50 µg/ml, 100 µg/ml and 200 µg/ml) and they were further inoculated with overnight grown bacterial strains. They were incubated at 37ºC in orbital shaker. One test tube was taken as control in which no mutagenic agent was added. Incubation of these test tubes for 2-6 hours was carried out in dark at 37°C and then further incubation was carried out in orbital shaker for 24 hours at 37°C for the growth of

mutated species. After incubation culture was spread over NA media, DSC-97 plates and incubation was carried out at 37^{o}C. Further total viable colony count was measured (Adwitiya *et al.,* 2008; Miller, 1992; Sreeju *et al.,* 2011; Fortin *et al.,* 1990).

These experiments will be carried out in order to improve the bacterial strains, in terms of PHB production.

5.14: PHB production and extraction from microbial cells

PHB production for mutated strains was carried out in 250 ml of conical flasks for analysis of any enhancement in quantity of PHB production. The culture was carried out in biphasic condition with utilization of 100 ml of nutrient agar medium and DSC-97 at the starting phase for production of maximum amount of cell biomass and utilization of mineral salt medium with 2% glucose in the secondary phase for maximum accumulation of PHB. All of the biphasic culture conditions were similar as described earlier. Extraction of PHB was performed by sodium hypochlorite and chloroform extraction method as discussed earlier in the above section from the mutated strains and wild strainS (Hahn *et al.,* 1993; Hahn *et al.,* 1995; Zakaria *et al.,* 2010).

5.15: Optimization of media and large scale fermenter production of bacterial extracted PHB from improved strains and its characterization for quality was the third objective and was achieved with following experiments

5.15.1. Media optimization protocol for improved strains

Media optimization was carried out for key ingredients required for growth and PHB production. Carbon and nitrogen sources play a major role during growth of the bacteria. Carbon optimization during secondary stage of biphasic culture for enhanced PHB production becomes very important. Different carbon sources can have variation on the level of PHB production. These variations were investigated by introducing different carbon sources at the time of PHB production induction. Nitrogen optimization was carried out by introducing different nitrogen sources in the mineral salt medium preparation. Negative control for PHB production was taken as the media in which no carbon or nitrogen source was included.

5.15.1.1: Effect of different carbon sources on PHB production

Different carbon sources sucrose, fructose, glucose, lactose and maltose were introduced during the induction stage to observe the maximum PHB production from microbial isolates as per protocols described by Elsayed *et al.,* 2013; Krithika *et al.,* 2011; Singh and Parmar, 2011.

The experiment was conducted in 250 ml of culture flasks. Flasks were taken with 100 ml of nutrient broth solution (DSC-97 for halophiles) as they are prescribed and they promote highest growth in terms of biomass production for different groups of bacteria taken. These flasks were inoculated with overnight grown bacterial culture and incubated at 37^{o}C. These flasks were observed for their stationary phases for achievement of maximum biomass by observing OD at 600nm as described in earlier part. Biphasic culture condition was introduced with 5 different carbon sources sucrose, fructose, glucose, lactose and maltose included in the mineral salt medium separately with conceNtration levels at 1%, 2% and 3%. This carbon source supplementation acts as inducer for maximum PHB production. Rest of the biphasic culture protocol as described earlier was followed. After 30 hrs of incubation bacterial cells were harvested through centrifugation. PHB was extracted and quantified. Extraction of PHB was performed by chloroform extraction method as discussed earlier in the above section (Hahn *et al.,* 1993; Hahn *et al.,* 1995; Zakaria *et al.,* 2010).

5.15.1.2: Effect of different nitrogen sources on PHB production

Isolates providing best yield in the carbon optimization protocol were further tested for best nitrogen source. Mineral medium described earlier was utilized for the purpose (Adwitiya *et al.,* 2009; Mercan *et al.,* 2002; Wang and Lee, 1997). Different nitrogen sources at 1% concentration were added additionally to the medium. The nitrogen sources added were yeast extract, peptone, ammonium chloride, ammonium sulphate and ammonium carbonate. Best carbon source with specific concentration obtained in the carbon optimization protocol according to the bacterial strain was also added in the medium. Rest of the biphasic culture protocol as described earlier was followed. Incubated was carried out at 37^{o}C till 30 hrs (Amoli *et al.,* 2013, Passanha *et al.,* 2013). After 30 hrs the culture were harvested and extraction of PHB was carried by chloroform extraction method and yield percentage was calculated (Saranya and Shenbagarathai, 2010). Negative control for PHB production was taken as the mineral media in which no carbon or nitrogen source was included. Positive

control was the mineral media with the best carbon source with specific concentration without any nitrogen source.

5.15.2: Large Scale production of Polyhydroxybutyrate (PHB) from improved microbial strain at optimized conditions

The highest PHB producing microorganism at optimized conditions was selected for large scale fermentation in fermenter vessel for large scale PHB production. Fermenter vessel (Bioage, Lab No. 29-403, LPU) was autoclaved at 121^{o}C at 15 psi for 20 minutes. 1 liter of Nutrient broth (DSC-97 for halophiles) with optimized salt molarity were sterilized and introduced in the fermenter vessel for fermentation experiment. Overnight grown bacterial culture was inoculated in the media with inoculum size of 2%. Aerobic conditions were provided with agitation speed of 150 rpm. Optimized best conditions obtained in biphasic culture condition with specific carbon and nitrogen source along with their concentration for the highest PHB production were provided at temperature of 37^{o}C. Rest all biphasic culture conditions were followed as described earlier. PHB was extracted by chloroform extraction method and was quantified as described earlier (Kshirsagar *et al.,* 2012; Heinrich *et al.,* 2012). Statistical analysis was carried out for highest PHB producing strains by using ANOVA SPSS 16.0 tool to find out the significant values.

Figure 5.2: Bioage fermenter installed at LPU [Lab no. 29-403]

5.16: Characterization for quality

Characterization and qualitative analysis of pure PHB extracted from bacterial strains was performed by techniques such as Fourier Transform Infra-red Spectroscopy. FTIR (Kirithika *et al.,* 2011; Pak *et al.,* 2013; Shah, 2011) measures the functional groups in the desired samples, Electrospray ionization- high resonance mass spectroscopy (Panda *et al.,* 2007; Elhottova *et al.,* 2000) measures the purity of the compound and separates different components of the mixture.

5.17: FTIR (Fourier Transform infrared spectroscopy) based Characterization of PHB

FTIR technique was utilized for the initial identification of PHB sample. 1 mg of PHB and 10 mg of spectral pure anhydrous potassium bromide crystal were mixed for IR analysis. The relative intensity of transmitted light was measured against the wavelength of absorption for the region. The Shimadzu FTIR facility in laboratory at Chemistry department of Lovely Professional University was utilized for the FTIR testing analysis (Rohini and Rawal, 2006). Standard highly purified PHB samples procured from Sigma Aldrich USA was also analyzed by FTIR facility. The graph of standard PHB from Sigma Aldrich would be utilized for comparison and analysis of the quality of the PHB obtained from bacterial isolates.

5.18: ESI-HRMS

Combination of electrospray ionizer (ESI) and mass spectroscopy (MS) was developed by Fern and co-workers (Yamashita and Fenn, 1984a; Fenn *et al.,* 1989; Yamashita and Fenn, 1984b). Non-fragmented multiple charged ions are generated by Electrospray ionization and hence allows the determination of mass of heavy biomolecules of few thousand m/z. Soft ionization without fragmentation of the analyte molecules is the key feature which causes immense use of electrospray in the analysis of non-volatile chargeable molecules such as proteins (Domon and Aebersold, 2006), nucleic acids (Nordhoff *et al.,* 1996) or whole viruses (Bothner and Siuzdak, 2004).

This test was performed for the final confirmation of quality. The samples that provided best yield and FTIR graph similar to standard were processed in SAIF Laboratories, CDRI, Lucknow for the ESI-HRMS analysis. The Model number Agilent 6520 Q-TOF was used by laboratory scientists for the analysis of samples. Standard highly purified PHB sample

procured from Sigma Aldrich USA was also sent for analysis at ESI-HRMS facility for ease of comparison of graphs for establishing the quality of PHB obtained.

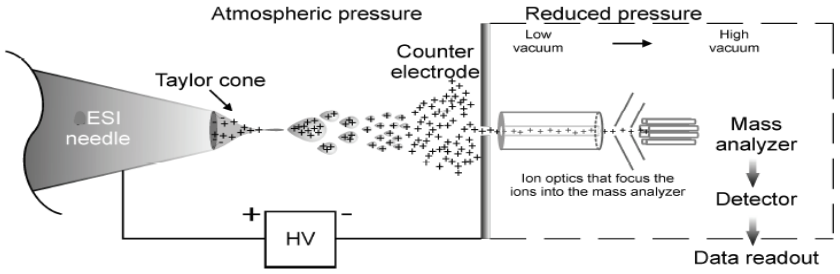


Figure 5.3: Principle of electrospray ionizer (ESI) and mass spectroscopy (MS)

5.19: Molecular characterization of bacterial isolates by 16s rRNA sequencing

16 rRNA sequencing was carried out at Yaazh genomics Mumbai.

5.19.1: DNA Extraction:

1. Bacterial Genomic DNA was isolated using the Insta Gene TM Matrix Genomic DNA isolation kit – The kit instructions were followed.
2. 1 ml of sterile water in microfuge was used to suspend bacterial colony.
3. Centrifugation was done to get the bacterial pellet for 1 minute at 10,000-12,000 rpm.
4. The pellet was then mixed with 200 µl of Insta Gene matrix at 56^{o}C for incubation time of 15 minutes.
5. Vortexing was done for 10 seconds and then place the microfuge tube at 100^{o}C in boiling water bath for 8 minutes.
6. Again vortex the bacterial content for 10 seconds and spinning was done at 10,000-12,000 rpm for 2 minutes.
7. 20µl of the supernatant DNA solution was obtained which was used per 50 µl of PCR reaction.

5.19.2: PCR Protocol

16S rRNA Universal primers sequence was utilized for amplification procedure. The sequence is given below. MJ Research Peltier Thermal Cycler was utilized for amplification process.

5.19.2.1: Primer Details

Primer Name	Sequence Details	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

Addition of 11 µL of template DNA in 200µL of PCR reaction solution. 1 µL of 27F/1492R primers were used for amplification procedure, and further PCR reaction was performed with conditions as follows:

Initial Denaturation cycle 94°C for 2 min and then 35 amplification cycles at 94°C for 45 sec, 55°C for 60sec, and 72°C for 60 sec. Final Extension at 72°C for 10 min. DNA fragments are amplified were about 1,400bp in the case of bacteria. A positive control (*E.coli* genomic DNA) and a negative control in the PCR were included.

5.19.3: Purification of PCR products

Montage PCR cleaning kit (Millipore) was used to remove the unincorporated dNTPs and PCR primers. The primers used for sequencing the PCR product was 518F/800R primers.

Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

5.19.3.1: Sequencing Primer Details:

Primer Name	Sequence Details	Number of Base
518F	CCAGCAGCCGCGGTAATACG	20
800R	TACCAGGGTATCTAATCC	18

Sequence data was aligned and analyzed for Identifying the Sample.

5.19.5. Bioinformatics protocol:

1. The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

2. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar, 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana, 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.
3. The program Tree Dyn 198.3 was used for tree rendering. (Dereeper *et al.,* 2008).

5.20:Visualization of bacterial PHB accumulation by Transmission Electron Microscopy (TEM)

5.20.1: Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) is a microscopy technique whereby a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen while passing through it. TEM was carried out at SAIF Labs, Punjab University, Chandigarh using the Hitachi (H-7500) 120KW instrument equipped with CCD camera, highest resolution magnification of 6 lakhs. The selected bacterial strains were grown under optimized conditions as discussed earlier. After 30 hrs of induction of PHB production, cells were harvested and sample for visualization were prepared as given below.

5.20.2: Preparation of 0.2 M phosphate buffer (pH 7.4)

3.12 gm of Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$; MW 156) was dissolved in 100 ml of distilled water taken in 250 ml of glass conical flask and marked as solution A. 2.84 gm of Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4; MW 142) was dissolved in 100 ml of distilled water taken in 250 ml of glass conical flask and marked as solution B. Mix 19 ml of solution A with 81 ml of solution B. Adjust the pH with HCl and NaOH. Similar protocol was followed for the preparation of 0.1 M phosphate buffer as per its molecular weight.

5.20.3: Preparation of fixative for electron microscopy

4 gram of dry powder of paraformaldehyde was added in 100ml of distilled water and heated at 60^{o}C until powder becomes completely dissolved in distilled water. Mouth of conical flask was covered with aluminum foil. 0.1 N sodium hydroxide was used if not dissolved properly. The solution was cooled and filtered. Equal amount of 0.2 M phosphate buffer (pH 7.2) was

added to the paraformaldehyde solution to make it 2% solution in 0.1 M phosphate buffer. 10 ml of glutaraldehyde solution was added to 90ml of 2% paraformaldehyde solution. The final concentration of the fixative was 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer.

5.20.4: Sample preparation:

The bacterial culture was centrifuged at 1000 rpm for 10 minutes. Supernatant was discarded. Bacterial pellet was suspended in 0.1 M of phosphate buffer (pH 7.4) and again centrifuged. Supernatant was discarded again. The pellet was again resuspended and fixed into the mixture of 2% paraformaldehyde and 2.5% of glutaraldehyde in buffer solution for 3-4 hrs at 4^{o}C. The pellet was centrifuged again for 10 minutes at 4^{o}C and fixative as supernatant was discarded. 0.1M phosphate buffer was added into the pellet and centrifuged for clear visualization (at least 2 mm in diameter).

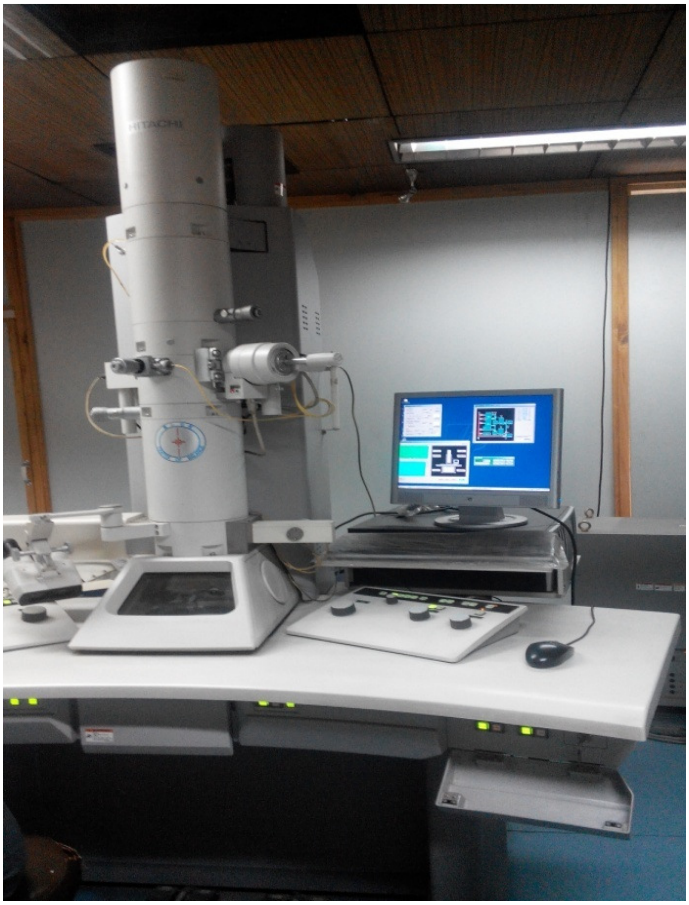


Figure 5.4: Transmission Electron Microscopy (TEM) installed at SAIF LAB Punjab University (Chandigarh) (Model no. Hitachi H-7500)

5.21: Exploration of pure form of PHB for production of nanocomposite biomaterial through mixing methods and its characterization through various nanomaterial characterization techniques was the fourth objective and was accomplished by following experiments

5.21.1: Blend preparation of PHB with nanoclay

Blends of PHB were prepared for overcoming its physical barriers and increasing its applicability for industrial applications. Many approaches have been utilized for the preparation of PHB blends/nanocomposites by various workers (El-Taweel, 2004; Liau *et al.,* 2014; Maiti *et al.,* 2007). Solvent casting method was utilized in the current work for preparation of blends of varying concentrations.

High quality standard PHB procured from Goodfellow Chemical Supplier (UK) was utilized for the preparation of nanocomposite biomaterial. PHB produced during experimental procedure in the current work could have contaminants hence have not been utilized in the nanocomposite biomaterial preparation. High quality standard PHB from Goodfellow Chemical Supplier (UK) has been used as a model purified PHB for observing the changes in morphological, physical and mechanical properties during nanocomposite biomaterial formation.

The method for formation of exfoliated and intercalated nanocomposites with enhanced properties (such as polarity, molecular weight, hydrophobicity, reactive groups, charge density, etc.) is named as *solvent casting method* (Cheng *et al*., 2004). In the solvent casting technique, polymer and nanoclays are mixed together in the solvent i.e. chloroform, leading to insertion of the polymer chains into the galleries of the swollen clay. Polyhydroxybutyrate (PHB) was utilized as an organic polymer which acts as a matrix for the preparation of polymeric blend. Nanoclay employed for preparing nanocomposite was montmorillonite (MMT) surface modified with Di-methyl Di-alkyl Amine and Zeolite.

Table 5.1: Physical properties of Poly-hydroxy butyrate needing improvement

Property	PHB
Melting point (^{o}C)	175
Degradation temp (^{o}C)	200

Generally, introduction of clay into polymeric matrices can improve their thermal stabilities since the clay can hinder the permeability of volatile degradation products out of the materials. The dispersed clay generates a barrier which delays the release of thermal degradation products in comparison with PHB. The incorporation of clay causes pores into the surface of polymeric matrix. The intensity of pores plays an important role in biomedical application of PHB especially in the preparation of scaffolds (tissue engineering).

5.21.1.1: Solvent casting method

One grams of PHB was dissolved in 150 ml of $CHCl_3$ solution and was maintained at 60^oC for about 2 hours that facilitates the expansion of PHB matrix and dissolve into its natural solvent $CHCl_3$. The final volume of $CHCl_3$ and PHB left was 100 ml after intensive mixing on magnetic stirrer.

Further procedure was carried out separately for dried Zeolite and organo-modified MMT. Various amounts (1-10 wt%) of dried Zeolite and organo-modified MMT (OMMT) were added into the solution of PHB/$CHCl_3$ separately and the solutions were stirred at 60^oC for 2 hrs. The obtained PHB/Zeolite and PHB/OMMT nanocomposites were cast on a slide glass, maintained at room temperature for 24 hrs, and dried in a oven at 60^oC for next 24 hrs. Prepared nanocomposites were stored at room temperature for further analysis (Choi *et al.,* 2001; Ling *et al.,* 2009).

5.21.2: Characterization of produced blends/nano-composites through various nano-imaging and related techniques

Nanocomposite blends produced were characterized through following techniques

5.21.2.1: Field Emission Scanning electron microscopy (FE-SEM)

The technique was used for visualizing inside the ultrathin specimen by the transmission of electron beams which produce a very high resolution image as compared to a light microscope. SEM imaging systems are routinely used for observation of nanoscale structure of nanocomposite material and comparison of morphology (Marras *et al.,* 2007; Pak *et al.,* 2013; Sangkharak and Prasertsan, 2008; Rohini *et al.,* 2006). Scanning Electron Microscopy was done using FEI Quanta 200F with Oxford-EDS system IE 250 X Max 80; Netherlands operated at 15 kV available at SMITHA LAB, Department of textile technology at Indian

Institute of Technology, New Delhi. The surface of nanofilms was sputter coated with gold. The surface of the blend was examined for changes in its surface morphology and distribution of nanoclays in the matrix of PHB.

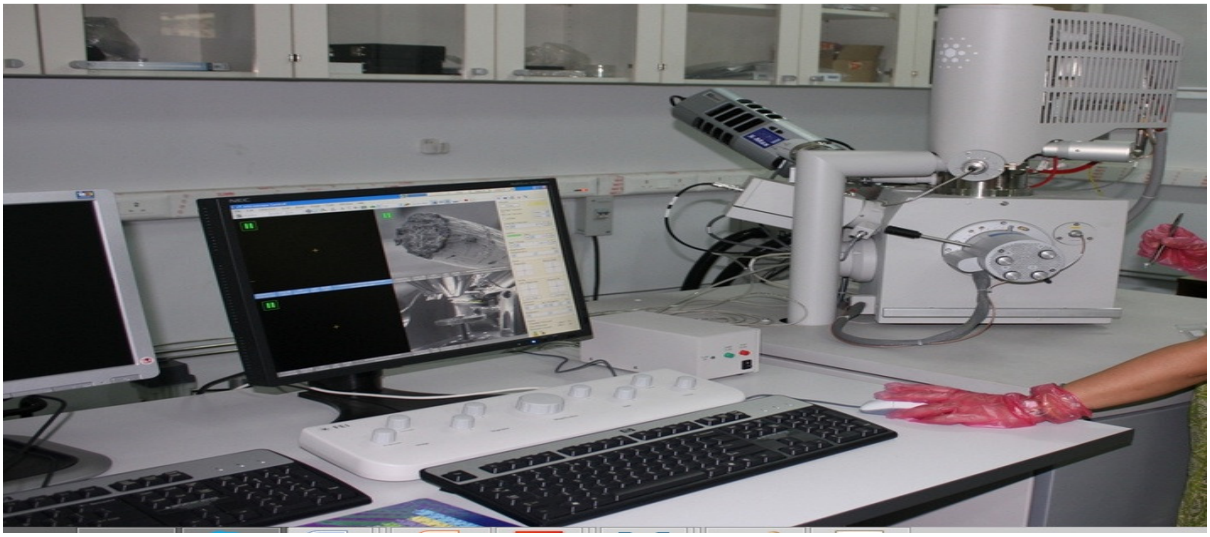


Figure 5.5: Field-Emission Scanning Electron Microscopy (FE-SEM) facility at Textile Technology Dept. IIT Delhi

5.21.2.2: Differential Scanning Calorimetry

The thermal behavior of the polymeric film or its polymer based nanocomposites was analysed by DSC analytical tool. Enthalpy of Melting (H_M), Melting temperature(T_m) is measured by this technique. Increment in the melting temperature shows the presence of inorganic silicates in the polymeric matrix system (El-Taweel *et al.,* 2004; Rohini *et al.,* 2005). DSC analysis was done by DSC Q200 V24.4 Build 116 available at SMITHA LAB, Department of textile technology at Indian Institute of Technology, New Delhi. DSC was performed to study the thermal behavior of the blends produced. The blended films obtained were prepared by dissecting them into squares of size 2mm×2mm and were placed in an aluminum cell by casting and were heated from -20°C to 200°C. DSC endothermic peaks analysed the melting temperature (T_m) apparent enthalpies of melting (ΔH_m). After keeping for 2 min at 200°C, the samples were cooled to room temperature. All the experiments were carried out in liquid nitrogen environment at a flow rate of 50 ml/min for preserving the matrix intact.

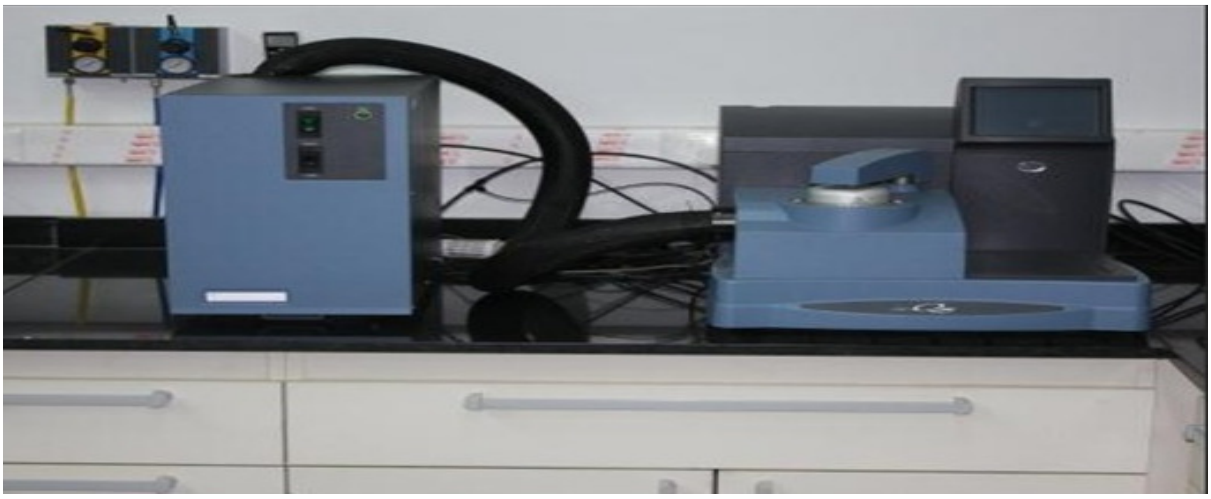


Figure 5.6: Differential Scanning Calorimetry (DSC) facility at Textile Technology Dept. IIT Delhi

5.21.2.3: Thermogravimetric Analysis: The mass change in the nanocomposite and its thermal degradation rates by the addition of inorganic silicates was measured by the quantitative measurement i.e. Thermogravimetric analysis (TGA). Variation in mass from decomposition and degradation with time and temperature is recorded by thermographs (Jandas *et al.,* 2014; Zuburtikudis *et al.,* 2008). Thermo gravimetric analysis was performed using a TGA Q500 V20.10 Build 36 available at SMITHA LAB, Department of textile technology at Indian Institute of Technology. The weight of the samples used was about 10 mg and were heated from 30 to 600°C at the heating rate of 20°C/min. The analysis was carried in nitrogen flow rate of 40 ml/min. The weight loss of samples were recorded and plotted as the function of temperature.

Figure 5.7: Thermo-gravimetric Analysis facility at Textile Technology Dept. IIT Delhi

5.21.2.4: Tensile testing: The mechanical analysis of films was done by instron micro tensile tester. Micro tensile tester is highly precise low force material testing system having two column load frame allowing both, horizontal and vertical test orientation. It can operate at 10 N and 2 kN load capacity. Instron Microtensile Tester, Model 5848, Singapore available at SMITHA LAB, Department of textile technology at Indian Institute of Technology, was used for measuring the mechanical properties of nanofilms i.e. tensile stress, strain, extension at max. Load, yield strength, modulus etc. (Pak *et al.,* 2013; Liu *et al.;* 2007; Shokrieh *et al.,* 2013). Statistical analysis were carried out by using ANOVA SPSS 16.0 tool to find out the significant values.

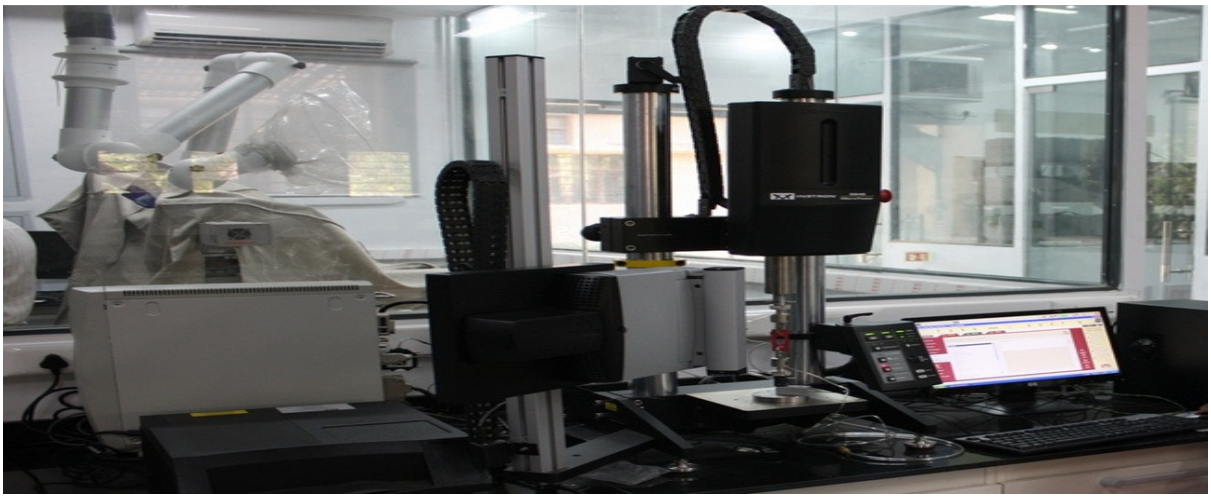


Figure 5.8: Instron Microtensile tester facility at Textile Technology Dept. IIT Delhi

5.22: Biodegradation study

5.22.1: Biodegradation by bacteria

In vitro biodegradation studies were carried out on PHB/OMMT, PHB/Zeolite blended nanocomposites and were compared with the degradation percentage of pure PHB film. As cited in literatures PHB can be degraded by many bacterial species like *E.coli*, *Pseudomonas*, *Bacillus*, *Azospirillum*, *Mycobacterium*, and *Streptomyces* (Bonartseva *et al.,* 2002; Bhatia *et al.,* 2014). For the current study *E.coli* (MTCC 40) species was utilized.

5.22.2: Preparation of Nutrient broth and Biodegradation assay by bacteria

Nutrient broth medium was prepared in order to culture the bacterial species. 28 grams of nutrient broth was added in 1000 ml of distilled water. For carrying out the biodegradation test, 100 ml of nutrient broth was poured in 250 ml of plastic conical flask. The media was

autoclaved under proper sterilized conditions. Each percentage of film i.e. Control, 1%, 3%, 5%, 7% and 10% was cut into small pieces and pre-weighted before carrying out the experiment. The films were then added into each conical flask along with 1 ml of bacterial inoculum. Flasks were incubated at 37^{o}C for a defined period of 21 days. Experiments were conducted in triplicate. The same protocols were followed for both the bacterial species. After 21 days, films were washed in distilled water and were weighted using weighing balance (Bonartseva *et al.,* 2002; Bhatia *et al.,* 2014).

5.22.3: Biodegradation study by PBS solution

In vitro degradation of pure PHB, PHB/OMMT and PHB/Zeolite blended films were carried in phosphate buffer saline.

Table 5.2: The Composition of PBS in 1000ml of distilled water are as follow:

S.No	Chemicals	Quantity (gm/1000ml)
1	NaCl	10
2	KCl	0.25
3	Na_2HPO_4	1.8
4	KH_2PO_4	0.3

The solution was maintained at 7.2 pH. The solution was sterilized in autoclave at 15 psi and at temperature121^{o}C for 20 minutes.

The biodegradation studies by phosphate buffer saline were carried out in 100 ml of plastic conical flasks. The films were cut into pieces and initial weight was noted. 100ml of PBS solution in each conical flask along with each percentage of film was set up. The conical flasks were incubated at 37^{o}C in rotary shaker for a definite period of 21 days. The films were filtered using whattman's filter paper and were washed with distilled water. The weight of the film was noted using weighing balance (Martínez-Valencia *et al.,* 2011; Murali *et al.,* 2013; Diba *et al.,* 2012).

The degradation percentage was calculated by using the formula given below:-

$$\% \text{ weight degradation} = \frac{\text{Initial Weight} - \text{Final Weight}}{\text{Initial Weight}} \times 100$$

5.23: SIMULATED BODY FLUID ASSAY

The bone-binding abilities of the biomaterials are evaluated by the development of Simulated body fluid assay, which is based on the conceptual approach of the formation of apatite like layer on the surface when implanted in living body.

The calcium phosphate ions are consumed by the material from the surrounding SBF fluid and stimulate the formation of apatite nuclei like layer on the material. The growth of calcium apatite layer on variety of biomaterial in laboratory conditions has very good application in tissue engineering especially in bone regeneration (Chavan *et al.,* 2010; Diba *et al.,* 2012).

The ionic concentration of Simulated body fluid prepared in laboratory conditions are nearly equal to human blood plasma as depicted in the table below:-

Table 5.3: Ion concentrations (mmol/dm3) of SBF and Human Blood plasma (Kokubo *et al.,* 1990)

Ionic concentration (mmol/dm^3)	**Simulated body fluid**	**Blood plasma**
Na^+	142	142
K^+	5.0	5.0
Mg^+	1.5	1.5
Ca^+	2.5	2.5
Cl^-	147.8	103.0
HCO_3^-	4.2	27
HPO_4^-	1.0	1.0
SO_4^-	0.5	0.5

Kokubo developed the procedure for the preparation of Simulated body fluid and is known as Kukubo method. The reagents used for the preparation of SBF fluid are as follow:
Reagents used for preparing SBF (pH 7.4) (Ohtsuki *et al.,* 1995).

Table 5.4: Preparation of Simulated Body fluid with different components

S. no	Reagent	Amount (g/l)
1	NaCl	7.996g
2	$NaHCO_3$	0.350g
3	KCl	0.224g
4	$K_2HPO_4.3H_2O$	0.228g
5	$MgCl_2.6H_2O$	0.305g
6	1M-HCl	40ml
7	$CaCl_2$	0.278g
8	Na_2SO_4	0.071g
9	$(CH_2OH)_3CNH_2$	6.057g

The reagents were dissolved in 1litre of double distilled water. The reagents were added one after another, ensuring that the earlier reagents were completely dissolved in the order given in the above table. pH was maintained at 7.4 and temperature of the fluid was maintained at 37^oC. Nanocomposites were added in the fluid for 7, 14, 21 days without the addition or refreshing of SBF solution. The films were removed from solution after the time lag and were dried at room temperature (Yan *et al.,* 2011; Khalil *et al.,* 2011). The formation of apatite layer was evaluated by FE-SEM technique (Model no. FEI QUANTA 200F, Netherlands operated at 15 kV) available at SMITHA LAB, Department of textile technology at Indian Institute of Technology, New Delhi.

RESULTS AND DISCUSSION

6.1: Isolation and screening of PHB producing strains from agriculture and saline environment sites.

First series of sites taken for collection of soil and analysis of bacteria were agricultural sites of different regions of Punjab. Geographically Punjab is divided into three regions Malwa Region, Doaba Region and Majha Region. Representative Samples were collected from all the three regions. Different sites of collection are shown in the map of Punjab (Figure 6.1.1) under given.

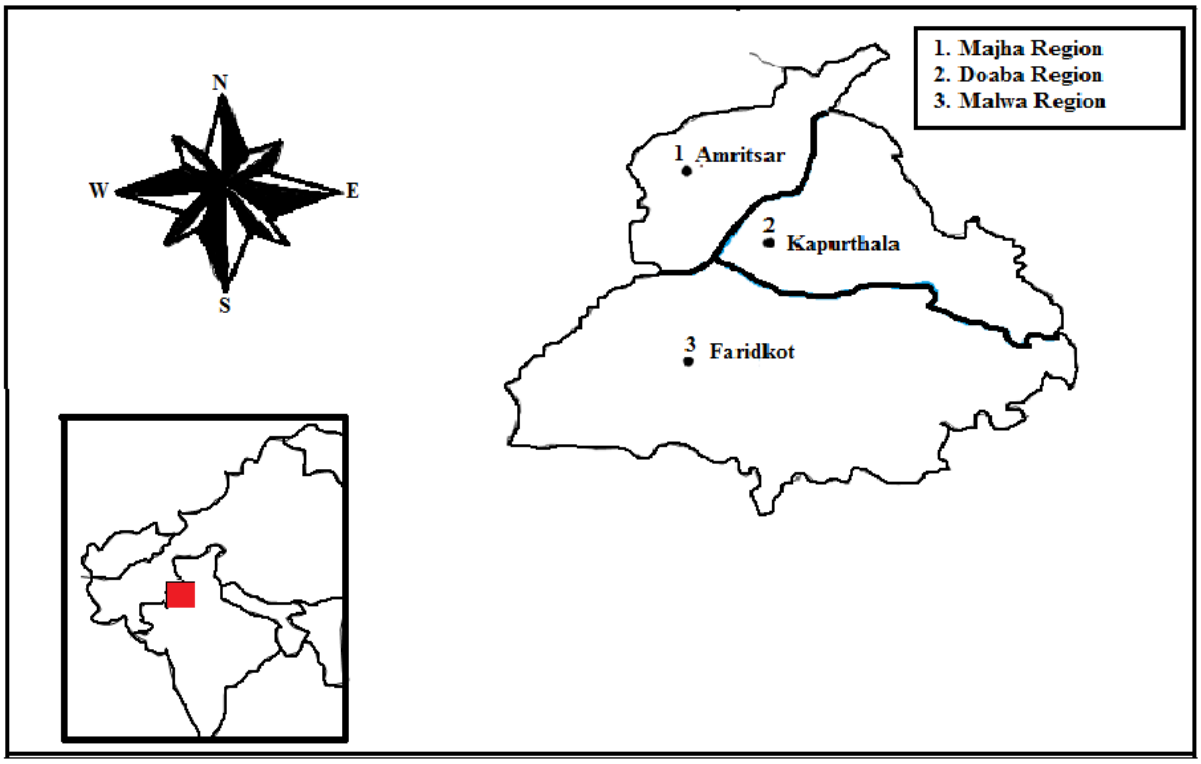


Figure 6.1.1: Map of Punjab showing geographical location of soil sample collection sites

6.1.1: Agriculture sites: Various agriculture sites selected for soil samples collection are given.

6.1.1.1: Agriculture sites (Malwa Region of Punjab) 30.6^{o}N 74.7^{o}E

Faridkot District was taken as representative site for Malwa Region of Punjab. Dried agriculture sites were selected for collection of soil samples.

Figure 6.1.2: Faridkot District Agriculture sites (Malwa Region of Punjab)

6.1.1.1a: Sample Collection Photographs

Figure 6.1.3: Site 1

Figure 6.1.4: Site 2

Figure 6.1.5: Site 3

Figure 6.1.6: Site 4

6.1.1.2: Agriculture sites (Majha Region of Punjab) 31.58°N 74.98°E

Amritsar District was taken as representative site for Majha Region of Punjab. Dried agriculture sites were selected for collection of soil samples. 3 different representative sites were chosen for soil sample collection. Figures showing sample collection sites are shown below.

Figure 6.1.7: Amritsar District agriculture sites (Majha Region of Punjab)

6.1.1.2a: Sample Collection Photographs

Figure 6.1.8: Site 1

Figure 6.1.9: Site 2

Figure 6.1.10: Site 3

Figure 6.1.11: Site 4

6.1.1.3: Agriculture sites (Doaba Region of Punjab) 31.37°N 75.38°E

Kapurthala district agricultural sites and Lovely Professional University agricultural sites were taken as representative sites for Doaba Region of Punjab. Dried agriculture sites were selected for collection of soil samples. 4 different representative sites were chosen for soil sample collection. Figures of sample collection are shown below.

Figure 6.1.12: Kapurthala District Agriculture sites (Doaba Region of Punjab)

6.1.1.3a: Sample Collection Photographs

Figure 6.1.13: Site 1

Figure 6.1.14: Site 2

Figure 6.1.15: Site 3

Figure 6.1.16: Site 4

6.1.2.: Saline sites: Various Saline sites selected for soil samples collection are given below

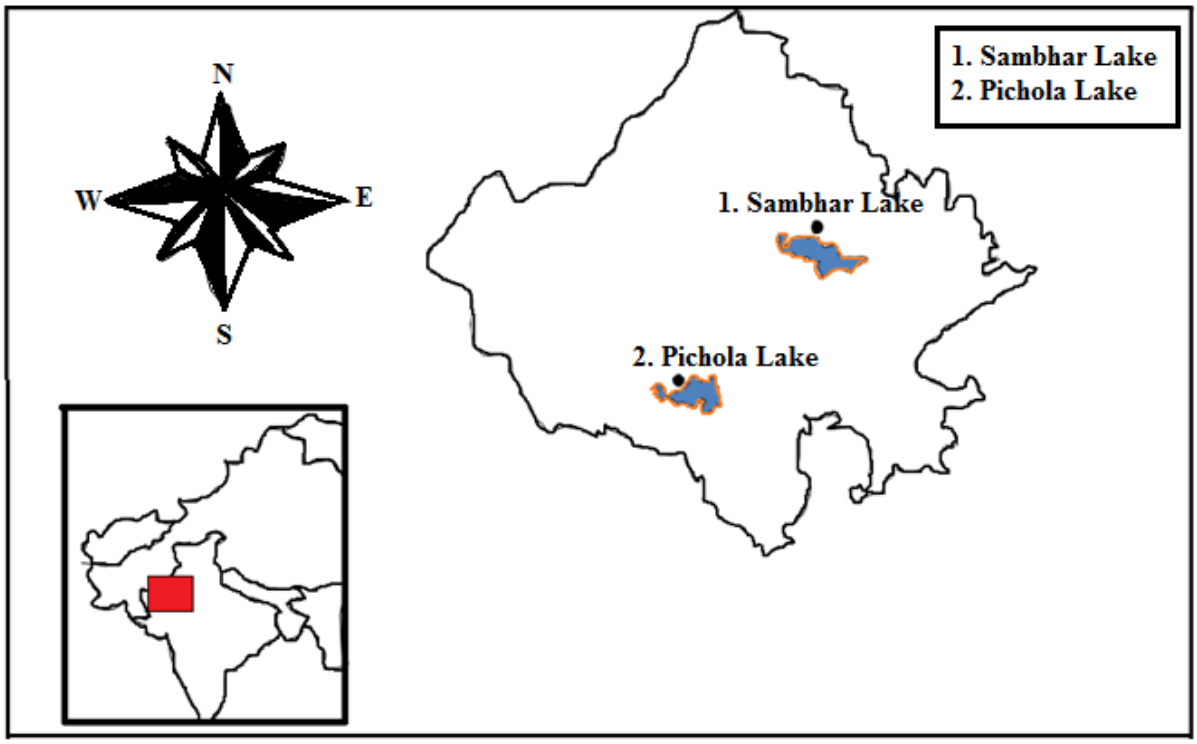


Figure 6.1.17: Map of Rajasthan showing geographical location of Soil sample collection sites

State of Rajasthan contains saline lake geographical sites of India. Different sites were selected and collection method was followed as described in material and method section.

6.1.2.1: Sambhar lake (96 km south west of the city Jaipur, Rajasthan) 26.96^{o}N 75.08^{o}E

Figure 6.2.18: Sambhar lake (96 km south west of the city Jaipur, Rajasthan)

6.1.2.1a: Sample Collection Photographs

Figure 6.1.19: Site 1

Figure 6.1.20: Site 2

Figure 6.1.21: Site 3

Figure 6.1.22: Site 4

Figure 6.1.23: Site 5

Figure 6.1.24: Site 6

Figure 6.2.25: Site 7

Figure 6.2.26: Site 8

6.1.2.2: Pichola lake (Udaipur, Rajasthan) 24.57°N, 73.67°E

Figure 6.1.27: Pichola lake (Udaipur, Rajasthan)

6.1.2.2a: Sample Collection Photographs

Figure 6.1.28: Site1

Figure 6.1.29: Site 2

Table 6.1: Physio-chemical analysis of Soil samples from different sites

Sample Collection Sites	pH	Electrical conductivity
Majha Site-1	7.9	9.14 µs/cm
Malwa Site-1	8.12	17.62 µs/cm
Malwa Site-2	8.01	34.10 µs/cm
Malwa Site-3	8.44	48.2 µs/cm
Doaba Site-1	7.44	7.41 µs/cm
Doaba Site-2	8.08	17.08 µs/cm
Pichola Site-1	7.77	54.3 µs/cm
Sambhar Site-1	9.31	125.2 ms/cm
Sambhar Site-3	9.94	28.5 ms/cm
Sambhar Site-5	8.94	157.5 ms/cm
Sambhar Site-8	9.64	12.68 ms/cm

6.1.3: Analysis of Physio-chemical testing values

6.1.3.1: Analysis of pH values

It was observed from the physio-chemical analysis that the pH value of Majha region Site-1 (7.9) and Daoba Region Site-1 (7.44) was almost near to the neutral value. The analysis also shows that Malwa region Site-1 (8.12), Site-2 (8.01) and Site-3 (8.44) was slightly alkaline in nature. The pichola site samples was having close to neutral pH of 7.77, it shows that despite of being a salty lake, still it is having less alkaline soil. The analysis of Sambhar samples justified the well-established fact that it is a true salt lake and due to continuous evaporation of water, the aqueous environment of the lake is considerably alkaline (Sambhar Site-1: 9.31 pH, Site-3: 9.94 pH , Site-5: 8.94 pH and Site-8: 9.64 pH.

6.1.3.2: Analysis of Electrical conductivity values

Different sites electrical conductivity analysis shows the electron conductance value OH^- of the Majha region Site-1 (9.14 µs/cm), Malwa region Site-1 (17.62 µs/cm), Site-2 (34.10 µs/cm), Site-3 (48.2 µs/cm) and Doaba Region Site 1 (7.41 µs/cm), Site 2 (17.08 µs/cm). The Pichola Site samples were having an electrical conductivity of 54µs/cm. The analysis of Sambhar Lake samples Site 1 (125 ms/cm) and Site 5 (157.5 ms/s) have a high electrical conductivity, as compared to other samples, whereas two other sites of Sambhar Lake i.e. Site 3 (28.5 ms/cm) and Site 8 (12.68 ms/cm). The analysis shows that the Sambhar lake samples were having a high electrical conductivity due to its alkaline nature.

6.1.4: Isolated bacteria from different sites of Punjab Agriculture Region and Salty Lake sites

Bacteria were isolated from different soil samples as detailed above. Nutrient Agar media along with different salt level modifications, selective media for halophiles (DSC-97 and Halobacterium medium) were utilized in the isolation protocols along with temperature and other conditions as discussed in material and method section. Purified bacteria further were sub-cultured from the mixed preliminary isolates as described in material and method section

6.1.4.1: Photographs of Purified Bacterial isolates from different agriculture sites of Punjab

6.1.4.1a: Malwa Region

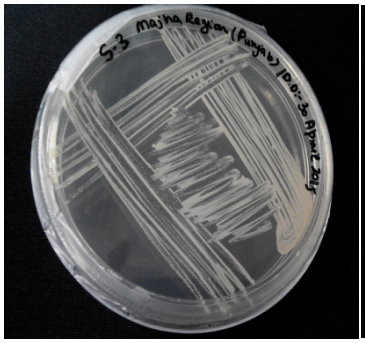


Figure 6.1.30: S3 Site-1

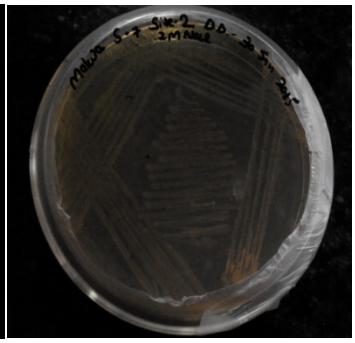


Figure 6.1.31: S7 Site-2

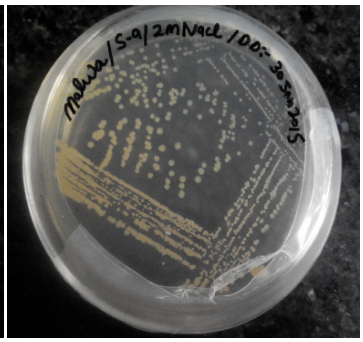


Figure 6.1.32: S9 Site-3

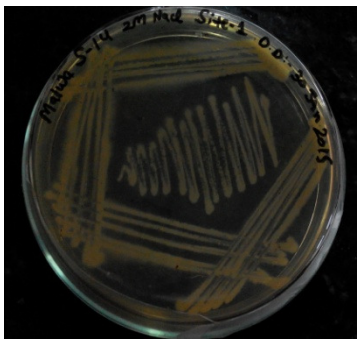


Figure 6.1.33: S14 Site-1

6.1.4.1b: Majha Region

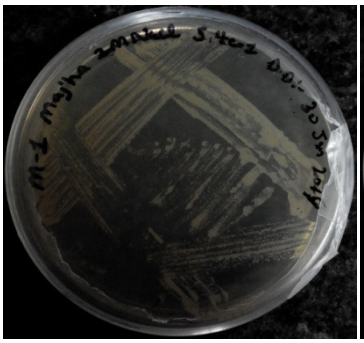


Figure 6.1.34: M1 Site-2

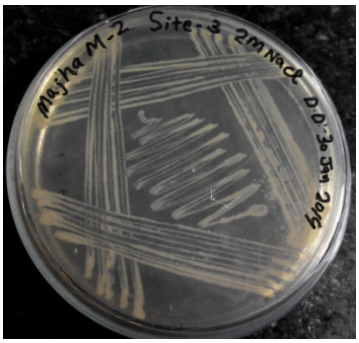


Figure 6.1.35: M2 Site-3

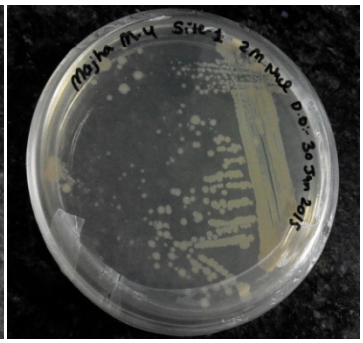
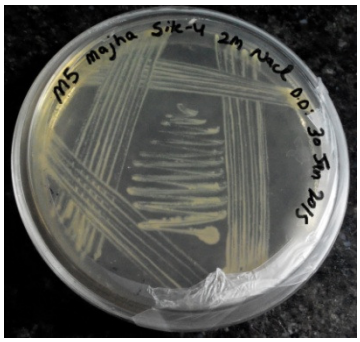
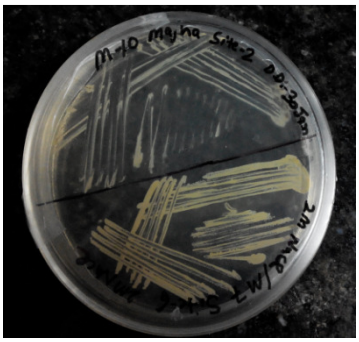


Figure 6.1.36: M4 Site-1





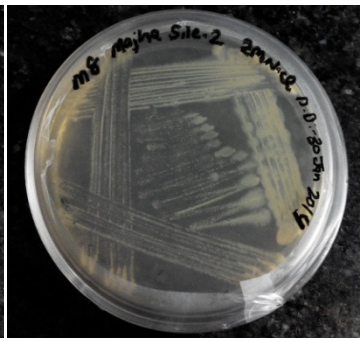
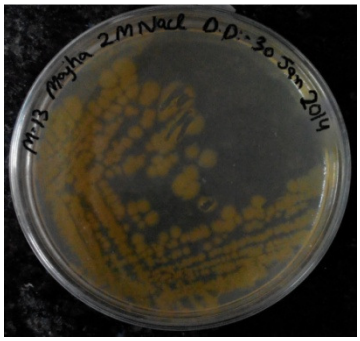


Figure 6.1.37: M5 Site-4 **Figure 6.1.38:** M7 and M10 Site-2 **Figure 6.1.39:** M8 Site-2



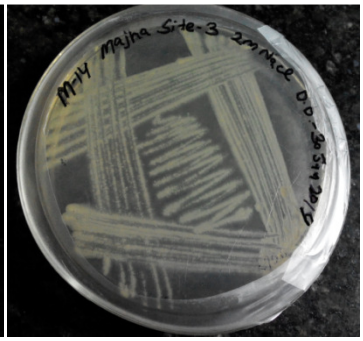
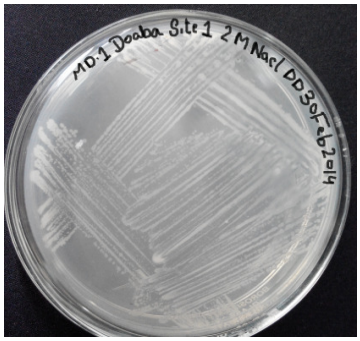
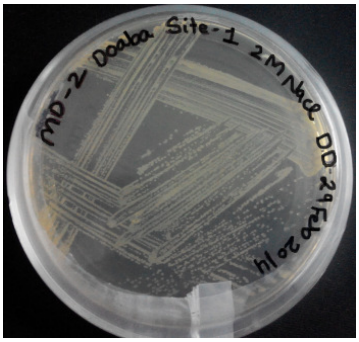


Figure 6.1.40: M13 Site-4 **Figure 6.1.41:** M14 Site-3

6.1.4.1c: Doaba region





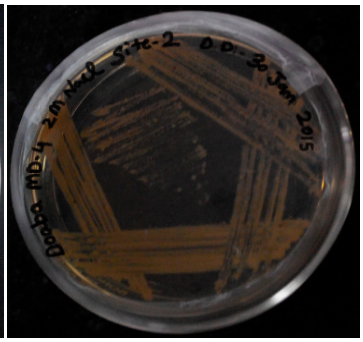
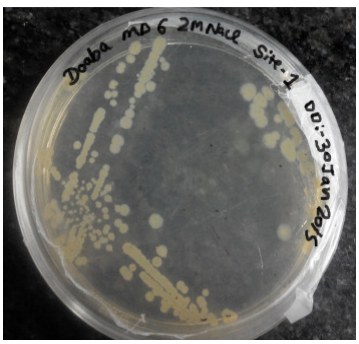


Figure 6.1.42: MD1 Site-1 **Figure 6.1.43:** MD2 Site-1 **Figure 6.1.44:** MD4 Site-2



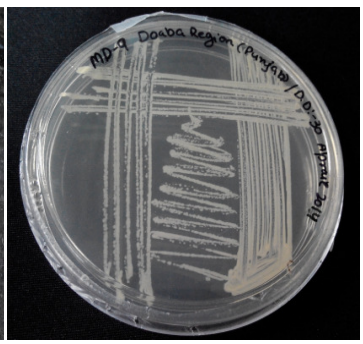


Figure 6.1.45: MD5 Site-3 **Figure 6.1.46:** MD6 Site-1 **Figure 5.1.47:** MD9 Site-4

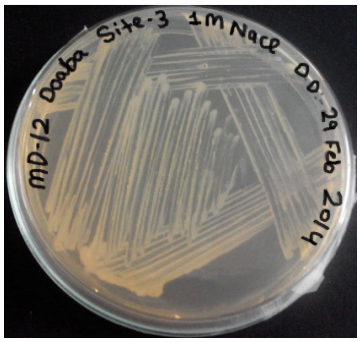
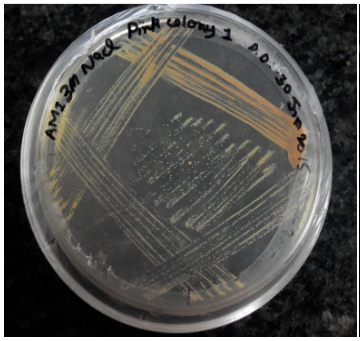
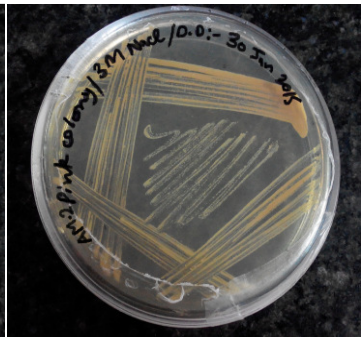


Figure 5.1.48: MD12 Site-3

6.1.4.1d: Purified bacterial isolates from different sites of Sambhar Region





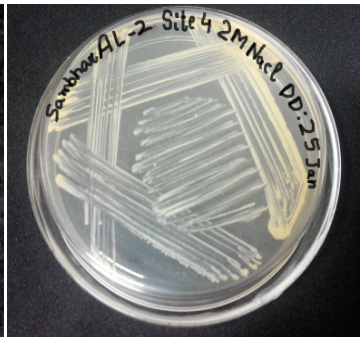
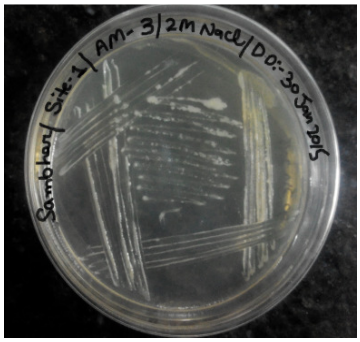
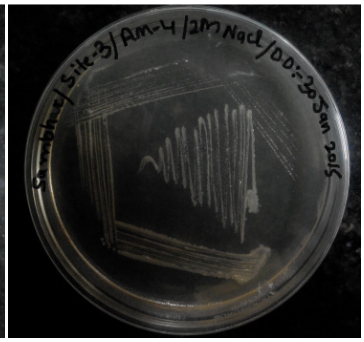


Figure 6.1.49:AM1 Site-1 **Figure 6.1.450:**AM2 Site-1 **Figure 6.1.51:** AL2 Site-4





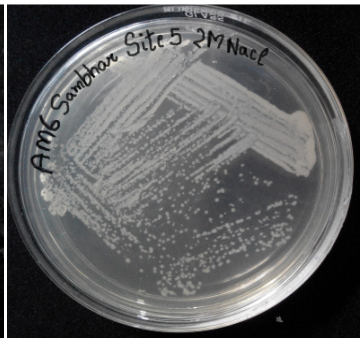
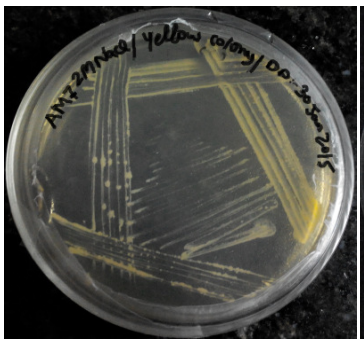


Figure 6.1.52:AM3 Site-1 **Figure 6.1.53:** AM4 Site-3 **Figure 6.1.54:** AM6 Site-5



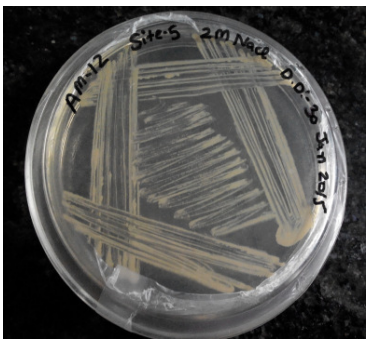


Figure 6.1.55: AM7 Site-4 **Figure 6.1.56:** AM9 Site-6 **Figure 6.1.57:** AM12 Site-5

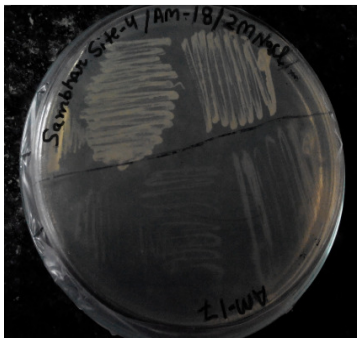
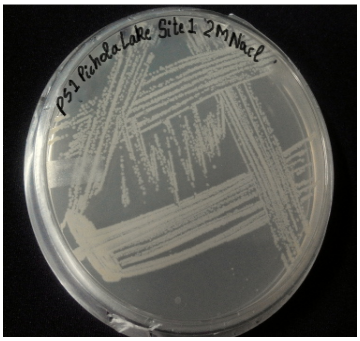
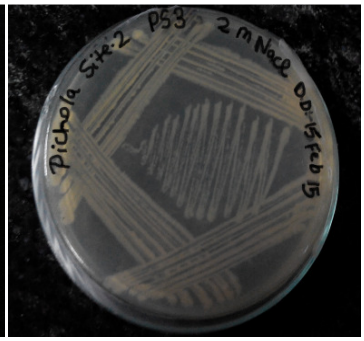


Figure 6.1.58: AM17 and AM18 Site-4

6.1.4.1e: Pichola Lake





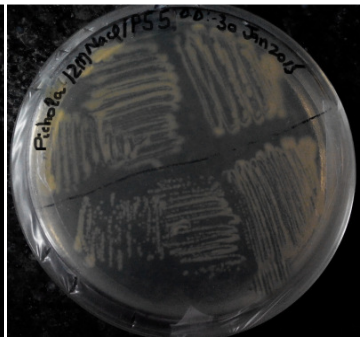


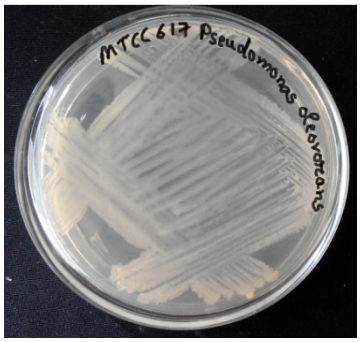
Figure 6.1.59: PS1 Site-1 **Figure 6.1.60:** PS3 Site-2 **Figure 6.1.61:**PS5 Site-2

6.1.5: Standard Microbial type culture collection (MTCC) strain for high Polyhydroxybutyrate (PHB) production

6.1.5.1: Pure MTCC strain cultured on plates

Pure MTCC strains were cultured on nutrient agar plates as described in Material and Method section

6.1.5.1a: Figures of pure MTCC strains cultured on Nutrient agar medium



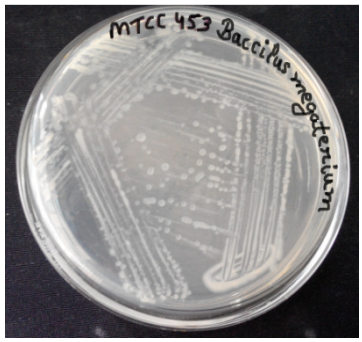


Figure 6.1.62: *Pseudomonas oleovorans* **Figure 6.1.63:** *Bacillus megaterium*

6.1.6: Screening for PHB producing bacteria

Isolated bacterial strains were further screened by Sudan Black B staining method as described in material & method section. Bacterial isolates, showing positive test for PHB production, are shown under.

6.1.6.1: Screening of isolates for PHB production from different regions of Punjab

6.1.6.1a: Malwa Region

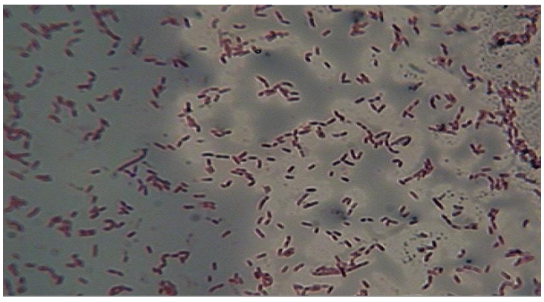


Figure 6.1.64: Malwa Region S1

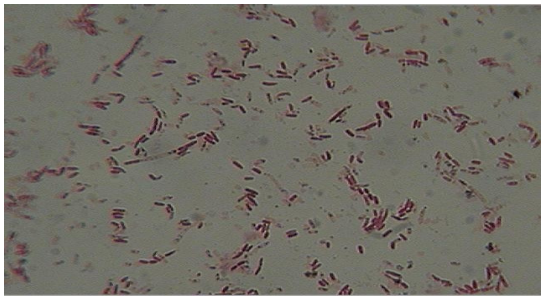


Figure 6.1.65: Malwa Region S3

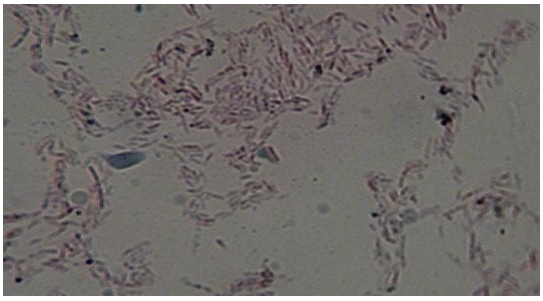


Figure 6.1.66: Malwa Region S8

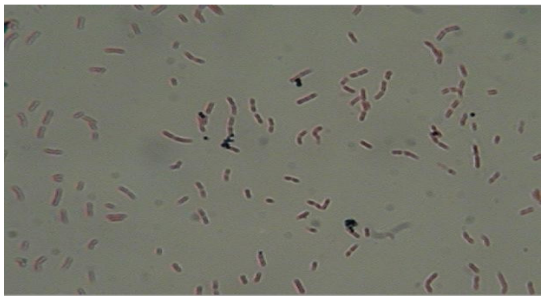


Figure 6.1.67: Malwa Region S10

6.1.6.1b: Majha Region

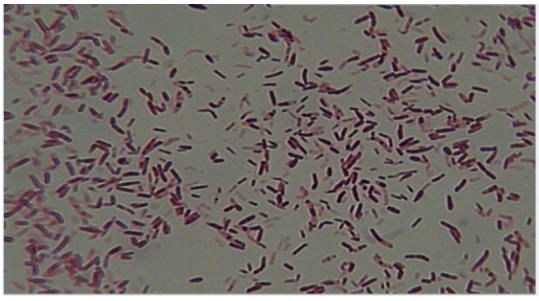


Figure 6.1.68: Majha Region M1

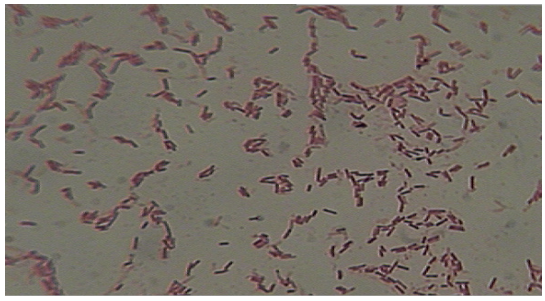


Figure 6.1.69: Majha Region M2

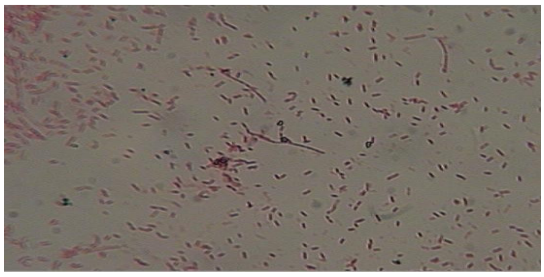


Figure 6.1.70: Majha Region M10

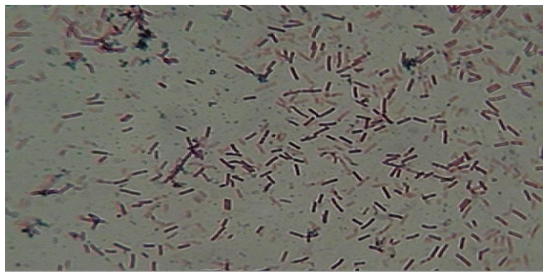


Figure 6.1.71: Majha Region M10

6.1.6.1c: Doaba Region

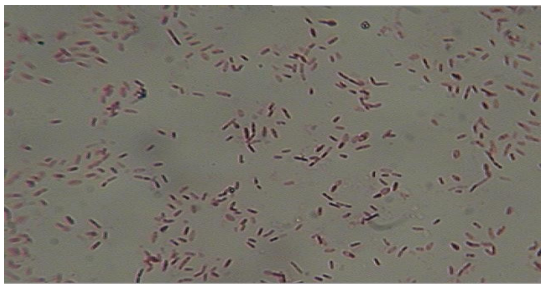


Figure 6.1.72: Doaba Region MD1

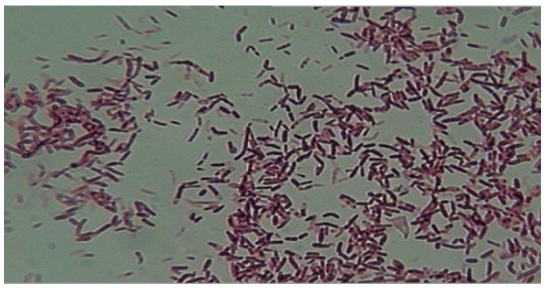


Figure 6.1.73: Doaba Region MD2

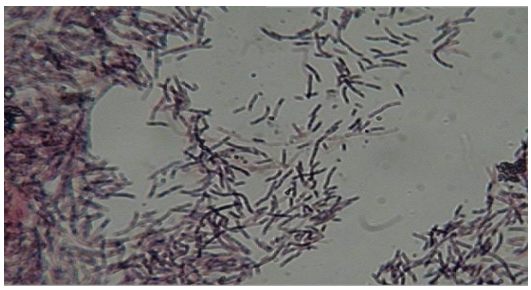


Figure 6.1.74: Doaba Region MD5

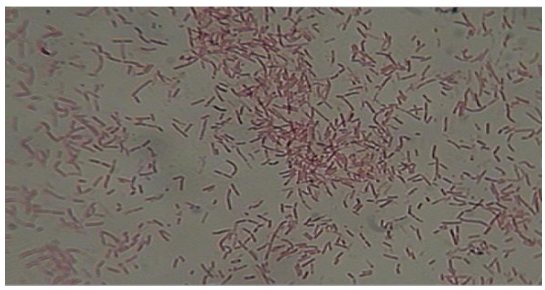


Figure 6.1.75: Doaba Region MD14

6.1.6.2: Screening of Isolates from Saline Salt Lake Sites

6.1.6.2a: Sambhar Lake (Rajasthan)

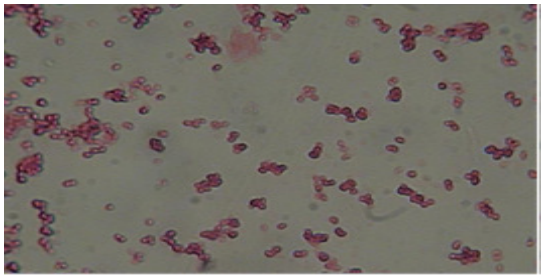


Figure 6.1.76: Sambhar Site-1 AM1

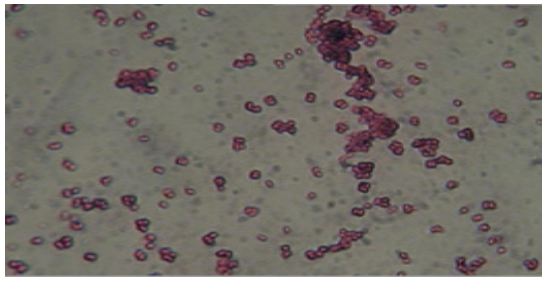


Figure 6.1.77: Sambhar Site-1 AM2

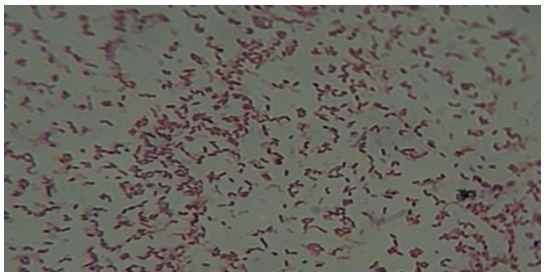


Figure 6.1.78: Sambhar Site-1 AM6

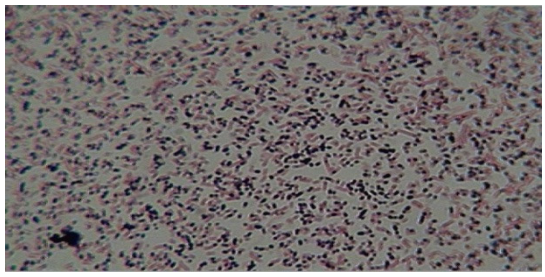


Figure 6.1.79: Sambhar Site-1 AM9

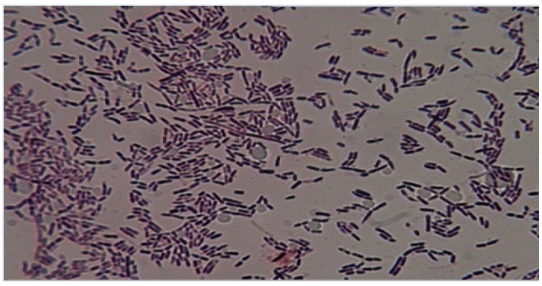


Figure 6.1.80: MTCC 617

6.2: Strain improvement protocols for isolated strains and standard strains of PHB production through mutagenesis

6.2.1: Investigation for maximum PHB production of isolated strains through induction.

The induction protocol defined in material and method section required first step of investigation of growth curve. Conditions of growth curve determination were maintained as described in material and method section. Growth curves of different bacterial strains are shown in figures.

6.2.1.1: Growth curves of different bacterial isolates

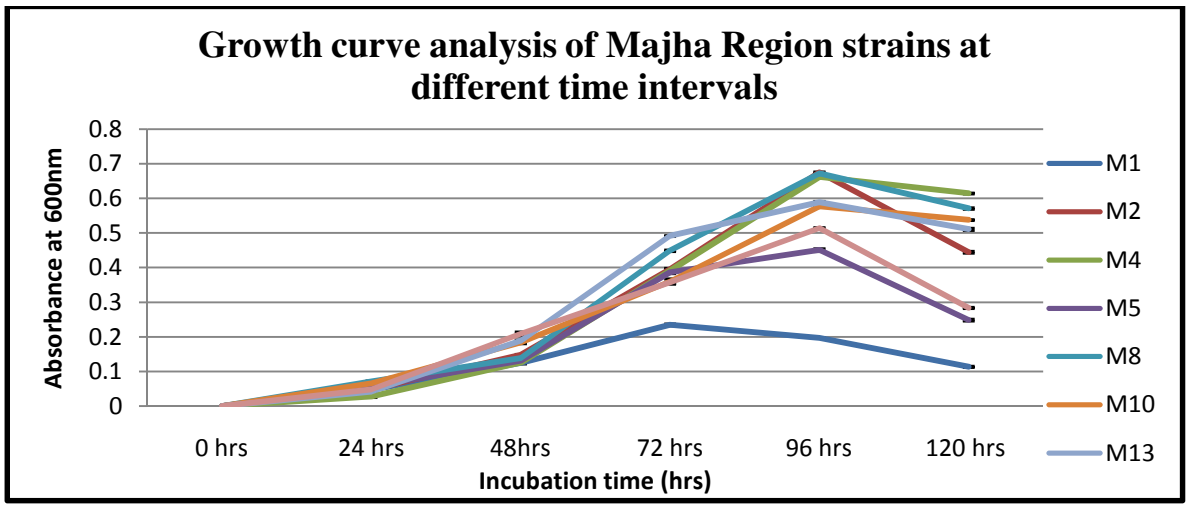


Figure 6.2.1: Data represents the growth curve of different isolates of Majha Region (Punjab)

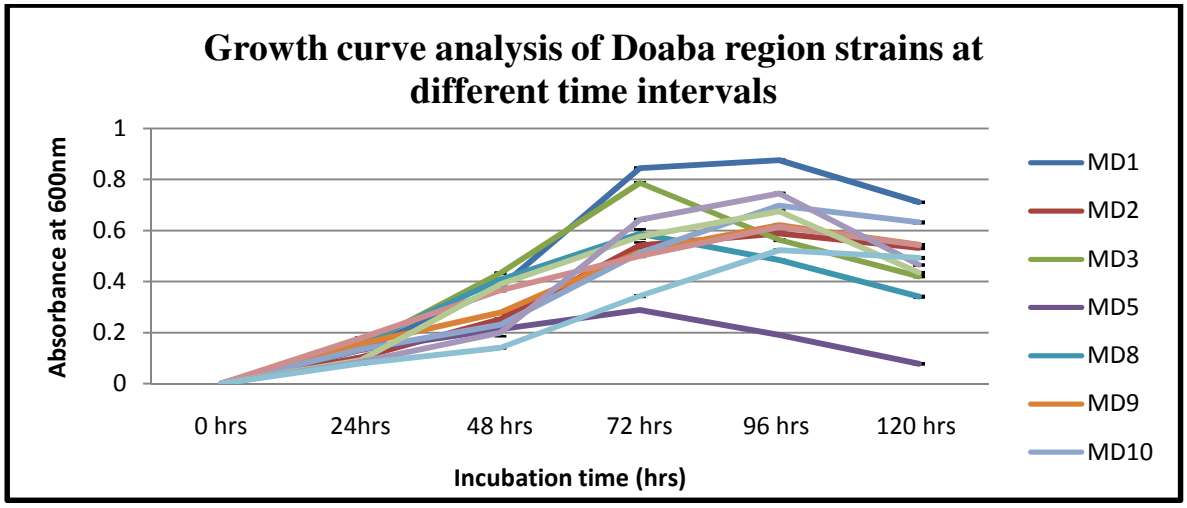


Figure 6.2.2: Data represents the growth curve of different isolates of Doaba Region (Punjab)

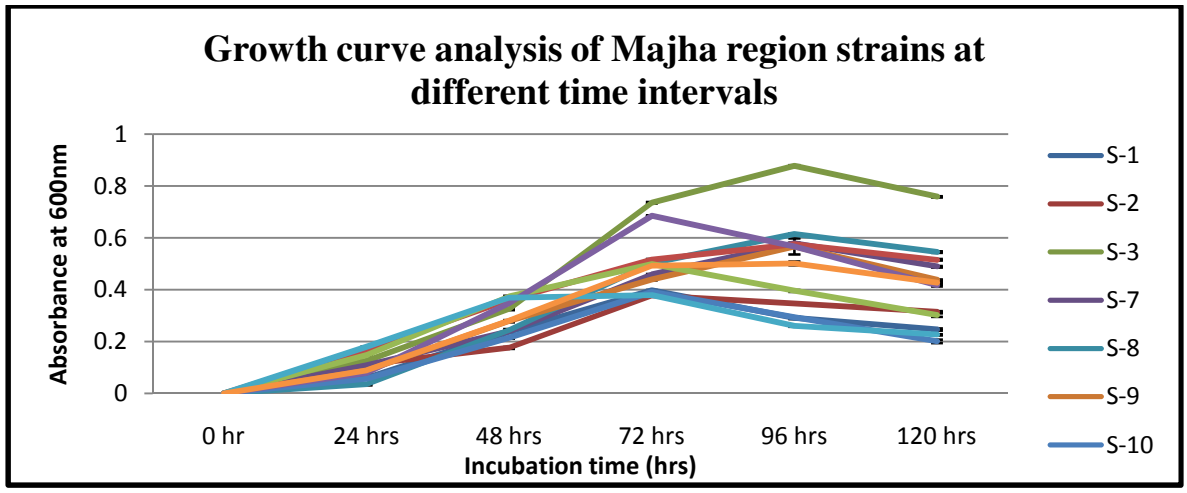


Figure 6.2.3: Data represents the growth curve of different isolates of Malwa Region (Punjab)

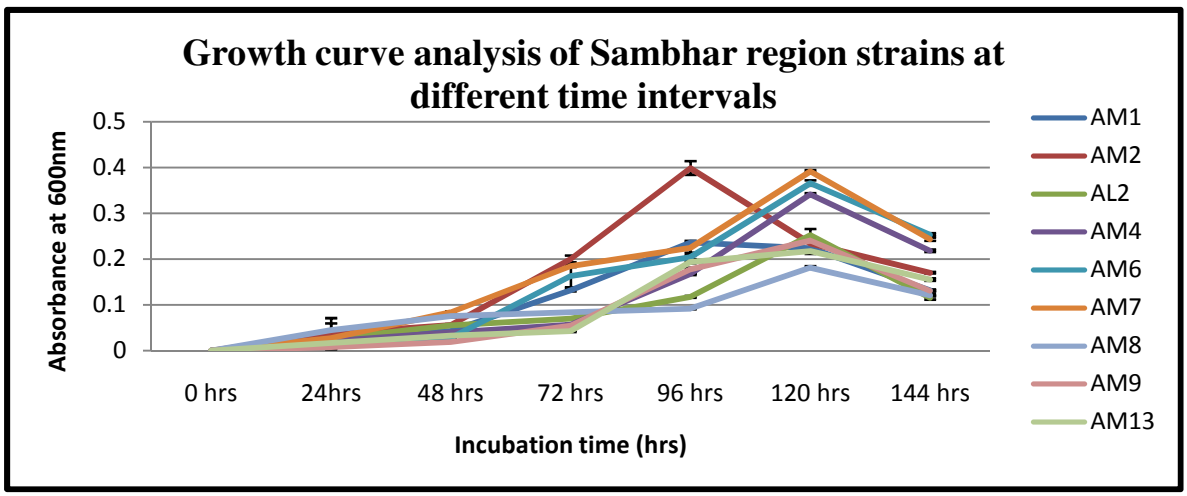


Figure 6.2.4: Data represents the growth curve of different isolates of Sambhar Lake (Rajasthan).

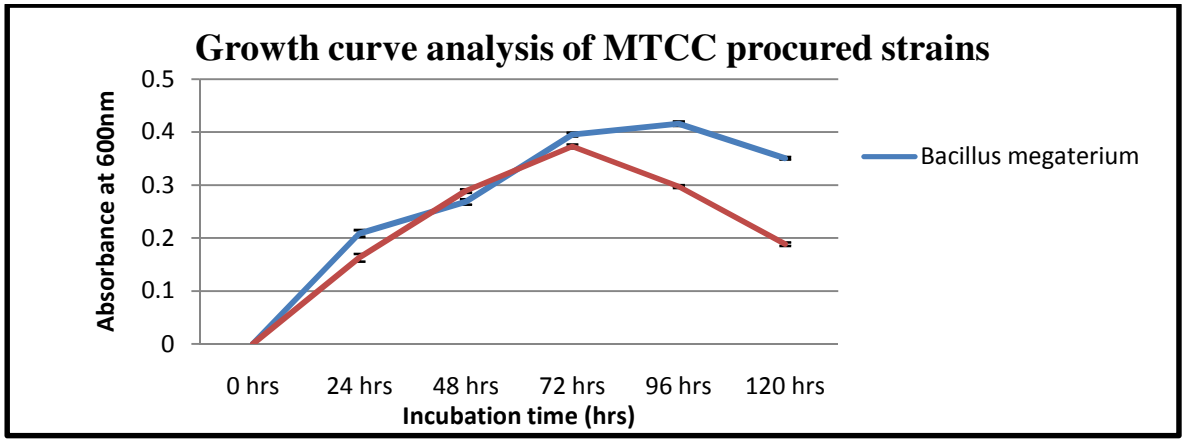


Figure 6.2.5: Data represents the growth curve of MTCC strains

6.2.1.2: PHB extraction and quantification from bacterial strains isolated from different geographical areas

6.2.1.2a: PHB quantification and extraction from strains isolated from Malwa Region (Punjab)

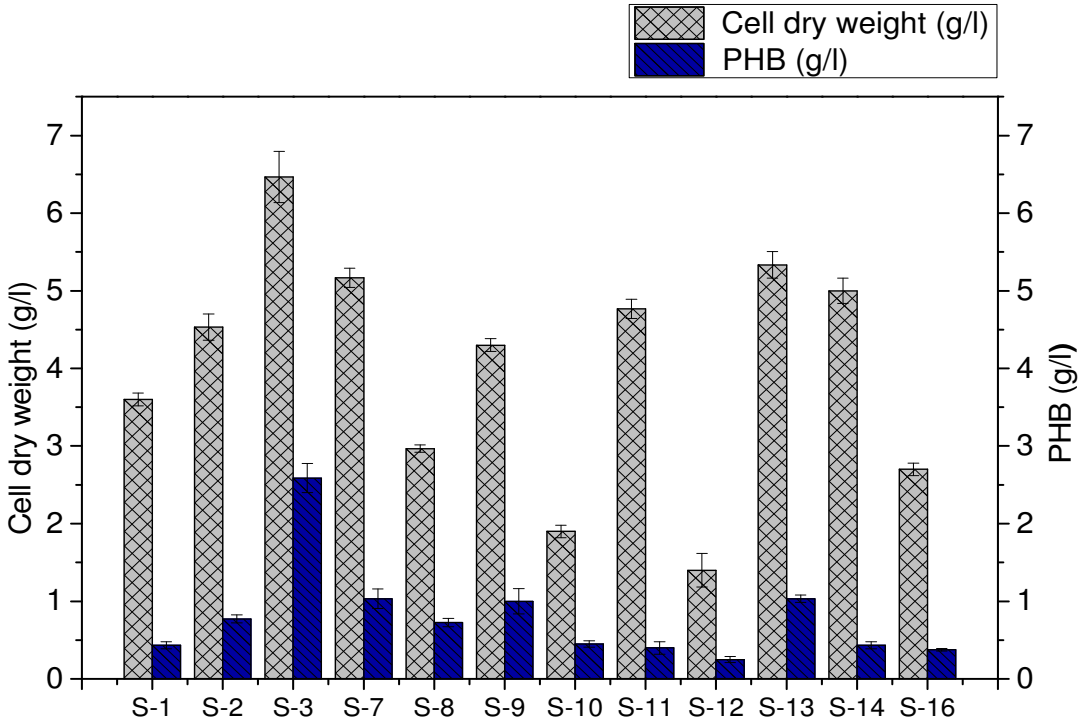


Figure 6.2.6: Cell dry weight (g/l) and PHB (g/l) of the bacterial strains isolated from Malwa Region (Punjab)

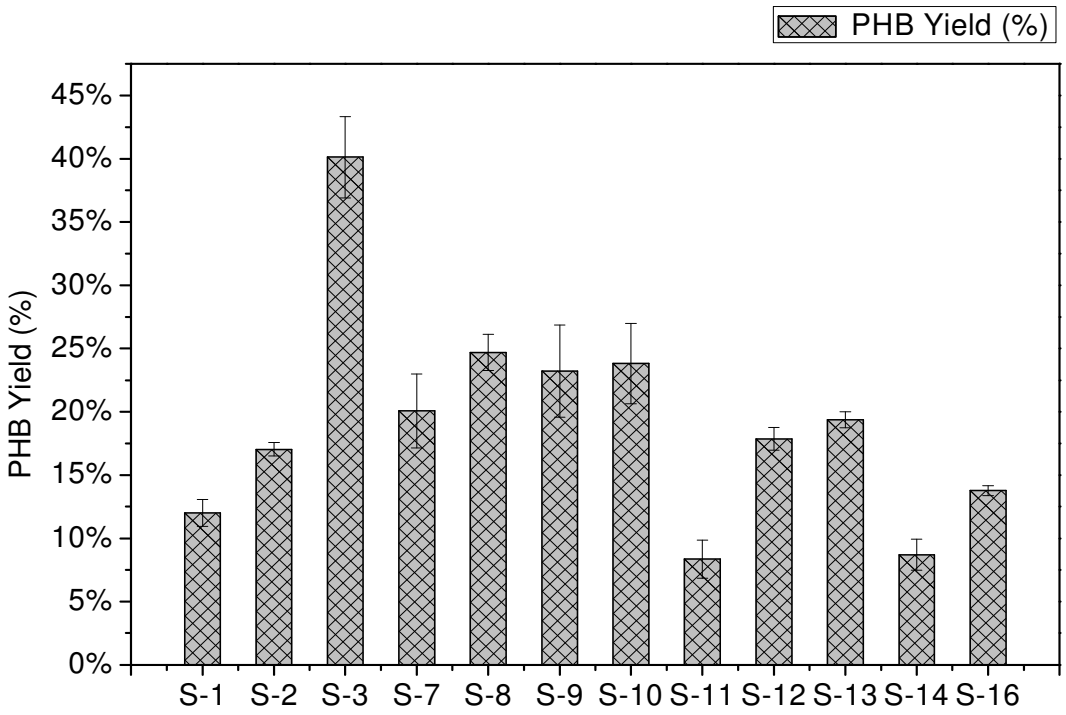


Figure 6.2.7: PHB yield (%) from the bacterial strains isolated from Malwa Region (Punjab)

6.2.1.1a (i): Observation of PHB quantification from strains of Malwa Region (Punjab)

PHB quantification was carried out for the isolates from Malwa Region of Punjab, which were screened and have shown positive results by Sudan Black Staining technique. PHB extraction protocol was carried out as described in material and method section. S-3 isolate yielded a cell dry weight (cdw) of 6.46 g/l and S-13 isolate yielded a cell dry weight (cdw) of 5.3 g/l. Highest accumulation of PHB production was observed in S-3 with PHB yield of 2.588 g/l from culture broth and S-13 with PHB yield of 1.03 g/l. Percentage yield of PHB in S-3 isolate was 40.13% and S-13 was 19.37%

According to the analysis of literature available there are certain conditions defined for high PHB production, the first necessary condition is achievement of high cell mass density and second is high percentage yield of PHB product. On the basis of these conditions from Malwa region 1 isolate namely S-3 was selected.

6.2.1.2c: PHB quantification and extraction from strains isolated from Doaba Region (Punjab)

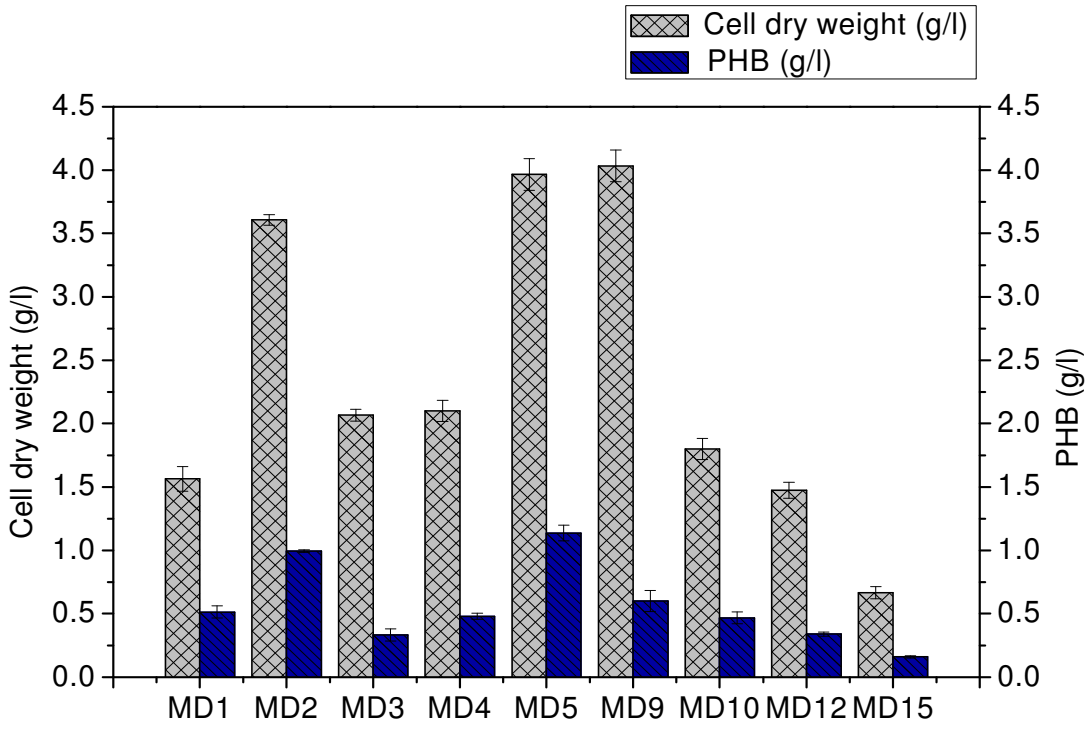


Figure 6.2.10: Cell dry weight (g/l) and PHB (g/l) of the bacterial strains isolated from Doaba Region (Punjab)

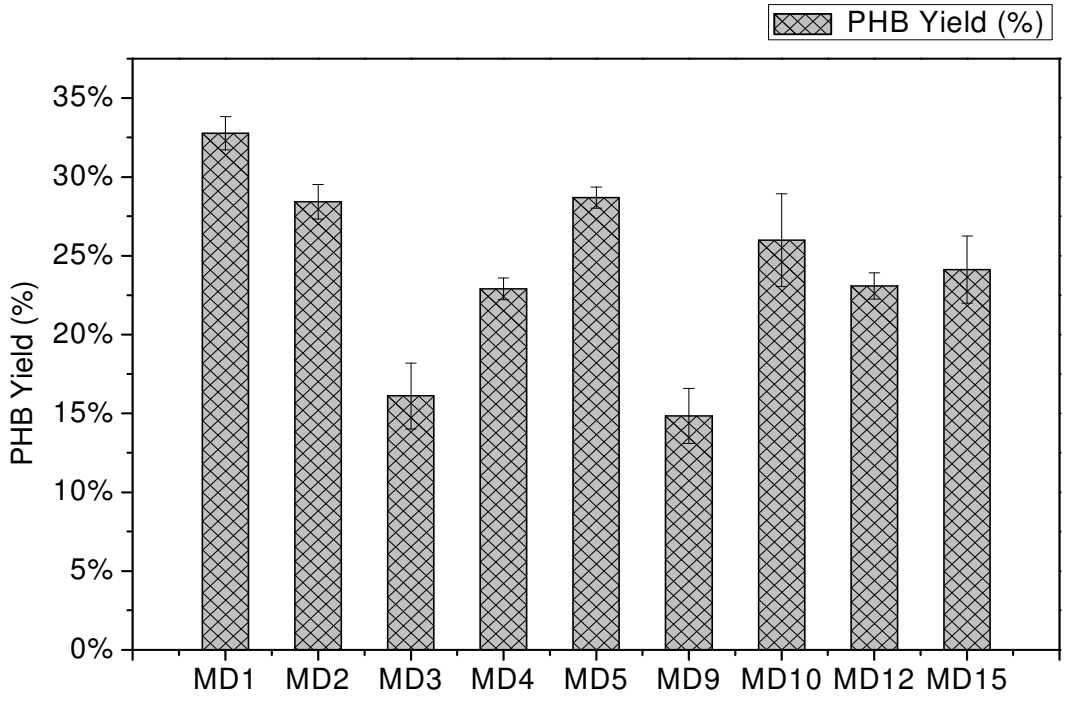


Figure 6.2.11: PHB yield (%) from the bacterial strains isolated from Doaba Region (Punjab)

6.2.1.2a(i): Observation of PHB quantification from strains of Doaba Region (Punjab)

PHB quantification was carried out for the isolates from Doaba Region of Punjab, which were screened and have shown positive results by Sudan Black Staining technique. PHB extraction protocol was carried out as described in material and method section. MD1 isolate yielded a cell dry weight (cdw) of 1.563 g/l, MD2 isolate yielded a cell dry weight (cdw) of 3.66 g/l and MD5 isolate yielded a cell dry weight (cdw) of 3.606 g/l. It was observed that PHB yield in MD5 was 1.1373 g/l from culture broth, MD2 with PHB yield of 0.993 g/l and MD1 with PHB yield of 0.5133 g/l. Percentage yield of PHB in MD1 isolate was 32.77%, MD2 28.41% and MD5 28.69%.

According to the analysis of literature available there are certain conditions defined for high PHB production, the first necessary condition is achievement of high cell mass density and second is high percentage yield of PHB product. On the basis of these conditions from Doaba regions 3 isolates namely MD1, MD2 and MD5 were selected.

6.2.1.3b: PHB quantification and extraction from strains isolated from Majha Region (Punjab)

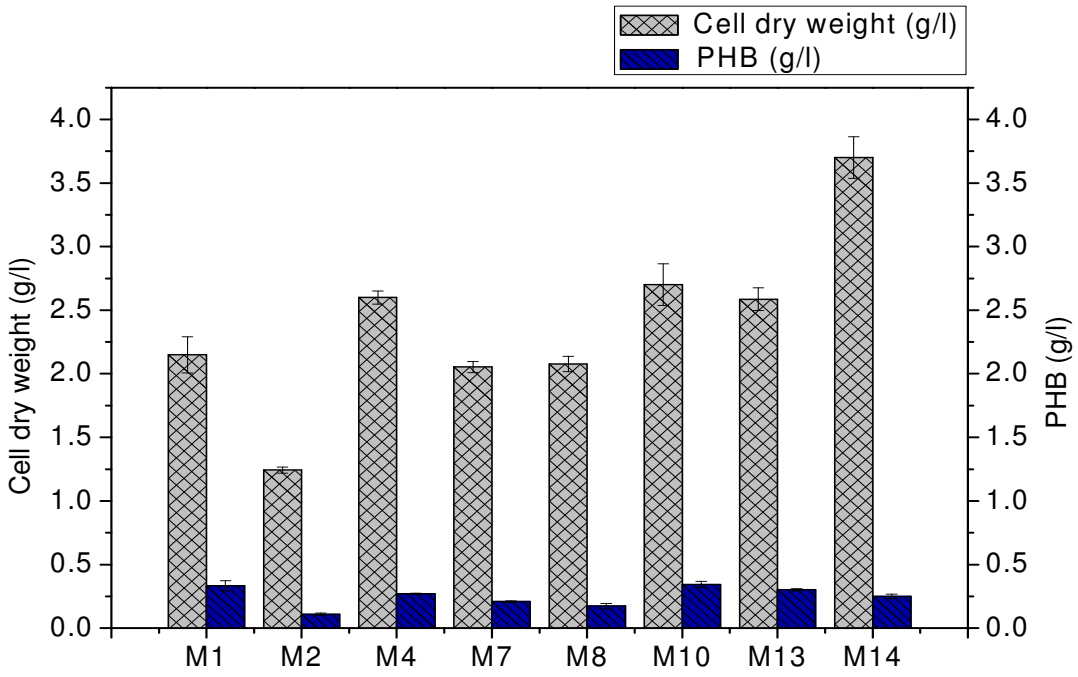


Figure 6.2.8: Cell dry weight (g/l) and PHB (g/l) of the bacterial strains isolated from Majha Region (Punjab)

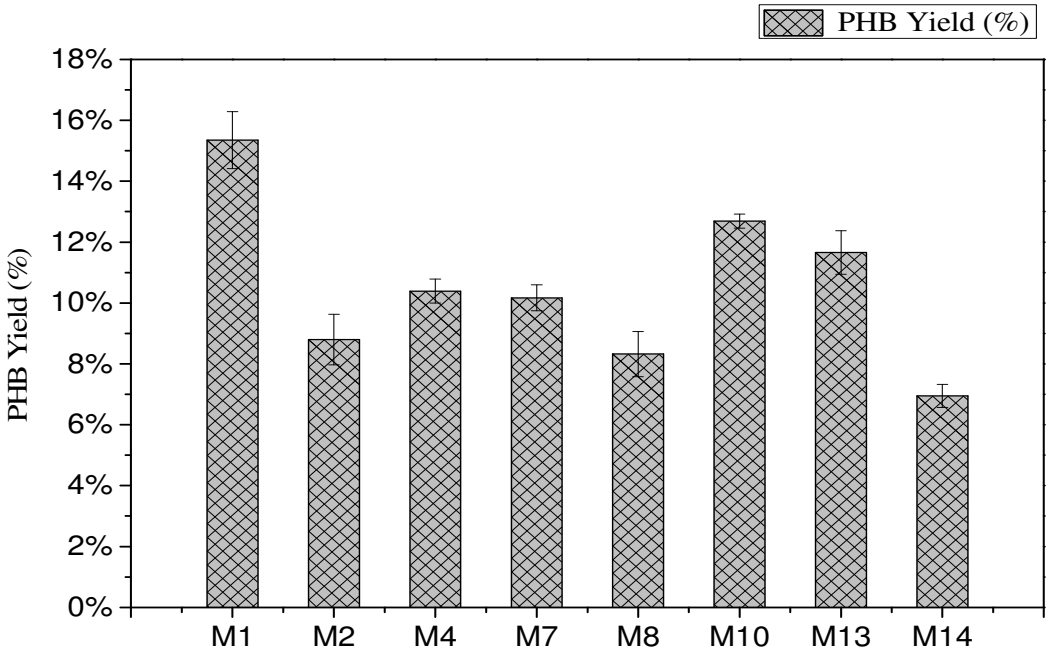


Figure 6.2.9: PHB yield (%) from the bacterial strains isolated from Majha Region (Punjab)

6.2.1.3b(i): Observation of PHB quantification from strains of Majha Region (Punjab)

PHB quantification was carried out for the isolates from Majha Region of Punjab, which were screened and have shown positive results by Sudan Black Staining technique. PHB extraction protocol was carried out as described in material and method section. M1 isolate yielded a cell dry weight (cdw) of 2.15 g/l, M7 isolate yielded a cell dry weight (cdw) of 2.05 g/l and M10 isolate yielded a cell dry weight (cdw) of 2.7 g/l. Highest accumulation of PHB production was observed in M1 with PHB yield of 0.33 g/l from culture broth, M7 with PHB accumulation of 0.208 g/l and M10 with PHB accumulation of 0.343 g/l. Percentage yield of PHB in M1 isolate was 15.34%, M10 12.69% and M7 10.17%.

According to the analysis of literature available there are certain conditions defined for high PHB production, the first necessary condition necessary is achievement of high cell mass density and second is high percentage yield of PHB product. On the basis of these conditions from Majha regions 2 isolates namely M1 and M10 were selected.

6.2.1.4d: PHB quantification and extraction from strains isolated from Salty Sambhar lake sites (Rajasthan)

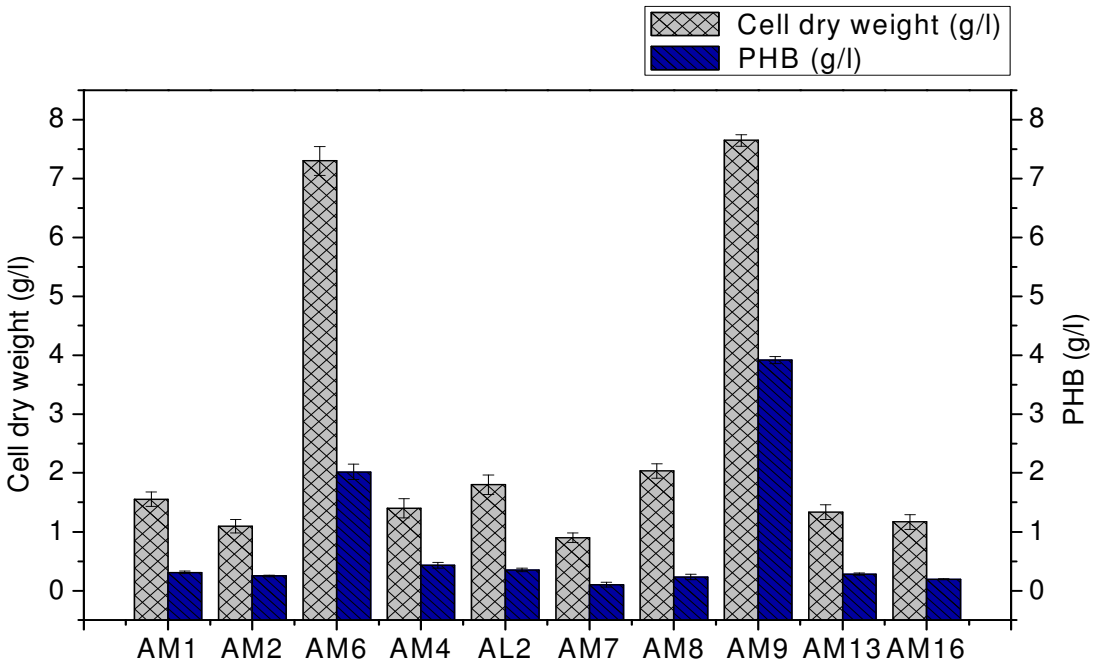


Figure 6.2.12: Cell dry weight (g/l) and PHB (g/l) from the bacterial strains isolated from Sambhar Lake Region (Punjab)

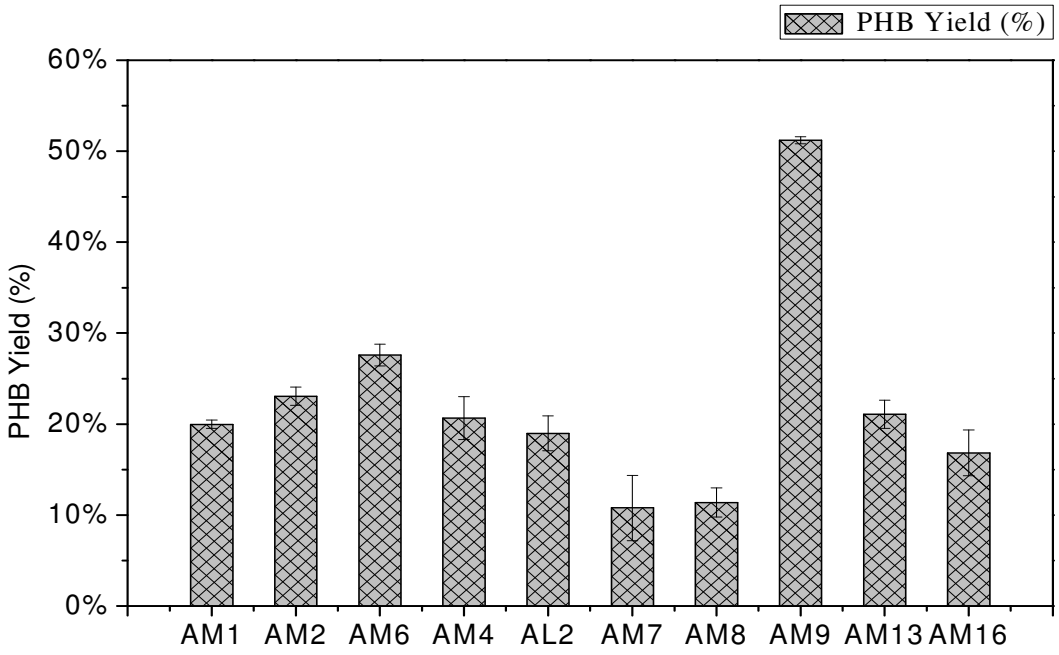


Figure 6.2.13: PHB yield (%) of the bacterial strains isolated from Sambhar Lake Region Region (Punjab)

6.2.1.4a(i): Observation of PHB quantification from strains of Salty Sambhar Lake Region (Rajasthan)

PHB quantification was carried out for the isolates from Malwa Region of Punjab, which were screened and have shown positive results by Sudan Black Staining technique. PHB extraction protocol was carried out as described in material and method section. AM1 isolate yielded a cell dry weight (cdw) of 1.553 g/l, AM2 isolate yielded a cell dry weight (cdw) of 1.093 g/l, AM6 isolate yielded a cell dry weight (cdw) of 7.3 g/l, AM4 isolate yielded a cell dry weight (cdw) of 1.4 g/l, AL2 isolate yielded a cell dry weight (cdw) of 1.8 g/l and AM9 isolate yielded a cell dry weight (cdw) of 7.64 g/l. Highest accumulation of PHB production was observed in AM9 with PHB of 3.91 g/l from culture broth, AM6 with PHB yield of 2.016 g/l, AM4 with PHB of 0.433 g/l, AL2 with PHB accumulation of 0.353 g/l, AM1 with PHB of 0.31 g/l and AM2 with PHB accumulation of 0.251 g/l. Percentage yield of PHB in AM6 was 27.60%, AM9 51.19%, AM1 19.97%, AM2 23.06% AM4 20.66%, and AL2 19%.

According to the analysis of literature available there are certain conditions defined for high PHB production, the first necessary condition is achievement of high cell mass density and second is high percentage yield of PHB product. On the basis of these conditions from Sambhar regions 2 isolates namely AM6 and AM9 were selected.

On the basis of performance for PHB production, yield, less time consumption for growth finally the following given bacteria were selected for further work from all the regions.

Table 6.2: Selected strains for further analysis of PHB production with their isolaton site and optimum salt concentration for their growth

S.no	Isolate name	Isolated Site	Salt molar concentration for growth	pH for growth
1.	S-3	Malwa Region Site-1	2 Molarity	7.2
2.	M1	Majha Region Site-2	1 Molarity	7.2
3.	M10	Majha Region Site-2	2 Molarity	7.2
4.	MD1	Doaba Region Site-1	2 Molarity	7.2
5.	MD2	Doaba Region Site-1	2 Molarity	7.2
6.	MD5	Doaba Region Site-3	1 Molarity	7.2
7.	AM6	Sambhar Lake Region Site-5	2 Molarity	7.4
8.	AM9	Sambhar Lake Region Site-6	2 Molarity	7.4

6.2.1.2e: PHB quantification and extraction from strains procured from MTCC (Chandigarh)

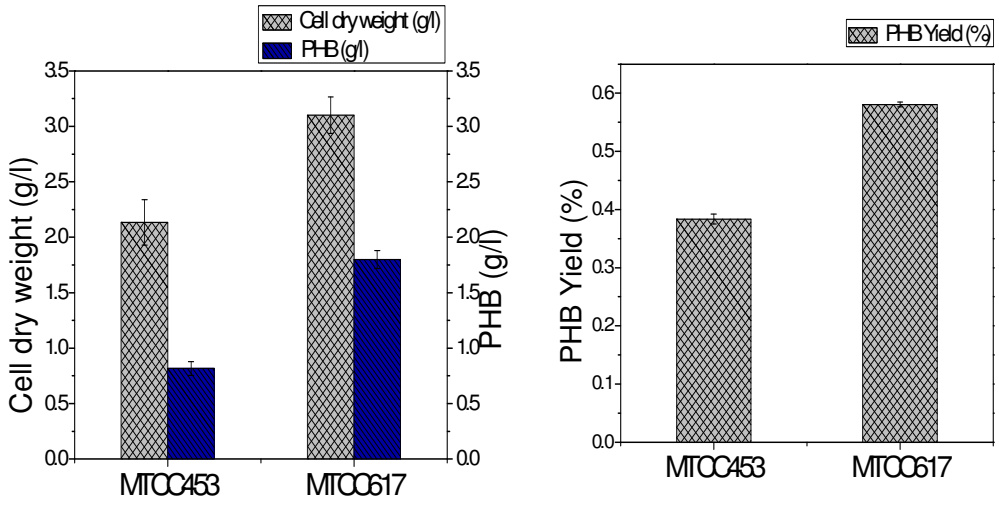


Figure 6.2.14: Cell dry weight (g/l), PHB (g/l) and PHB yield (%) from the MTCC strains (*Bacillus megaterium* MTCC 453 and *Pseudomonas oleoverans* MTCC 617)

6.2.1.2e (i): Observation of PHB quantification from standard strains procured from IMTECH CHANDIGARH

PHB quantification was carried out on standard PHB producer strains procured from IMTECH Chandigarh. The strains were MTCC 453 (*Bacillus megaterium*) and MTCC 617 (*Pseudomonas oleoverans*). PHB extraction protocol was carried out as described in material and method section. MTCC 453 yielded a cell dry weight (cdw) of 9.616 g/l, MTCC617 yielded a cell dry weight (cdw) of 8.566 g/l. Highest accumulation of PHB production was observed in MTCC617 with PHB yield of 4.166 g/l from culture broth. MTCC 453 with PHB accumulation of 3.543 g/l. Percentage yield of PHB in MTCC 617 isolate was 48.44% and MTCC453 36.85%.

6.2.2: Strain improvement through Mutagenesis

Strain improvement protocols were followed as described in the material and method section.

6.2.3: Results of mutagenesis experiments

6.2.3.1: Number of colonies in control & mutated samples after Exposure to different mutagenic agents [S-3 bacterial isolate from Malwa Region Punjab]

Table 6.3: Differential Exposure of Acridine orange mutagen and Total viable count (S-3) observed on petri-plate

Control	Mean colony count at 50 µg/ml	Mean colony count at 100 µg/ml	Mean colony count at 200 µg/ml
More than 200	17	3	0

Table 6.4: Differential Exposure of Sodium azide mutagen and Total viable count (S-3) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	17	7	2

Table 6.5: Differential Exposure of Ethidium bromide and Total viable count (S-3) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	178	60	10

Table 6.6: Differential Exposure of Ethyl methane sulphonate (EMS) and Total viable count (S-3) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	94	76	4

Table 6.7: Differential Exposure of Ultra-violet radiation mutagen and Total viable count (S-3) observed on petri-plate

Control	Mean colony count of 1 min	Mean colony count of 3 min	Mean colony count of 5 min	Mean colony count of 7 min
More than 200	157	102	77	8

6.2.3.1a: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate S-3

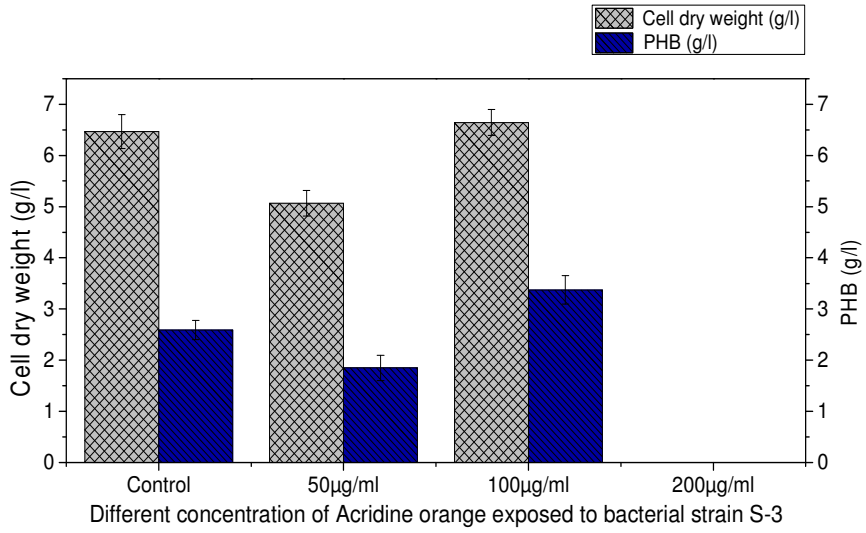


Figure 6.2.15: Cell dry weight (g/l) and PHB (g/l) of S-3 strain exposed to Acridine orange mutagen

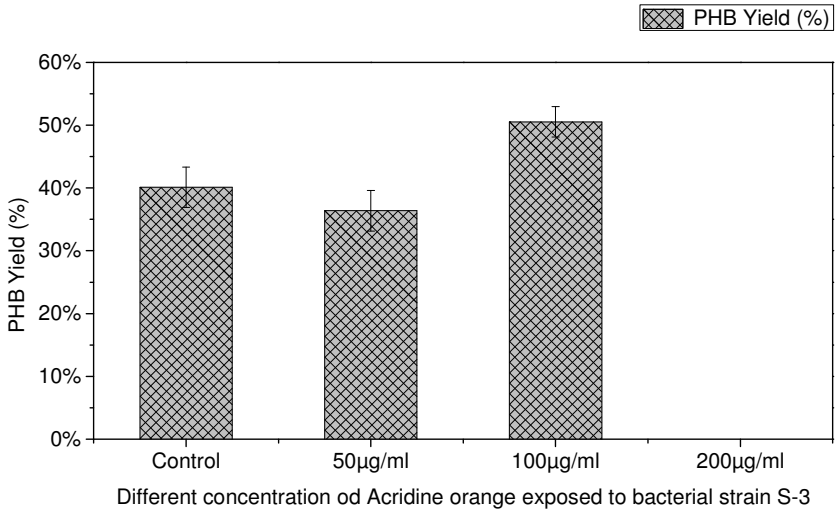


Figure 6.2.16: PHB Yield (%) of S-3 strain exposed to Acridine orange mutagen

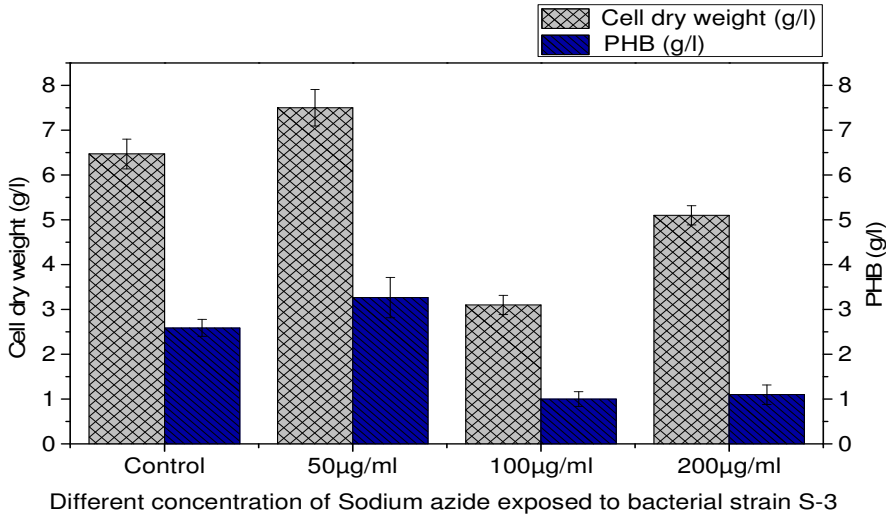


Figure 6.2.17: Cell dry weight (g/l) and PHB (g/l) of S-3 strain exposed to Sodium azide mutagen

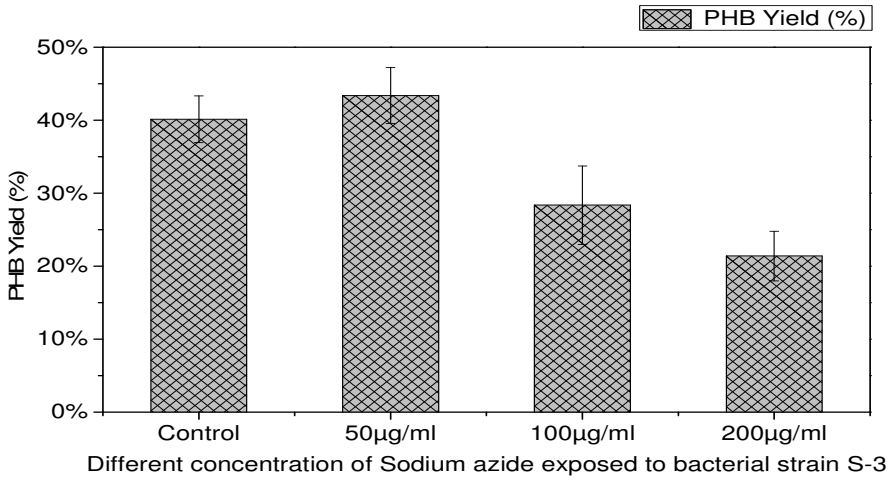


Figure 6.2.18: PHB Yield (%) of S-3 strain exposed to Sodium azide mutagen

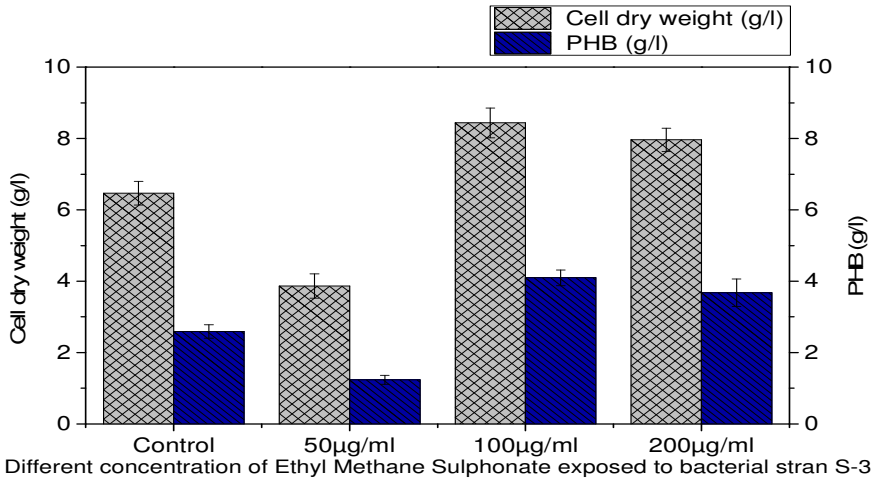


Figure 6.2.19: Cell dry weight (g/l) and PHB (g/l) of S-3 strain exposed to EMS mutagen

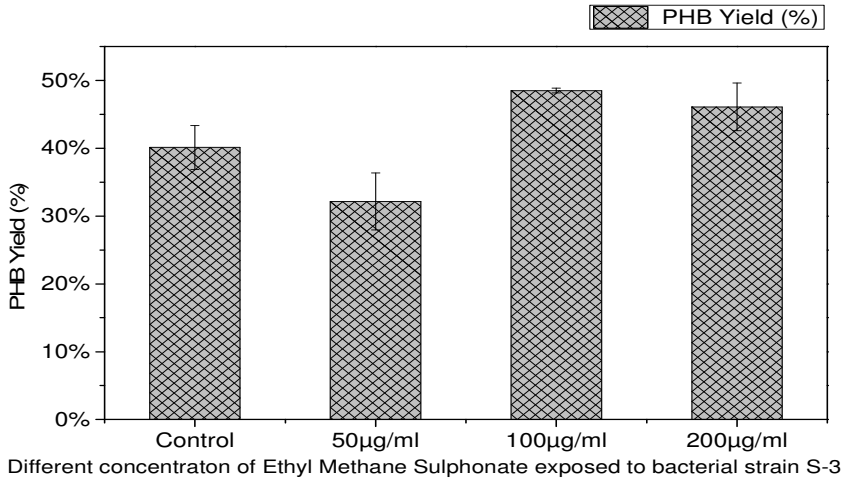


Figure 6.2.20: PHB Yield (%) of S-3 strain exposed to EMS mutagen

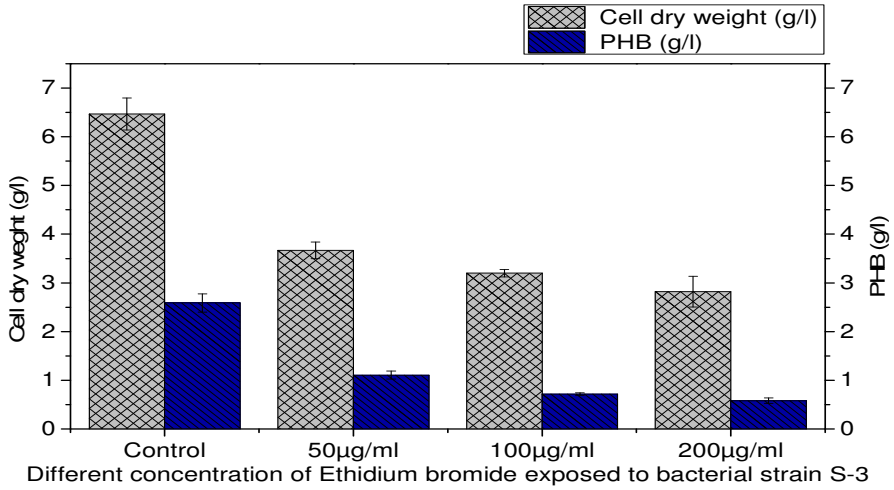


Figure 6.2.21: Cell dry weight (g/l) and PHB (g/l) of S-3 strain exposed to EtBr mutagen

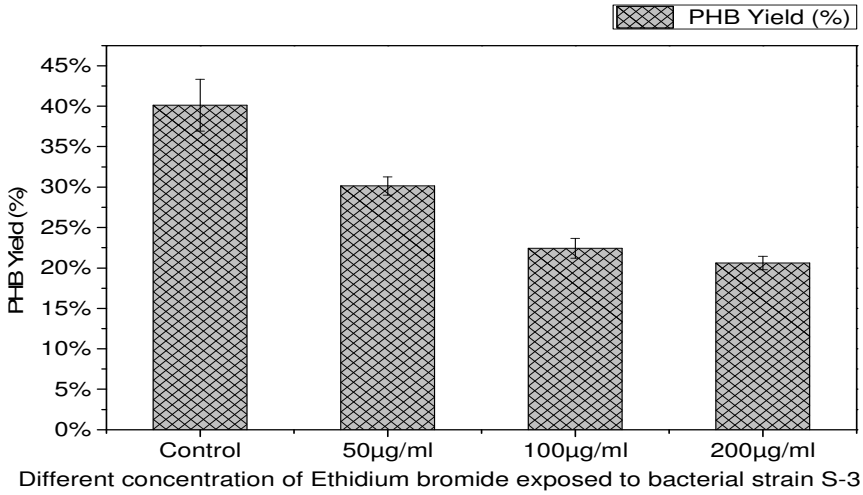


Figure 6.2.22: PHB Yield (%) of S-3 strain exposed to Ethidium bromide (EtBr) mutagen

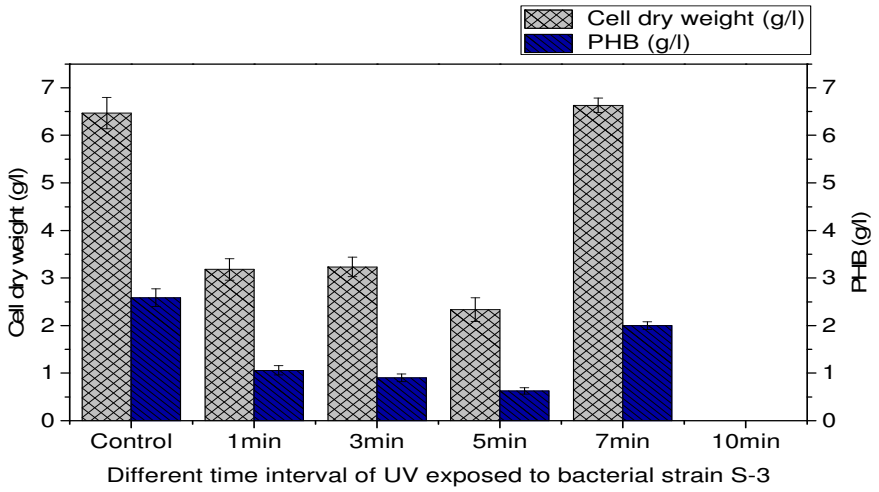


Figure 6.2.23: Cell dry weight (g/l) and PHB (g/l) of S-3 strain exposed to UV radiation

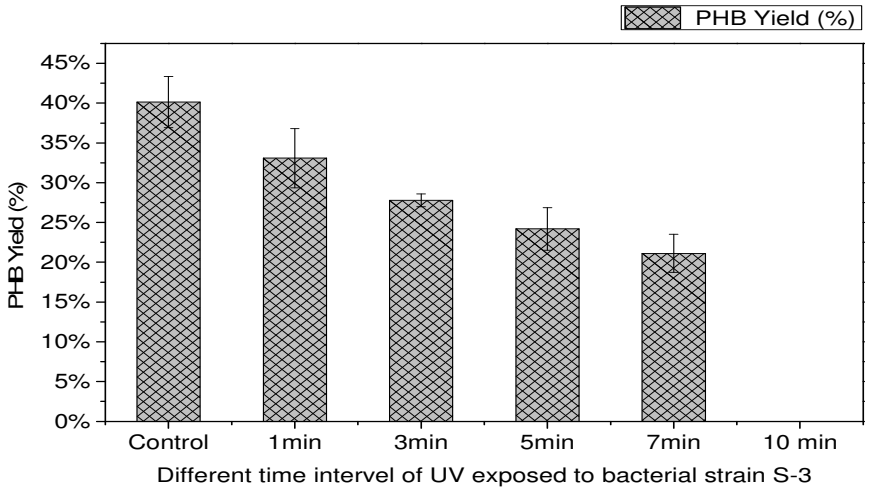


Figure 6.2.24: PHB Yield (%) of S-3 strain exposed to Ultra-violet (UV) radiation mutagen

6.2.3.1a (i): Observation of Mutagenesis Studies using different Mutagenic agents in isolate S-3

Mutagenic investigations were carried out with the above described agents to the isolate S-3. Isolate S3 initial PHB production was 40.13%, which increases to 50.53% with 100 µg/ml t of Acridine orange treatment. In case of control, cdw was 6.46 g/l and in 50 µg/ml it was 5.4 g/l. PHB accumulation was 2.588 g/l (control), 1.85 g/l (50 µg/ml) and 3.372 g/l in 100 µg/ml treatment of acridine orange. The highest PHB yield was observed in 100 µg/ml A.O concentrations treatment i.e. 50.53%, 36.38% in 50 µg/ml and 40.13% in control. From the overall observation, it was found that in terms of PHB yield 100 µg/ml was the best exposure for high PHB yield. The overall analysis shows the results that 100 µg/ml treated strain accumulated the PHB around 3.86 g/l. The main aim of the study was to get high PHB yield in g/l.

Sodium azide treatment at 50 µg/ml concentration yields PHB production level of 43.39%. In terms of cell dry weight, maximum cell mass was observed in case of 50 µg/ml (7.5 g/l) as compared to other exposed concentrations of sodium azide. Cell mass was less in case of 100 µg/ml (3.1 g/l), 200 µg/ml (5.1 g/l) and control (6.46 g/l) as compared to 50 µg/ml (7.5 g/l). PHB accumulation was 2.588 g/l (control), 1g/l (100 µg/ml) and 1.096 g/l (200 µg/ml). Maximum accumulation was observed in 50 µg/ml i.e. 3.266 g/l. The highest PHB yield was observed at 50 µg/ml sodium azide treatment i.e. 43.39%. The other concentrations were analysed for PHB yield and were 28.36% in 100 µg/ml, 21.37% in 200 µg/ml and 40.13% in control. From the overall observation, it was found that in terms of PHB yield 50 µg/ml was the best exposure concentration for high PHB yield.

Ethyl methane sulphonate (EMS) treatment at 100 µg/ml concentration yields PHB production level of 48.52%. In terms of cell dry weight, maximum cell mass was observed at 100 µg/ml (8.44 g/l) as compared to other exposed concentrations of EMS. Cell mass was less in case of 50 µg/ml (3.86 g/l), 200 µg/ml (7.96 g/l) and control (6.46 g/l). PHB accumulation was 2.588 g/l (control), 1.23 g/l (50 µg/ml), 4.09 g/l (100 µg/ml) and 3.67 g/l (200 µg/ml). The highest PHB yield was observed at 100 µg/ml sodium azide concentrations i.e. 48.52%. The others were 32.16% in 50 µg/ml, 46.11% in 100 µg/ml and 40.13% in control. From the overall observation, it was found that in terms of PHB yield 100 µg/ml was the best exposure treatment for high PHB yield i.e. 43.39%.

Ethidium bromide treated bacterial isolate S-3 at different concentration shows the maximum cdw in control i.e. 6.46 g/l. The high concentration of EtBr affects the cell growth rate. It was observed that cdw of 50 µg/ml was 3.66 g/l, 100 µg/ml was 3.196 g/l and 200 µg/ml was 2.82 g/l. The PHB accumulation also gets reduced by the effect of mutagenic agent on the stored bacterial lipid granules. The analysis shows that in control PHB accumulation was 2.588 g/l, which was maximum as compared to the mutagenic affected strains. The accumulation of PHB was 1.106 g/l in 50 µg/ml, 0.716 g/l in 100 µg/ml and 0.58 g/l in 200 µg/ml. The PHB yield was highest in control i.e. 40.13%. The other yield percentage was 30.15% in 50 µg/ml, 22.44% in 100 µg/ml and 20.61% in 200 µg/ml. The overall analysis shows that maximum PHB accumulated in control sample per litres of culture medium i.e. 2.588 g/l, which shows that in terms of PHB production, control strain was better among the other mutated strains.

For UV treatment PHB yield was 40.13% observed in case of control. In terms of cell dry weight, maximum cell mass was observed in case of control i.e. 6.46 g/l as compared to other UV exposed timing. Cell mass was reduced in case of 1 minute UV exposure i.e. (3.18 g/l), 3 min (3.23 g/l), 5 min (2.33 g/l) and 7 min (2.09 g/l). No growth was observed at 10 min exposure. The observation for PHB accumulation in terms of exposure time was 1.09 g/l (1 min), 0.9 g/l (3 min), 0.63 g/l (5 min) and 0.44 g/l (7 min). Maximum PHB accumulation was observed in control i.e. The highest PHB yield was observed at control, which was 40.13%. The other exposure yielded PHB of 33.08% in 1 min, 27.78% in 3 min, 24.19% in 5 min and 21.12% in 7 min UV exposure. From the overall observation, it was found that in terms of PHB yield control shows a high PHB yield. The results are in coherence with Adwitiya *et al.,* 2009.

6.2.3.2: Number of colonies in control & mutated samples after Exposure to different mutagenic agents [M1 bacterial isolate from Majha Region Punjab]

Table 6.8: Differential Exposure of Acridine orange and Total viable count (M1) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
67	39	17	-

Table 6.9: Differential Exposure of Ultra-violet radiation and Total viable count (M1) observed on petri-plate

Control	Mean colony count of 1 min	Mean colony count of 3 min	Mean colony count of 5 min	Mean colony count of 7 min	Mean colony count of 10 min
More than 200	56	28	15	6	2

6.2.3.2a: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate M1

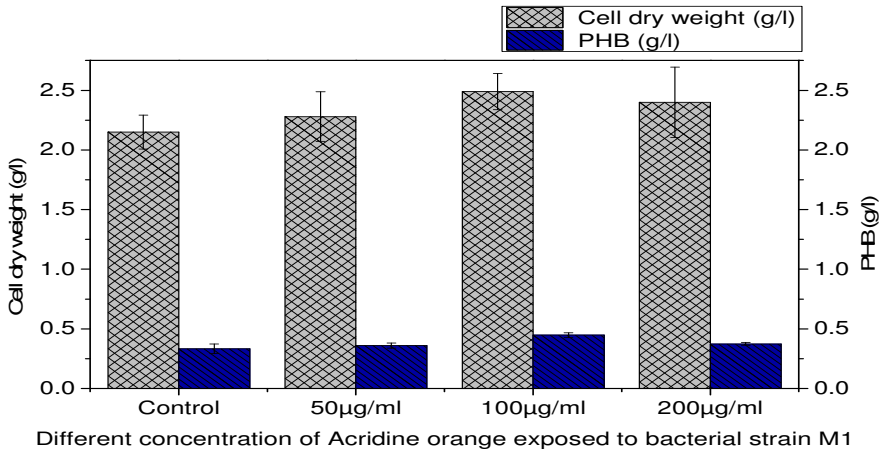


Figure 6.2.25: Cell dry weight (g/l) and PHB (g/l) of M1 strain exposed to Acridine orange mutagen

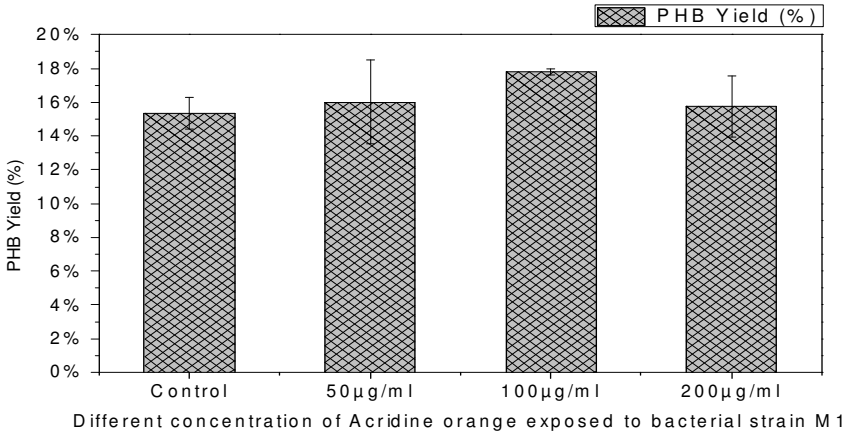


Figure 6.2.26: PHB Yield (%) of M1 strain exposed to Acridine orange mutagen

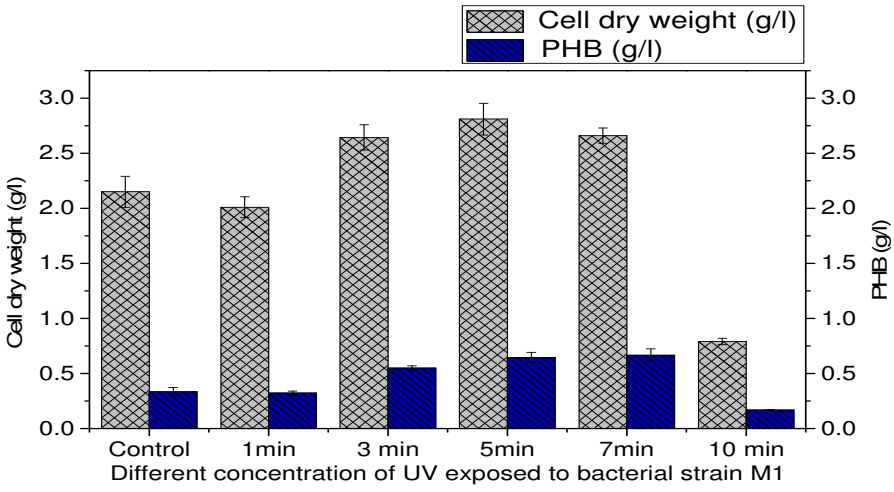


Figure 6.2.27: Cell dry weight (g/l) and PHB (g/l) of M1 strain exposed to UV radiation mutagen

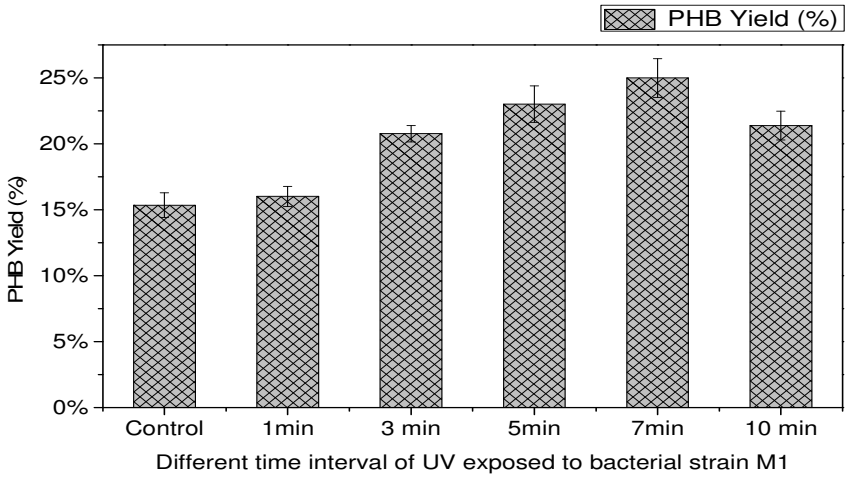


Figure 6.2.28: PHB Yield (%) of bacterial isolate M1 exposed to UV radiation mutagen

6.2.3.2a (i): Observation of Mutagenesis Studies using different Mutagenic agents in isolate M1

Mutagenic investigations were carried out with different agents on the isolate M1. Isolate M1 initial PHB production was 15.34%, which increases to 17.80% with 200 µg/ml of Acridine orange treatment.

It was observed that cdw was maximum in case of 100 µg/ml i.e. 2.49 g/l. The other cdw was 2.28 g/l in 50 µg/ml, 2.4 g/l in 200 µg/ml and 2.15 g/l in case of control. The PHB accumulation was highest in case of 100 µg/ml i.e. 0.446 g/l. The PHB accumulation in 50 µg/ml was 0.36 g/l, 200 µg/ml (0.373 g/l) and control (0.33 g/l). The PHB yield was highest in 100 µg/ml i.e. 17.80%. The PHB yield observed in other concentrations was 16.01% (50 µg/ml), 15.75% (200 µg/ml) and 15.34% (control). The overall analysis demonstrated that 100µg/ml exposure produced the best results in both aspects of PHB accumulation and PHB yield.

UV mutagenesis, 7 minutes exposure yields PHB at 24.99%. In terms of cell dry weight, maximum cell mass was observed in case of 7 min UV exposure i.e. 2.66 g/l as compared to other mutagen exposed strains. Cell mass was reduced in case of 1 minute UV exposure i.e. (2.01 g/l), 3 min (2.643 g/l), 5 min (2.81 g/l), 10 min (0.79 g/l) and control (2.15 g/l). PHB accumulation was maximum in 7 min UV exposure i.e. 0.6656 g/l. The observation shows that the PHB accumulation was 0.3216 g/l (1 min), 0.548 g/l (3 min), 0.646 g/l (5 min), 0.168 g/l (10 min) and 0.33 g/l (control). The highest PHB yield was observed at 7 min UV exposure, which was 24.99%. The other exposure yielded PHB of 16.01% in 1 min, 20.77%

in 3 min, 23.01% in 5 min, 21.39% in 10 min and 15.34% in control. From the overall observation, it was found that in terms of PHB yield 7 min UV exposed strain shows a high PHB yield i.e. 24.99% with high accumulation of PHB i.e. 0.646 g/l. The results are in coherence with Adwitiya *et al.,* 2009.

No effect of mutagenic agents was observed in the case of Ethidium bromide, Ethyl methane sulphonate (EMS) and sodium azide at different concentrations as no reduction in number of colonies was observed as compared with the control.

6.2.3.3: Number of colonies in control & mutated samples after Exposure to different mutagenic agents [M10 bacterial isolate]

Table 6.10: Differential Exposure of Acridine orange mutagen and Total viable count (M10) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	65	21	7

Table 6.11: Differential Exposure of Sodium azide mutagen and Total viable count (M10) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	44	16	8

Table 6.12: Differential Exposure of Ethidium bromide (EtBr) mutagen and Total viable count (M10) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	165	7	2

Table 6.13: Differential Exposure of Ethyl Methane Sulphonate (EMS) and Total viable count (M10) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	14	5	2

Table 6.14: Differential Exposure of Ultra-violet radiations (mins) and Total viable count (M10) observed on petri-plate

Control	Mean colony count of 1 min	Mean colony count of 3 min	Mean colony count of 5 min	Mean colony count of 7 min	Mean colony count of 10 min
More than 200	125	111	29	4	0

6.2.3.3a: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate M10

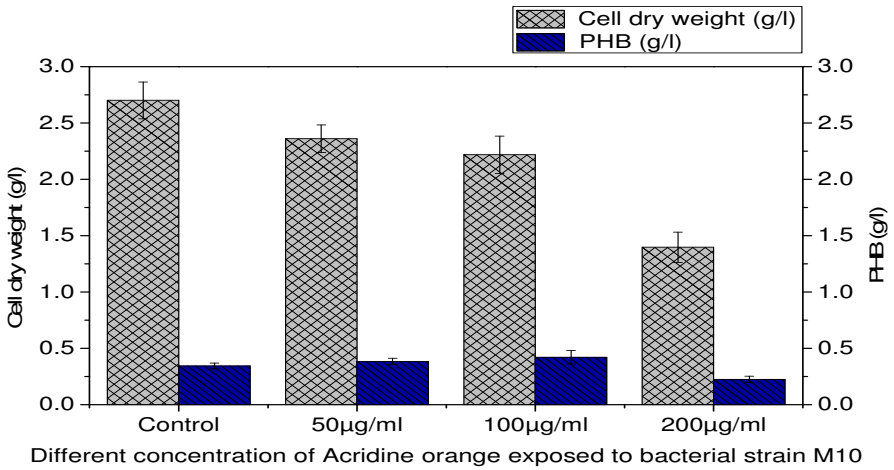


Figure 6.2.29: Cell dry weight (g/l) and PHB (g/l) of M10 strain exposed to Acridine orange mutagen

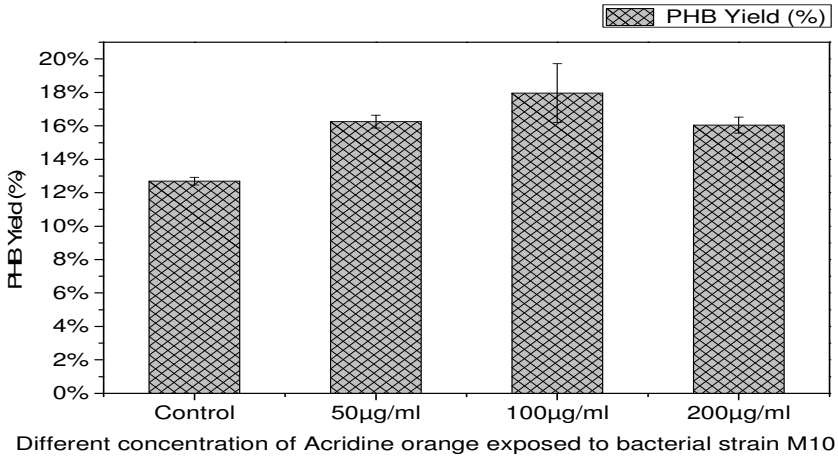


Figure 6.2.30: PHB Yield (%) of M10 strain exposed to Acridine orange mutagen

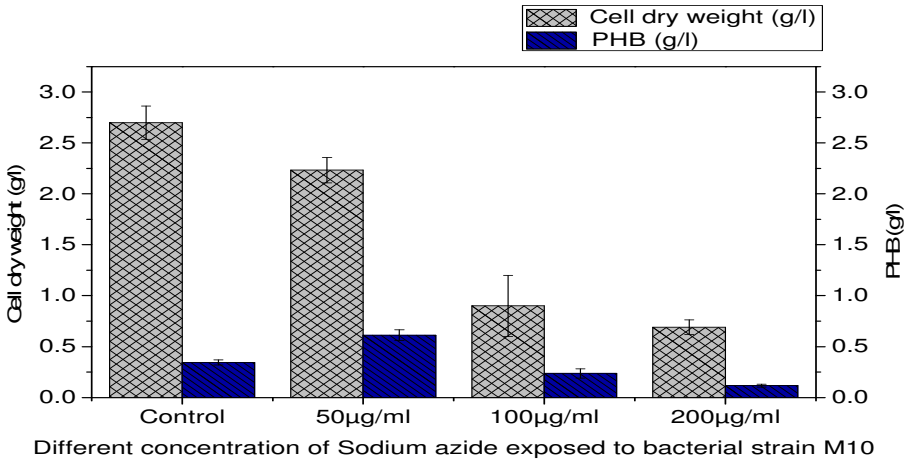


Figure 6.2.31: Cell dry weight (g/l) and PHB (g/l) of M10 strain exposed to Sodium azide mutagen

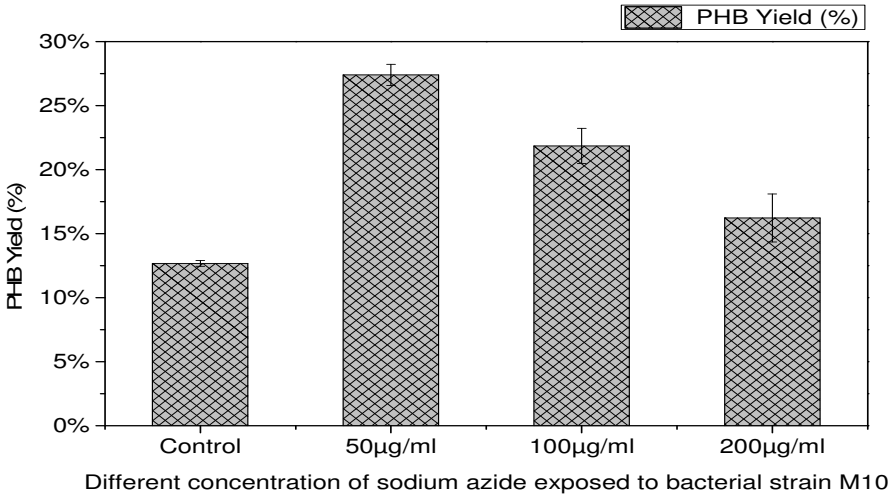


Figure 6.2.32: PHB Yield (%) of M10 strain exposed to Sodium azide mutagen

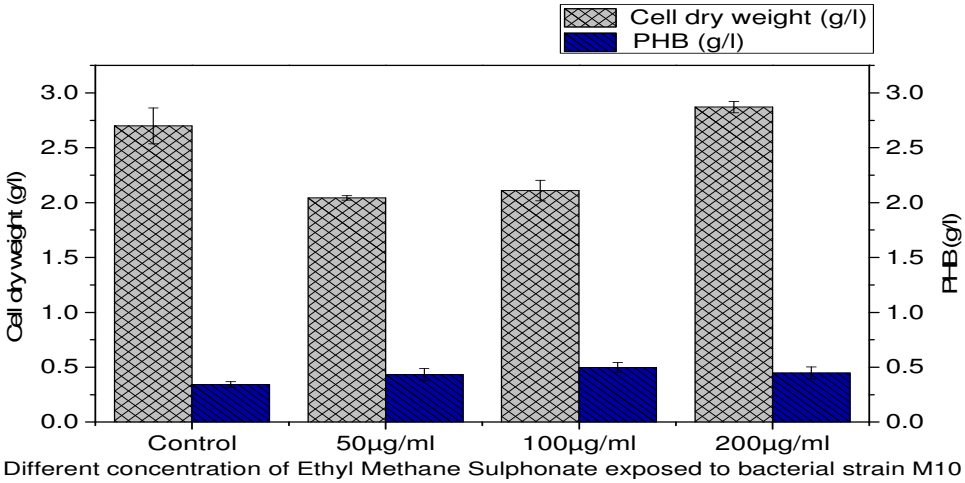


Figure 6.2.33: Cell dry weight (g/l) and PHB (g/l) of M10 strain exposed to EMS mutagen

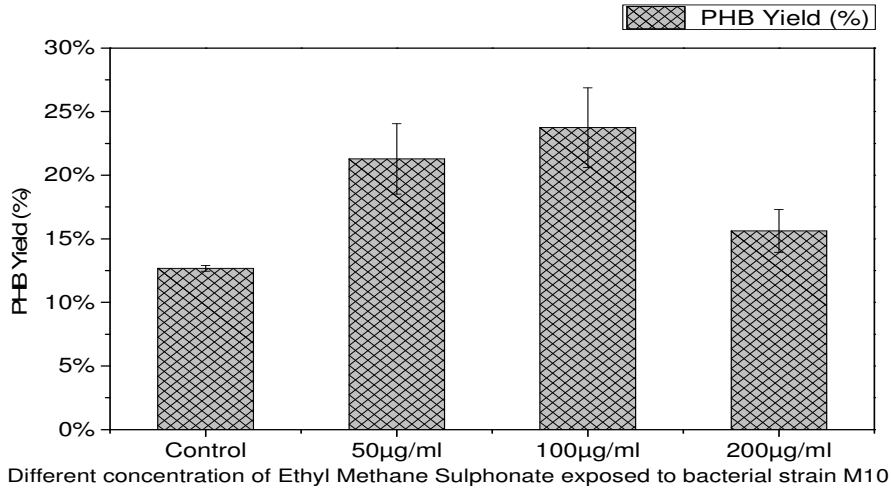


Figure 6.2.34: PHB Yield (%) of M10 strain exposed to EMS mutagen

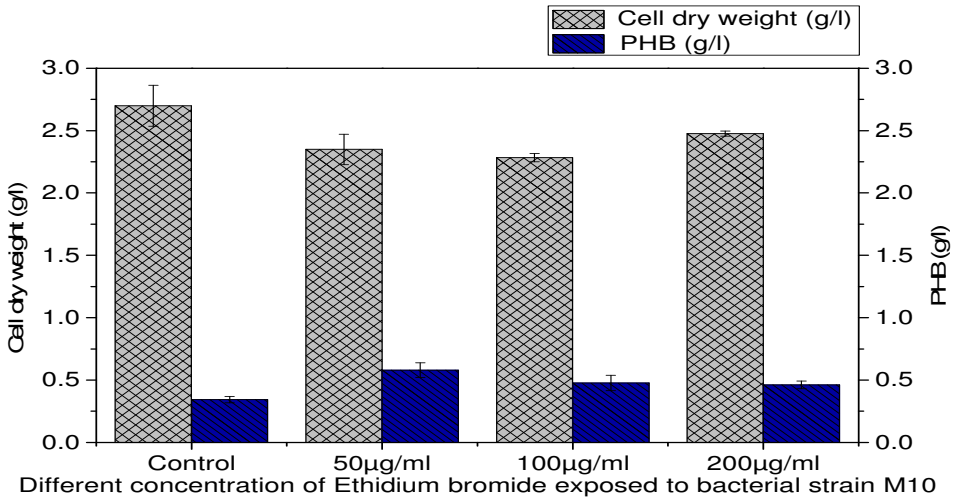


Figure 6.2.35: Cell dry weight (g/l) and PHB (g/l) of M10 strain exposed to EtBr mutagen

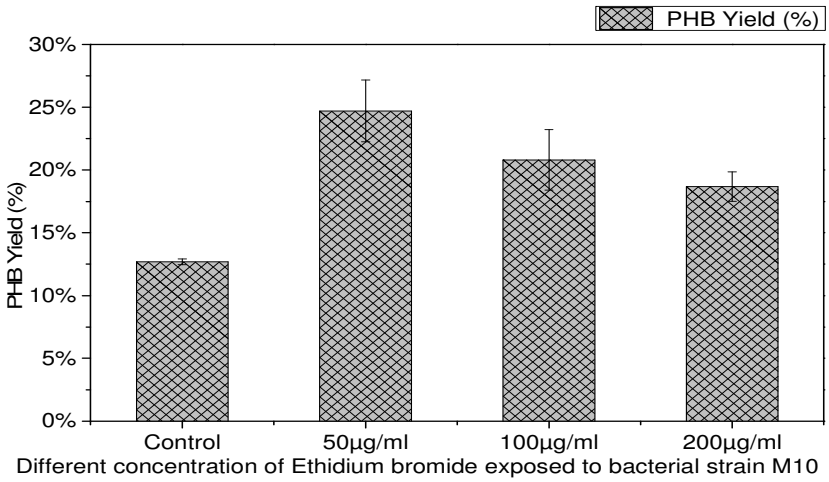


Figure 6.2.36: PHB Yield (%) of M10 strain exposed to EtBr mutagen

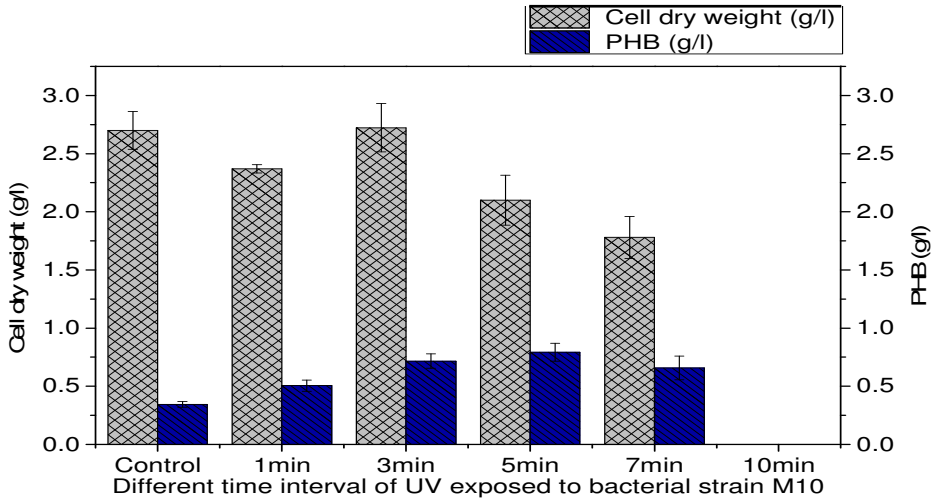


Figure 6.2.37: Cell dry weight (g/l) and PHB (g/l) of M10 strain exposed to UV radiation mutagen

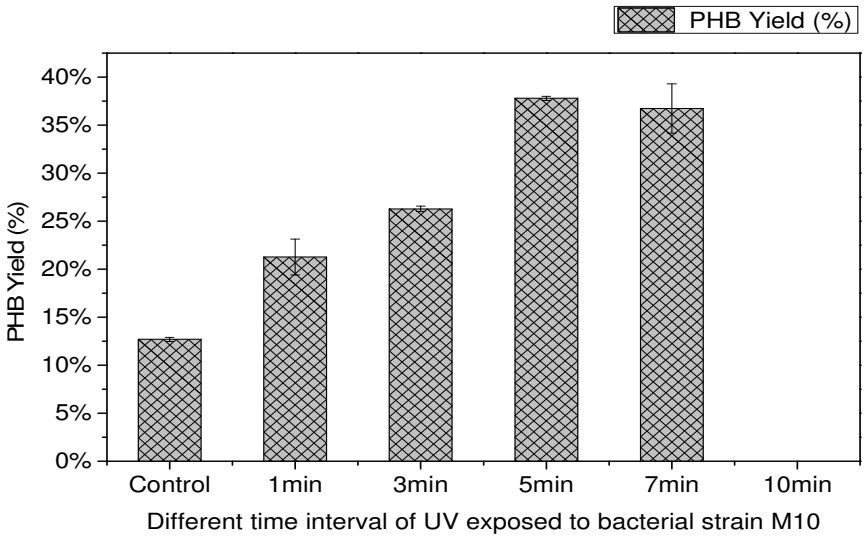


Figure 6.2.38: PHB Yield (%) of M10 strain exposed to Ultra-violet (UV) radiation mutagen

6.2.3.3a (i): Observation of Mutagenesis Studies using different Mutagenic agents in isolate M10

Mutagenic investigations were carried out with the different agents on the isolate M10. Isolate M10 initial PHB production was 12.69%, which increases to 18% with 100 µg/ml of Acridine orange treatment. In terms of cell dry weight, maximum cell mass was observed in case of 50 µg/ml (2.36 g/l) as compared to control. Cell mass was 200 µg/ml (1.39 g/l) and 100 µg/ml 2.216 g/l, whereas cdw in control was 2.7 g/l. Maximum PHB accumulation was 0.42 g/l in 100 µg/ml. PHB accumulation in other treatment was 0.343 g/l (control), 0.384 g/l (50 µg/ml) and 0.224 g/l (200 µg/ml). The highest PHB yield was observed in 100 µg/ml A.O concentrations. The other exposed bacterial strain yield PHB 12.69% in control, 16% in 50 µg/ml and 16% in 200 µg/ml treatment. From the overall observation, it was found that in terms of PHB yield 100 µg/ml was the best exposed concentration for high yield. The main aim of the study was to get high PHB g/l, which was achieved in 100 µg/ml of Acridine orange treatment.

Sodium azide treatment at 50 µg/ml concentration yields PHB production level of 27%. In terms of cell dry weight, maximum cell mass was observed in case of control (2.7 g/l) as compared to other exposed concentrations of sodium azide treatment. Cell mass was reduced in case of 50 µg/ml (2.23 g/l), 100 µg/ml (0.9 g/l) and 200 µg/ml (0.69 g/l). PHB accumulation was 0.343 g/l (control), 0.236 g/l (100 µg/ml) and 0.116 g/l (200µg/ml). Maximum accumulation was observed in 50 µg/ml i.e. 0.612 g/l. The highest PHB yield was observed at 50 µg/ml sodium azide concentrations i.e. 27%. The other concentrations were analysed for PHB yield and were 22% in 100 µg/ml, 16% in 200 µg/ml and 12.69% in control. From the overall observation, it was found that in terms of PHB yield and accumulation 50 µg/ml was the best exposed concentration.

Ethyl methane sulphonate (EMS) treatment at 100 µg/ml concentration yields PHB production level of 23.74%. In terms of cell dry weight, maximum cell mass was observed in case of 200 µg/ml (2.87 g/l) as compared to other exposed concentrations of EMS. Cell mass was reduced in case of 50 µg/ml (2.04 g/l), 100 µg/ml (2.11 g/l) and control (2.7 g/l). PHB accumulation was 0.343 g/l (control), 0.434 g/l (50 µg/ml) and 0.448 g/l (200 µg/ml). Maximum PHB accumulation was observed in 100 µg/ml treatment i.e. 0.498 g/l. The highest PHB yield was observed at 100 µg/ml sodium azide concentrations i.e. 23.74%. The

others were 21.28% in 50 µg/ml, 15.62% in 200 µg/ml and 12.69% in control. From the overall observation, it was found that in terms of PHB yield 100 µg/ml was the best exposed concentration for high PHB yield i.e. 23.74%

Ethidium bromide treated bacterial isolate M10 at different concentration shows the maximum cdw at control i.e. 2.7 g/l. It was observed that cdw at 50 µg/ml was 2.35 g/l, 100 µg/ml was 2.283 g/l and 200 µg/ml was 2.47 g/l. The analysis shows that in 50 µg/ml PHB accumulation was 0.579 g/l, which was maximum as compared to all mutagenic affected strains. The accumulation of PHB was 0.461 g/l in 200 µg/ml, 0.475 g/l in 100 µg/ml and 0.343 g/l in control. The PHB yield was highest in 50 µg/ml concentration of EtBtr i.e. 24.70%. The other yield percentage was 18.68% in 200 µg/ml, 20.81% in 100 µg/ml and 12.96% in control. The overall analysis shows that maximum PHB accumulated in 50 µg/ml exposed concentration i.e. 0.579 g/l.

The PHB yield was 37.80% observed in case of 5 min UV exposure. Cell mass was reduced in case of 1 min UV exposure i.e. (2.37 g/l), 3 min (2.723 g/l), 5 min (2.1 g/l) and 7 min (1.78 g/l). No Colony was observed in 10 min UV exposure. PHB accumulation was maximum in 5 min UV exposure i.e. 0.7933 g/l. The observation shows that the PHB accumulation was 0.5043 g/l (1 min), 0.7166 g/l (3 min), 0.656 g/l (7 min) and 0.343 g/l (control). The highest PHB yield was observed at 5 min UV exposure, which was 37.80%. The other exposure yielded PHB of 21.26% in 1 min, 26.29% in 3 min, 36.74% in 7 min and 12.69% in control. From the overall observation, it was found that in terms of PHB yield and PHB accumulation 5 min UV exposed strain shows a high PHB yield i.e. 37.80% and PHB accumulation i.e. 0.7933 g/l. The results are in coherence with Adwitiya *et al.,* 2009.

6.2.3.4: Number of colonies in control & mutated samples after Exposure to different mutagenic agents [MD1 bacterial isolate from Doaba Region Punjab]

Table 6.15: Differential Exposure of Acridine orange and Total viable count (MD1) observed on petri-plate observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
43	16	10	3

Table 6.16: Differential Exposure of Sodium azide and Total viable count (MD1)

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
43	12	2	1

6.2.3.4a: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate MD1

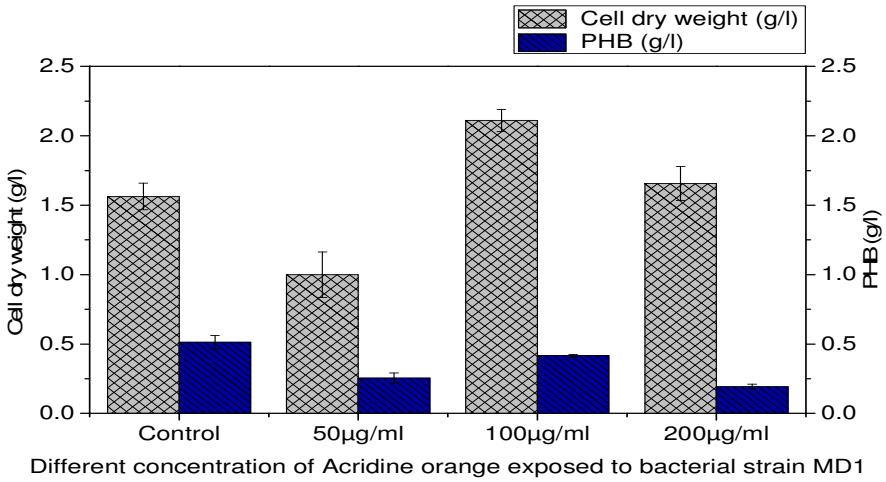


Figure 6.2.39: Cell dry weight (g/l) and PHB (g/l) of MD1 strain exposed to Acridine orange mutagen

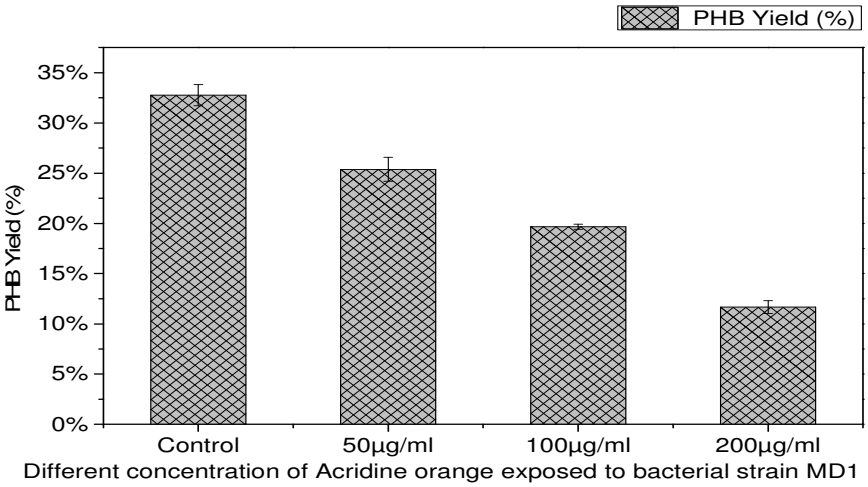


Figure 6.2.40: PHB Yield (%) of MD1 strain exposed to Acridine orange mutagen

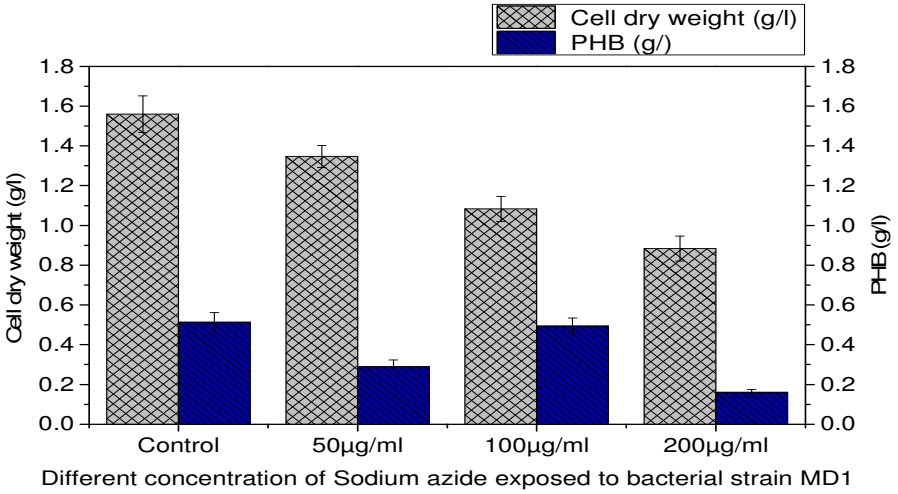


Figure 6.2.41: Cell dry weight (g/l) and PHB (g/l) of MD1 strain exposed to Sodium azide mutagen

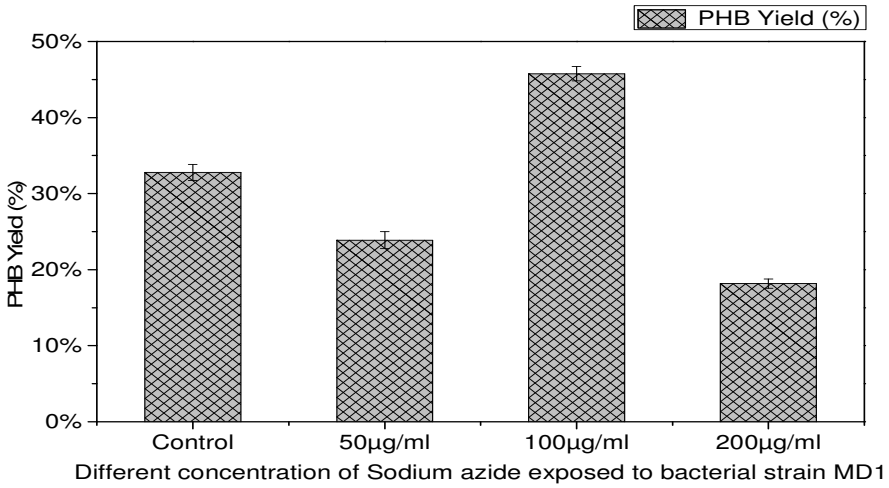


Figure 6.2.42: PHB Yield (%) of MD1 strain exposed to Sodium azide mutagen

6.2.3.4a (i): Observation of Mutagenesis Studies using different Mutagenic agents in isolate MD1

Mutagenic investigations were carried out with the different agents on the isolate MD1. Isolate MD1 initial PHB production was 32.77%, which was highest among all the exposed strain at different concentration of Acridine orange. It was observed that cdw was maximum in case of 100 µg/ml i.e. 2.11 g/l. The other cdw was 1.656 g/l in 200 µg/ml, 1.0 g/l in 50 µg/ml and 1.563 g/l in case of control. The PHB accumulation was highest in case of control i.e. 0.513 g/l. The PHB accumulation in 50 µg/ml was 0.253 g/l, 100 µg/ml (0.4148 g/l) and 200 µg/ml (0.193 g/l). The PHB yield was highest in control i.e. 32.77%. The PHB yield observed in other concentrations was 25.39% (50 µg/ml), 19.67% (100 µg/ml) and 11.67% (200 µg/ml). The overall analysis shows that the control was better in both aspect of PHB

accumulation and PHB yield. Negative effect of Acridine orange was observed, when exposed to MD1 strain.

Sodium Azide treatment analysis shows that there was an increment in PHB yield from 32.77% to 45.77% with 100 µg/ml treatment of sodium azide. Maximum cdw was observed in control i.e. 1.563 g/l. The cdw of other exposed strain was 1.346 g/l (50 µg/ml), 1.083 g/l (100 µg/ml) and 0.883 g/l (200 µg/ml). The PHB accumulation was maximum observed at control i.e.0.5133 g/l. PHB accumulation was also analysed in other concentration, which was 0.2889 g/l (50 µg/ml), 0.1605 g/l (200 µg/ml), and 0.494 g/l (100 µg/ml). PHB yield was 32.77% in control, 23.89% in 50 µg/ml and 18.16% in 200 µg/ml. Maximum yield was observed in 100 µg/ml i.e. 45.77%. The above investigation provides conclusive evidence that Sodium Azide treatment at 100 µg/ml produced high PHB yield of 45.77%. But in terms of PHB accumulation, best was observed in control i.e. 0.513 g/l.

No effect of mutagenic agents was observed in the case of Ethidium bromide, Ethyl methane sulphonate (EMS) and UV treatment at different concentrations as no reduction in number of colonies was observed as compared with the control.

Mutagenesis effect in isolate MD2 and MD5 was not observed as no decrease in the number of colonies were observed when treated with different mutagenic agents at different concentrations.

6.2.3.5: Number of colonies in control & mutated samples after Exposure to different mutagenic agents [AM6 bacterial isolate from Salty Sambhar Lake]

Table 6.17: Differential Exposure of Acridine orange mutagen and Total viable count (AM6) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	42	7	2

Table 6.18: Differential Exposure of Sodium azide mutagen and Total viable count (AM6) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	12	8	0

Table 6.19: Differential Exposure of Ultra-violet radiation mutagen and Total viable count (AM6) observed on petri-plate

Control	Mean colony count of 1 min	Mean colony count of 3 min	Mean colony count of 5 min	Mean colony count of 7 min
More than 200	28	5	4	0

6.2.3.5a: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate AM6

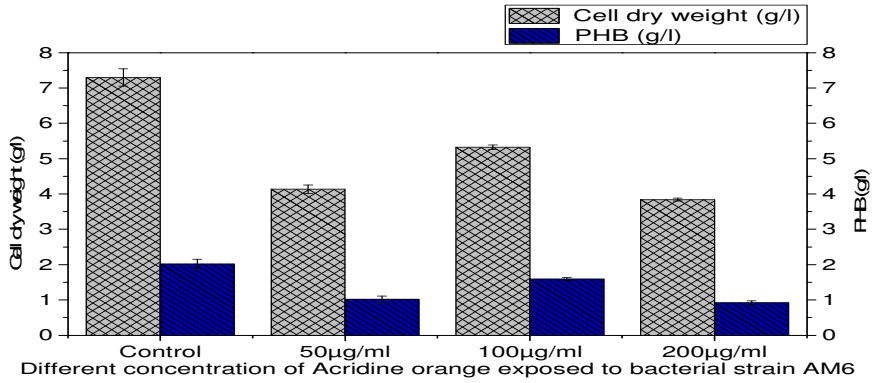


Figure 6.2.43: Cell dry weight (g/l) and PHB (g/l) of AM6 strain exposed to Acridine orange mutagen

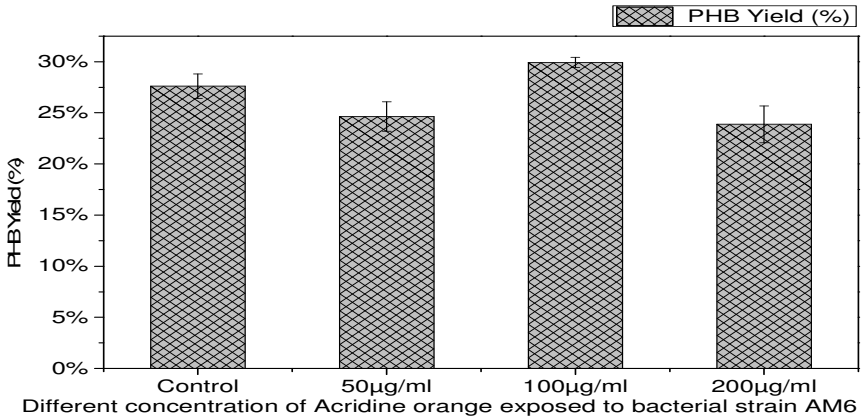


Figure 6.2.44: PHB Yield (%) of AM6 strain exposed to Acridine orange mutagen

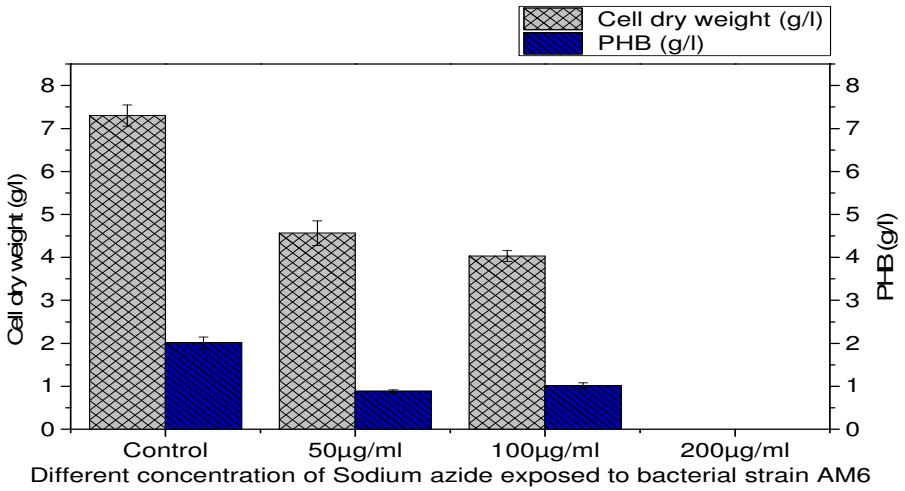


Figure 6.2.45: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate AM6 exposed to Sodium azide mutagen

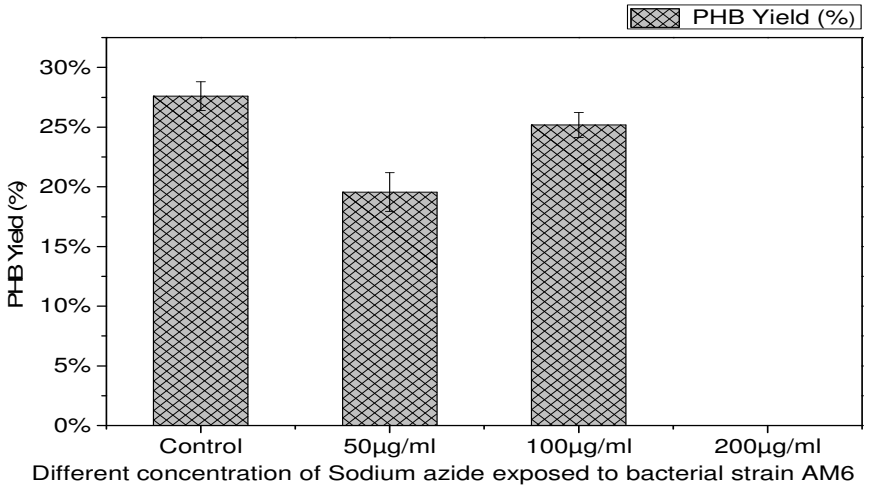


Figure 6.2.46: PHB Yield (%) of AM6 strain exposed to Sodium azide mutagen

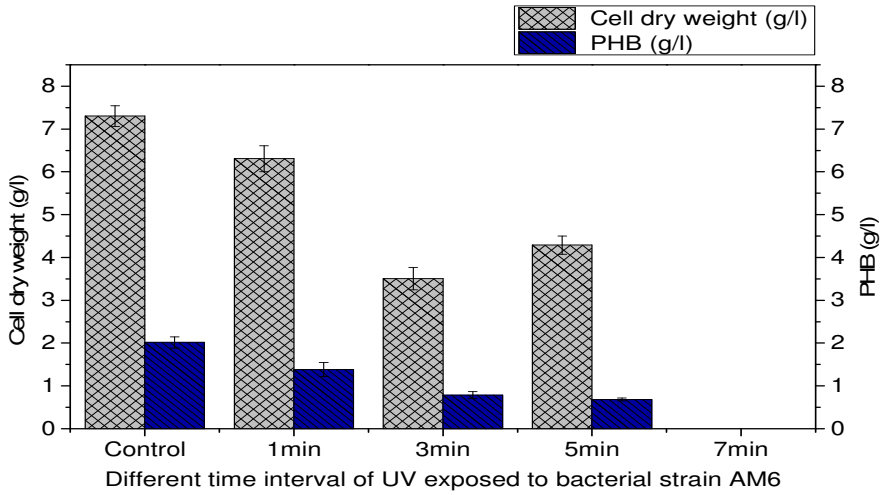


Figure 6.2.47: Cell dry weight (g/l) and PHB (g/l) of AM6 strain exposed to Ultra-violet radiation mutagen

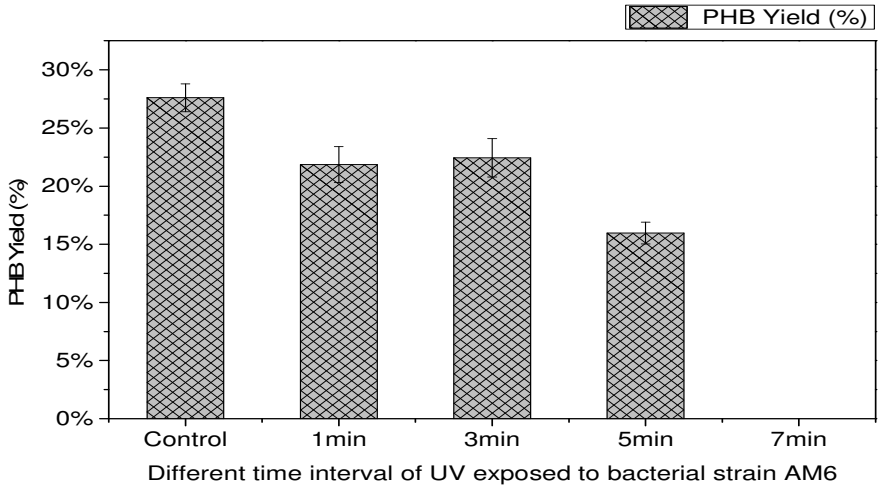


Figure 6.2.48: PHB Yield (%) of AM6 strain exposed to Ultra-violet radiation mutagen

6.2.3.5a(i): Observation of Mutagenesis Studies using different Mutagenic agents in isolate AM6

Isolate AM6 initial PHB production was 27.60% in control without A.O. treatment. In terms of cell dry weight, maximum cell mass was observed in case of control (7.3 g/l). Cell mass was reduced in case of 50 µg/ml (4.13 g/l), 100 µg/ml (5.323 g/l) and 200 µg/ml (3.84 g/l). PHB accumulation was 2.01 g/l (control), 1.02 g/l (50 µg/ml), 1.593 g/l (100 µg/ml) and 0.996 g/l (200 µg/ml). The highest PHB yield was observed in 100 µg/ml A.O concentrations i.e. 29.92%, 23.88% in 200 µg/ml, 24.63% in 50 µg/ml and 27.60% in control. From the overall observation, it was found that in terms of PHB yield 100 µg/ml was the best exposure of high PHB yield. The overall highest PHB accumulation was observed in control.

In Sodium azide treatment maximum PHB yield was observed in control 27.60%. In terms of cell dry weight, maximum cell mass was observed in case of control (7.3 g/l) as compared to other exposed concentrations of sodium azide. Cell mass was reduced in case of 50 µg/ml (4.56 g/l), 100 µg/ml (4.03 g/l) and in 200 µg/ml treatment no growth was observed. PHB accumulation was 2.01 g/l (control), 0.889 g/l (50 µg/ml) and 1.01 g/l (100 µg/ml). The highest PHB yield was observed in control 27.60%. The others were 19.57% in 50 µg/ml and 25.16% in 100 µg/ml treatment.

In UV exposure, maximum cell mass was observed in case of control (7.3 g/l) as compared to other mutagen exposures. Cell mass was reduced in case of 1 minute UV exposure i.e. (6.306 g/l), 3 min (3.506 g/l), 5 min (4.286 g/l) and in 7 min no growth was observed. PHB accumulation was 2.01 g/l (control), 1.383 g/l (1 min), 0.786 g/l (3 min) and 0.682 g/l in 5

min. The highest PHB yield was observed at control, which was 27.60%. The other exposure yielded PHB of 21.86% in 1 min, 22.43% in 3 min and 15.97% in 5 min. The overall analysis optimized the results that control strain accumulated the PHB of around 2.01 g/l., which was higher than the other exposed concentration. The results are in coherence with Adwitiya *et al.,* 2009. No effect of mutagenic agents was observed in the case of Ethidium bromide and Ethyl methane sulphonate (EMS) treatment at different concentrations as no reduction in number of colonies was observed as compared with the control.

6.2.3.6: Number of colonies in control & mutated samples after Exposure to different mutagenic agents [AM9 bacterial isolate from Saltren Sambhar Lake]

Table 6.20: Differential Exposure of Acridine orange mutagen and Total viable count (AM9)

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	64	15	1

Table 6.21: Differential Exposure of Sodium azide mutagen and Total viable count (AM9)

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	26	12	4

Table 6.22: Differential Exposure of Ethidium bromide (EtBr) mutagen and Total viable count (AM9)

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	10	-	-

Table 6.23: Differential Exposure of Ethyl Methane Sulphonate (EMS) mutagen and Total viable count (AM9)

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	33	16	5

Table 6.24: Differential Exposure of Ultra-violet radiation mutagen and Total viable count (AM9)

Control	Mean colony count of 1 min	Mean colony count of 3 min	Mean colony count of 5 min	Mean colony count of 7 min	Mean colony count of 10 min
More than 200	196	124	44	14	0

6.2.3.6a. Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate AM9

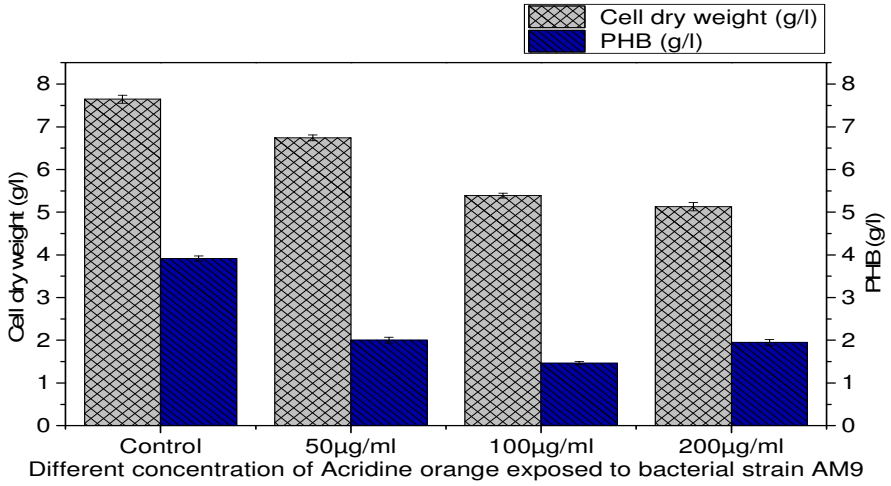


Figure 6.2.49: Cell dry weight (g/l) and PHB (g/l) of AM9 strain exposed to Acridine orange mutagen

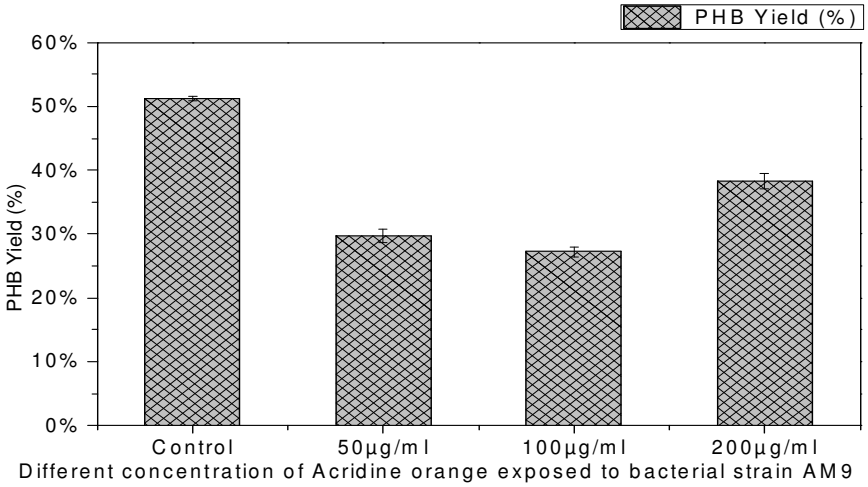


Figure 6.2.50: PHB Yield (%) of AM9 strain exposed to Acridine orange mutagen

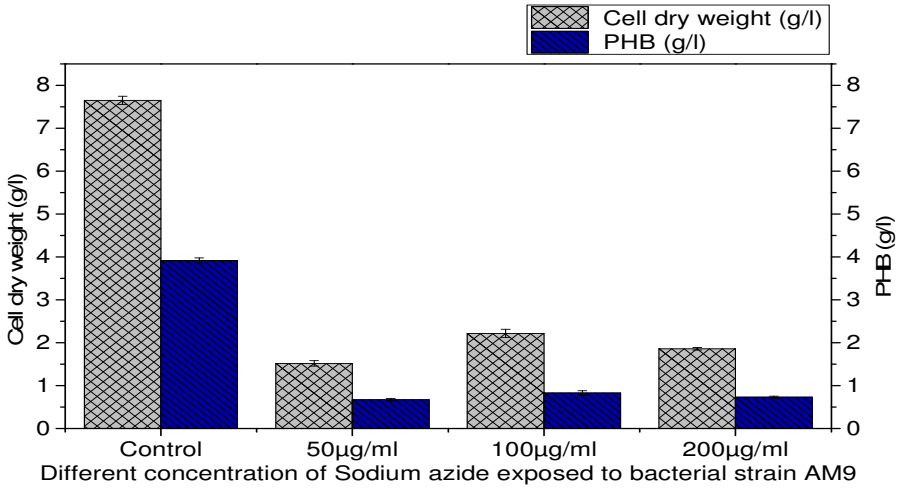


Figure 6.2.51: Cell dry weight (g/l) and PHB (g/l) of AM9 strain exposed to Sodium azide mutagen

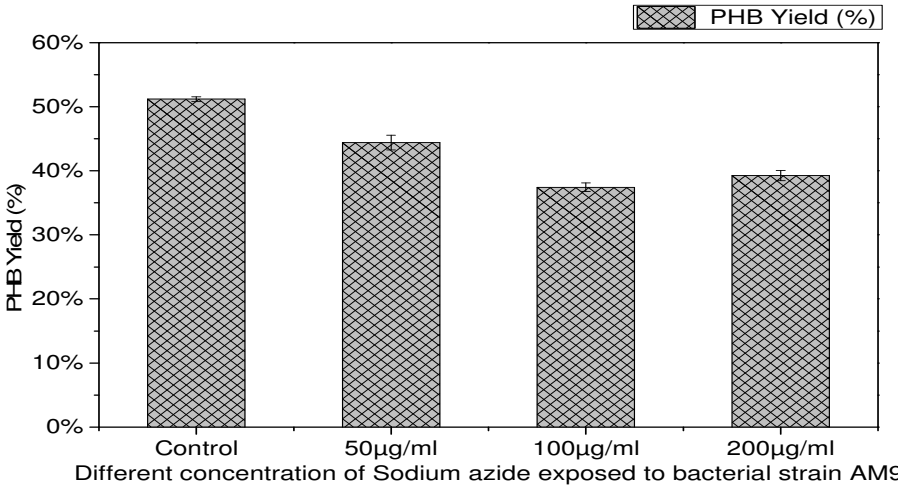


Figure 6.2.52: PHB Yield (%) of AM9 strain exposed to Sodium azide mutagen

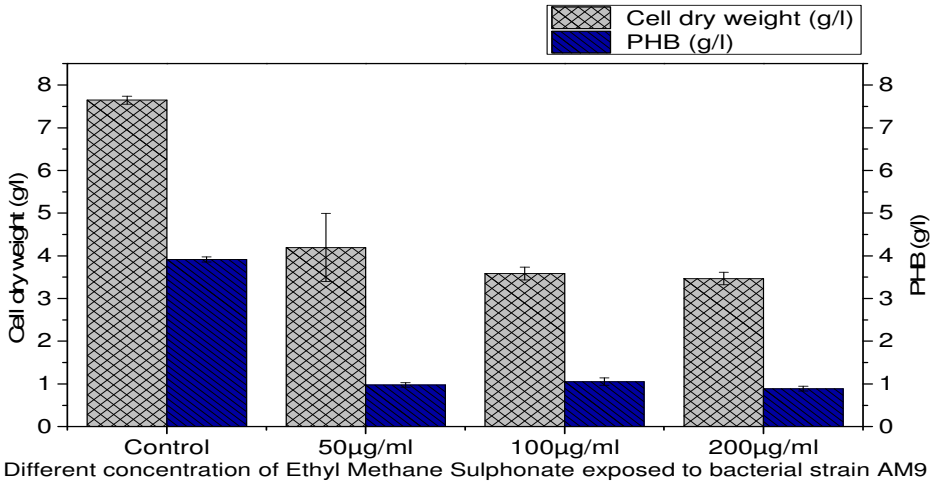


Figure 6.2.53: Cell dry weight (g/l) and PHB (g/l) of AM9 strain exposed to EMS mutagen

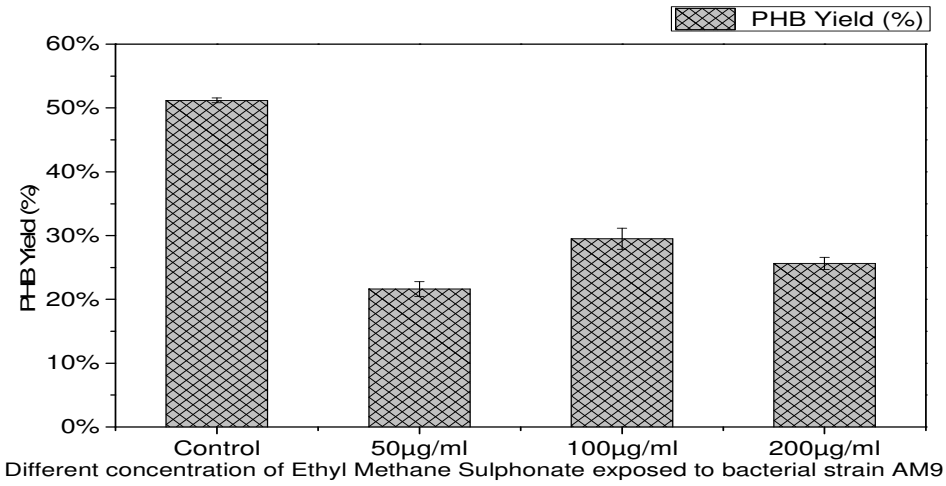


Figure 6.2.54: PHB Yield (%) of bacterial isolate AM9 exposed to EMS mutagen

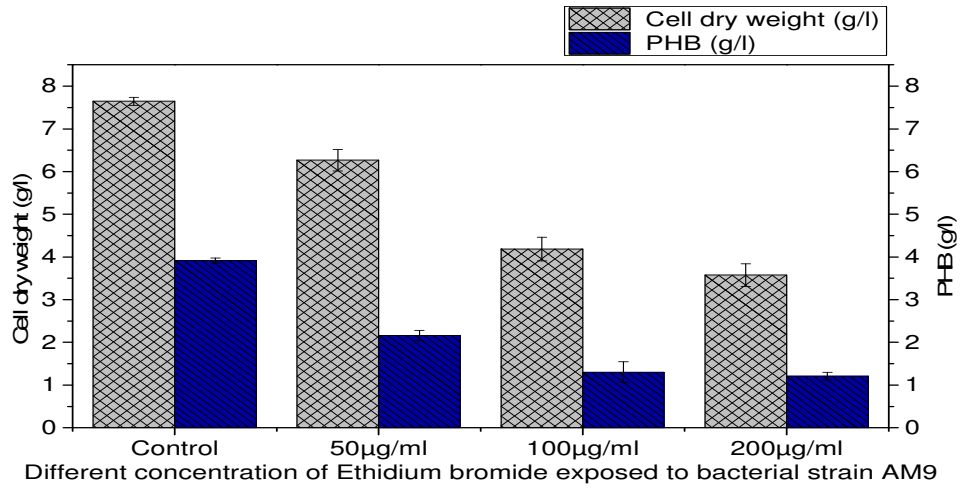


Figure 6.2.55: Cell dry weight (g/l) and PHB (g/l) of AM9 strain exposed to EtBr mutagen

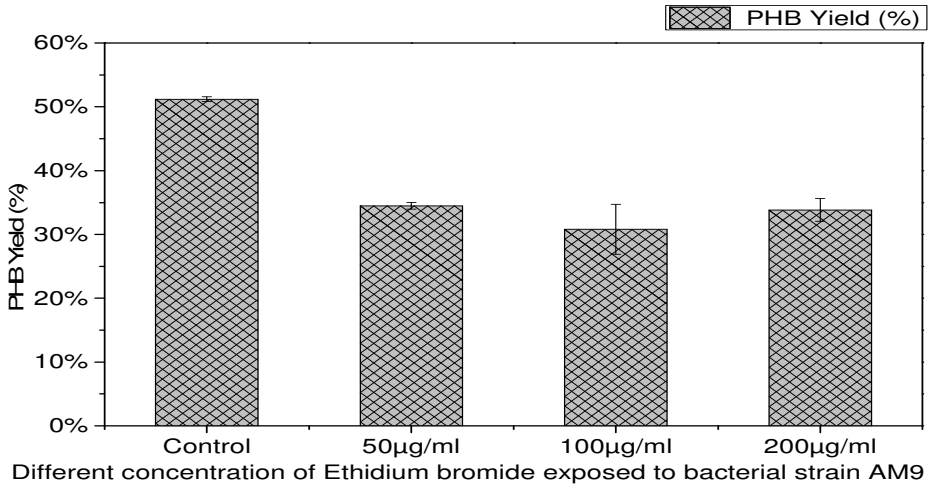


Figure 6.2.56: PHB Yield (%) of AM9 strain exposed to EtBr mutagen

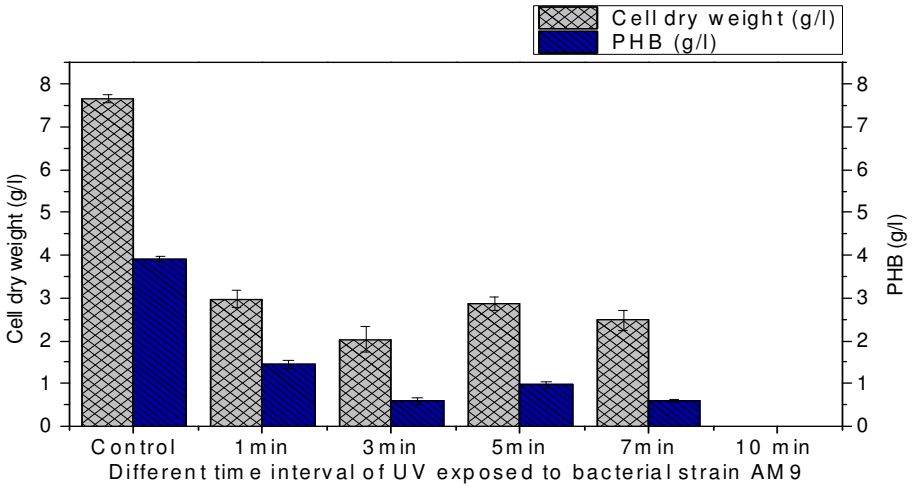


Figure 6.2.57: Cell dry weight (g/l) and PHB (g/l) of AM9 strain exposed to UV radiation mutagen

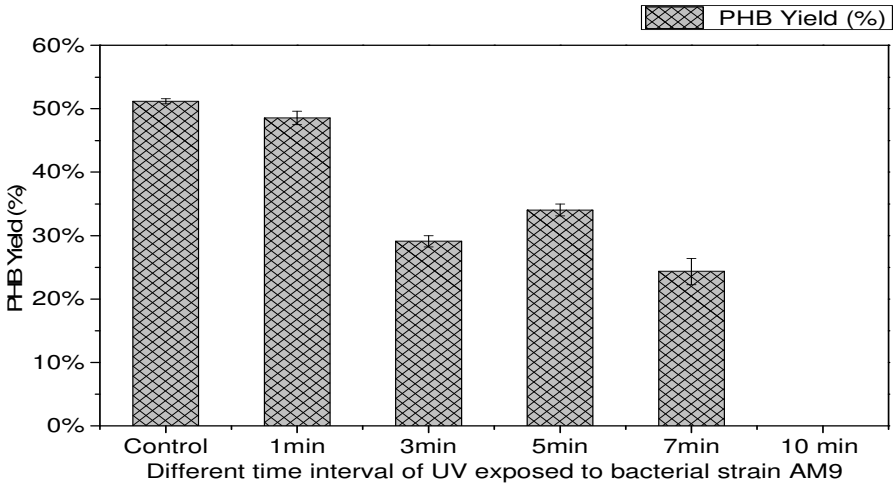


Figure 6.2.58: PHB Yield (%) of bacterial isolate AM9 exposed to UV radiation mutagen

6.2.3.6a(i): Observation of Mutagenesis Studies using different Mutagenic agents in isolate AM9

Mutagenic investigations were carried out with different agents to isolate AM9. Isolate AM9 initial PHB production was 51.19%. Negative effect of mutagenic agents was observed in the case of Acridine orange, Sodium Azide, Ethyl methane sulphonate, Ethidium bromide and UV treatment at different concentrations as reduction in PHB Yield was observed as compared with the control.

Initially without the treatment of Acridine orange, cdw for control was 7.646 g/l, PHB accumulation was 3.916 g/l and PHB yield was 51.19%. The effect of Acridine orange mutagen on cell dry weight shows that there was decrease in the cell mass density as the

concentration of mutagenic agent increases. It was observed that 50 µg/ml accumulated PHB of 2.004 g/l in cdw of 6.743 g/l, 100 µg/ml (1.463 g/l) in cdw of 5.39 g/l, 200 µg/ml (1.948 g/l) in cdw 5.126 g/l. The PHB yield was also decreased i.e. 50 µg/ml (29.74%), 100 µg/ml (27.20%) and 200 µg/ml (38%).

In case of sodium azide mutagenesis, as compared to control isolate AM9, it was observed that there was no increase in cdw, when treated with different concentrations of Sodium azide i.e 50 µg/ml, 100 µg/ml and 200 µg/ml. It was analyzed that the cell dry weight in 50 µg/ml of sodium azide concentration treatment was 1.516 g/l, 100 µg/ml (2.216 g/l) and 200 µg/ml (1.86 g/l), where as PHB accumulation was 50 µg/ml (0.673 g/l), 100 µg/ml (0.83 g/l) and 200 µg/ml (0.7302 g/l) and PHB yield was 44.04% (50 µg/ml), 37.41% (100 µg/ml) and 39.25% (200 µg/ml). The analysis shows that negative effect was observed in sodium azide treatment both in terms of cell dry weight and PHB accumulation.

The treatment of ethyl methane sulphonate (EMS) shows that there was no increase in cell dry weight of 50, 100 and 200 µg/ml concentration of EMS, which was observed to be 4.193 g/l, 3.586 g/l and 3.47 g/l. Reduction in PHB accumulation was observed in all the treated concentration i.e. 0.973 g/l (50 µg/ml), 1.052 g/l (100 µg/ml), 0.8899 g/l (200 µg/ml) and 3.91g/l (control). The negative effect was also observed in case of PHB yield, which was observed as 21.64% (50 µg/ml), 29.48% (100 µg/ml), 25.64% (200 µg/ml) as compared to control 51.19%.

Ethidium bromide treated bacterial isolate AM9 at different concentration also shows negative effect on cdw i.e. 6.266 g/l in case of 50 µg/ml EtBr treatment, 4.186 g/l in case of 100 µg/ml, 3.57 g/l in case of 200 µg/ml and 7.64 g/l in control. PHB accumulation was observed to be 2.162 g/l (50 µg/ml), 1.3 g/l (100 µg/ml), 1.206 g/l (200 µg/ml) and 3.916 g/l for control. The PHB yield was also reduced to 34.50% (50 µg/ml), 30.81% (100 µg/ml) and 33.82% (200 µg/ml) as compared to control i.e. 51.19%

Physical mutagenesis by Ultra-violet radiation at 254nm on PHB production shows that there was decrease in cdw i.e. 7.64 g/l in control sample, which was reduced to 2.966 g/l in case of 1 min UV exposure, 2.033 g/l in 3 min, 2.866 g/l in 5 min, 2.476 g/l in 7 min UV and no growth in 10 min exposure. The effect of UV exposure at different time intervals shows that

the PHB accumulation was also reduced to 1.44 g/l in 1 min treatment, 0.59 g/l (3 min), 0.976 g/l (5 min) and 0.597 g/l (7 min). The PHB yield was also decreases from 51.19% in case of AM9 control to 48.55% (1 min), 29.11% (3 min), 34.03 (5 min) and 24.34% (7 min). The above investigation provides conclusive evidence that control was better all mutated strains. The results are in coherence with Adwitiya *et al.,* 2009.

6.2.3.7: Number of colonies in control & mutated samples after Exposure to different mutagenic agents [MTCC 453 bacterial isolate] observed on petri-plate

Table 6.25: Differential Exposure of Acridine orange mutagen and Total viable count (MTCC 453) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	44	11	3

Table 6.26: Differential Exposure of Sodium Azide mutagen and Total viable count (MTCC 453) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	38	18	0

Table 6.27: Differential Exposure of Ethidium bromide mutagen and Total viable count (MTCC 453) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	21	8	0

Table 6.28: Differential Exposure of Ethyl Methane Sulphonate (EMS) mutagen and Total viable count (MTCC 453) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	24	12	0

6.2.3.7a: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate MTCC 453

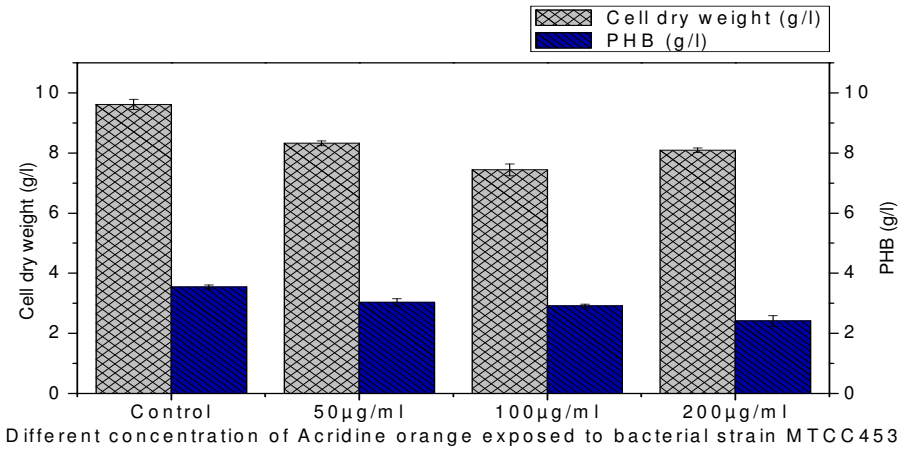


Figure 6.2.59: Cell dry weight (g/l) and PHB (g/l) of MTCC 453 strain exposed to Acridine orange mutagen

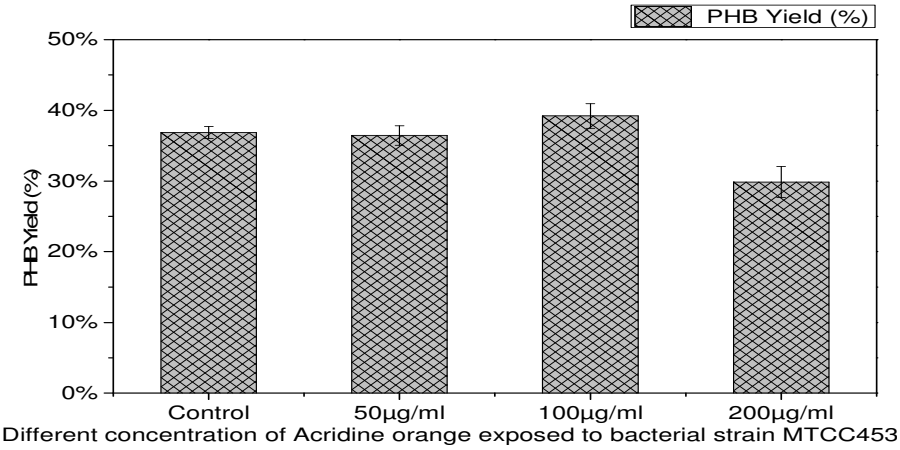


Figure 6.2.60: PHB Yield (%) of MTCC 453 strain exposed to Acridine orange mutagen

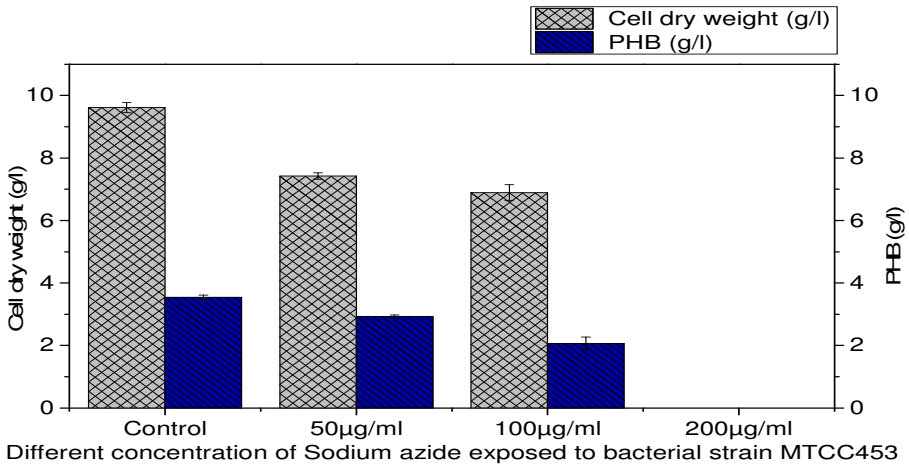


Figure 6.2.61: Cell dry weight (g/l) and PHB (g/l) of MTCC 453 strain exposed to Sodium azide mutagen

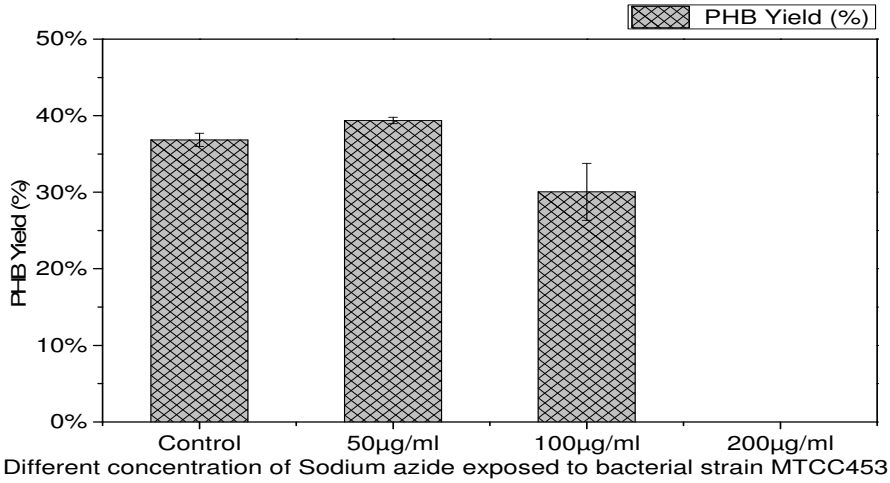


Figure 6.2.62: PHB Yield (%) of MTCC 453 strain exposed to Sodium azide mutagen

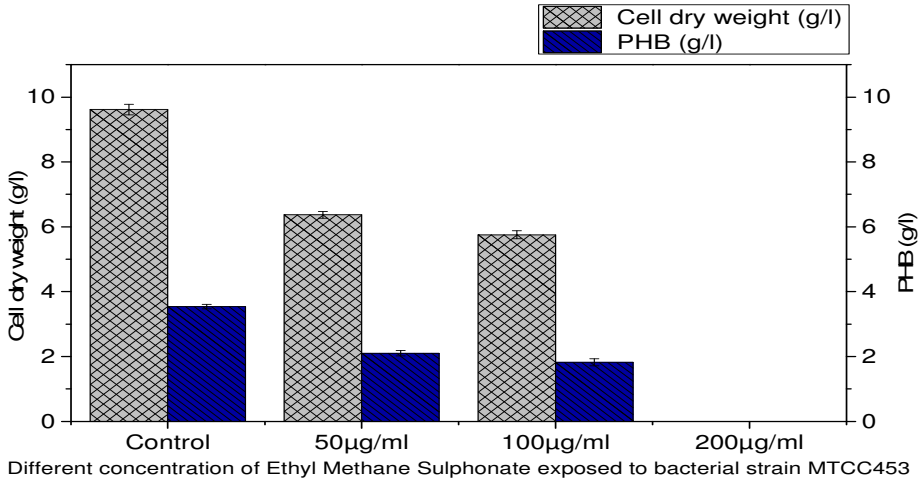


Figure 6.2.63: Cell dry weight (g/l) and PHB (g/l) of MTCC 453 strain exposed to EMS mutagen

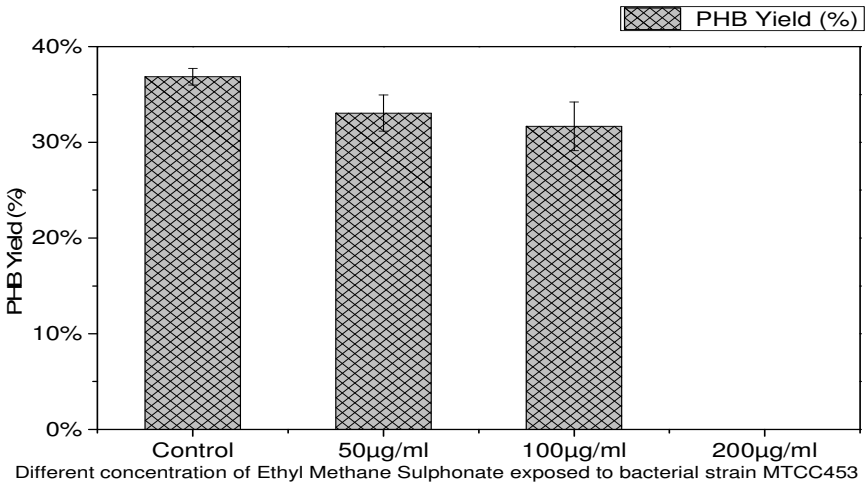


Figure 6.2.64: PHB Yield (%) of bacterial isolate MTCC 453 strain exposed EMS mutagen

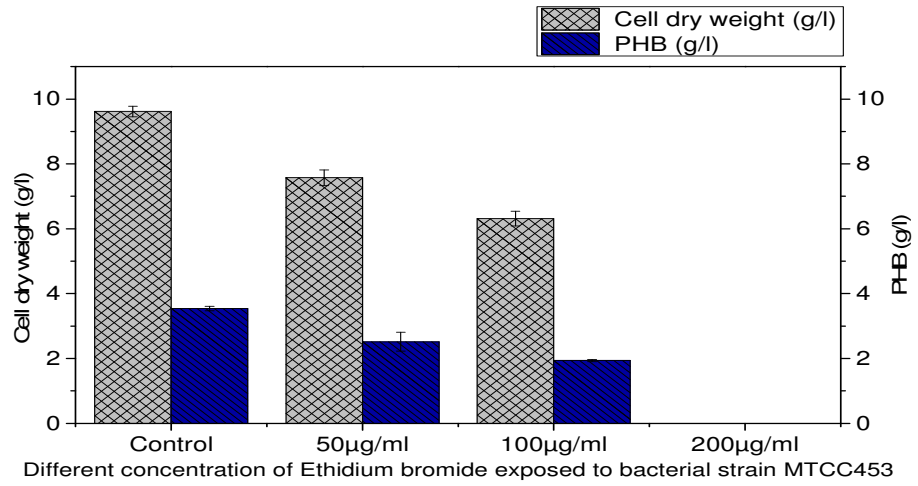


Figure 6.2.65: Cell dry weight (g/l) and PHB (g/l) of MTCC 453 strain exposed to EtBr mutagen

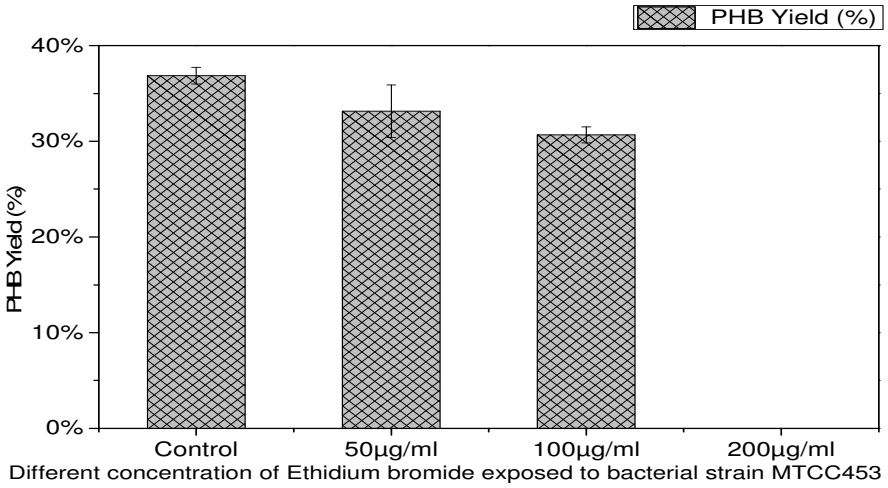


Figure 6.2.66: PHB Yield (%) of MTCC 453 strain exposed to EtBr mutagen

6.2.3.7A (i): Observations of Mutagenesis Studies using different Mutagenic agents in isolate MTCC 453

Mutagenic investigations were carried out with different agents on the isolate MTCC 453. Isolate MTCC 453 initial PHB production was 36.85%, which increases to 39.20% with 100 µg/ml of Acridine orange treatment. In terms of cell dry weight, maximum cell mass was observed in case of control (9.61 g/l) as compared to other exposed isolates. Cell mass was reduced in case of 50 µg/ml (8.323 g/l) and 100 µg/ml 7.44 g/l, whereas cdw in 200 µg/ml was 8.092 g/l. Maximum PHB accumulation was 3.543 g/l in control. The other PHB accumulation was 3.033 g/l (50 µg/ml l), 2.416 g/l (200 µg/ml) and 2.913 g/l (100 µg/ml). The highest PHB yield was observed in 100 µg/ml A.O concentrations i.e. 39.20%. The other exposed bacterial strain yield PHB of 34.44% in 50 µg/ml, 29.86% in 200 µg/ml and in

control 36.85%. In this studies, maximum PHB yield was observed in control strain i.e. 3.54 g/l.

Sodium azide treatment at different concentration analysed that maximum PHB yield was 39.38% in 50 µg/ml . In terms of cell dry weight, maximum cell mass was observed in case of control (9.61 g/l) as compared to other exposed concentrations of sodium azide. Cell mass was reduced in case of 50 µg/ml (7.423 g/l), 100 µg/ml (6.89 g/l) and no growth was observed in 200 µg/ml. Maximum accumulation was observed in control i.e. 3.543 g/l. The PHB accumulation was 2.923 g/l (50 µg/ml) and 2.066 g/l (100 µg/ml). The highest PHB yield was observed at 50µg/ml sodium azide concentrations i.e. 39.38%. The other concentrations were analysed for PHB yield and were 30.06% in 100 µg/ml and 36.85% in control. No bacterial growth was observed at 200 µg/ml concentration of Acridine orange. From the overall observation, it was found that in terms of PHB yield 50 µg/ml was the best exposed concentration for high PHB yield. Overall PHB accumulation was highest in control i.e. 3.543 g/l.

Ethyl methane sulphonate (EMS) treatment, maximum yield was observed at control i.e. 36.85%. In terms of cell dry weight, maximum cell mass was observed in case of control (9.61 g/l) as compared to other exposed concentrations of EMS. Cell mass was reduced in case of 50 µg/ml (6.366 g/l), 100 µg/ml (5.75 g/l) and in 200 µg/ml no growth was observed. PHB accumulation was 2.103 g/l (50 µg/ml) and 1.822 g/l (100 µg/ml). Maximum PHB accumulation was observed in control i.e. 3.543 g/l. The highest PHB yield was observed at control i.e. 36.85%. The PHB yield of other exposed strains was 33.06% in 50 µg/ml, 31.25% in 100 µg/ml. From the overall observation, it was found that control sample with no exposure of EMS was considered for high PHB production.

Ethidium bromide treated bacterial isolate MTCC 453 at different concentration shows the maximum cdw was observed in control i.e. 9.61 g/l. It was also analyzed that cdw at 50 µg/ml was 7.573 g/l, 100 µg/ml was 6.313 g/l and in 200 µg/ml no growth was observed. The analysis shows that in control PHB accumulation was 3.543 g/l, which was maximum as compared to the mutagenic affected strains. The accumulation of PHB was 2.516 g/l in 50 µg/ml and 1.935 g/l g/l in 100 µg/ml of EtBr exposure. The PHB yield was highest in control i.e. 36.85%. The other yield percentage was 33.14% in 50 µg/ml and 30.68% in 100 µg/ml.

The overall analysis shows that maximum PHB accumulated in control i.e. 3.54 g/l, which shows that control was better among the other mutated strains.

No effect of mutagenic agents was observed in the case of UV treatment at different time intervals as no reduction in number of colonies was observed as compared with the control.

6.2.3.8: Number of colonies in control & mutated samples after Exposure to different mutagenic agents [MTCC 617 bacterial isolate]

Table 6.29: Differential Exposure of Acridine orange mutagen and Total viable count (MTCC 617)

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	60	29	0

Table 6.30: Differential Exposure of Sodium azide mutagen and Total viable count (MTCC 617) observed on petri-plate

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	30	11	4

Table 6.31: Differential Exposure of Ethyl methane sulphonate mutagen and Total viable count (MTCC 617)

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	78	35	3

Table 6.32: Differential Exposure of Ethidium bromide mutagen and Total viable count (MTCC 617)

Control	Mean colony count of 50 µg/ml	Mean colony count of 100 µg/ml	Mean colony count of 200 µg/ml
More than 200	37	7	1

Table 6.33: Differential Exposure of Ultra-violet radiation and Total viable count (MTCC 617)

Control	Mean colony count of 1 min	Mean colony count of 3 min	Mean colony count of 5 min	Mean colony count of 7 min
More than 200	56	38	8	0

6.2.3.8a: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate MTCC 617

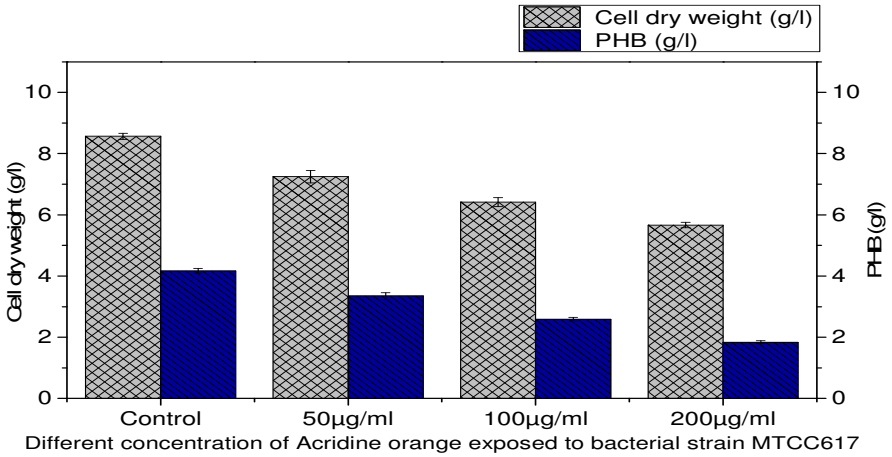


Figure 6.2.67: Cell dry weight (g/l) and PHB (g/l) of MTCC 617 strain exposed to Acridine orange mutagen

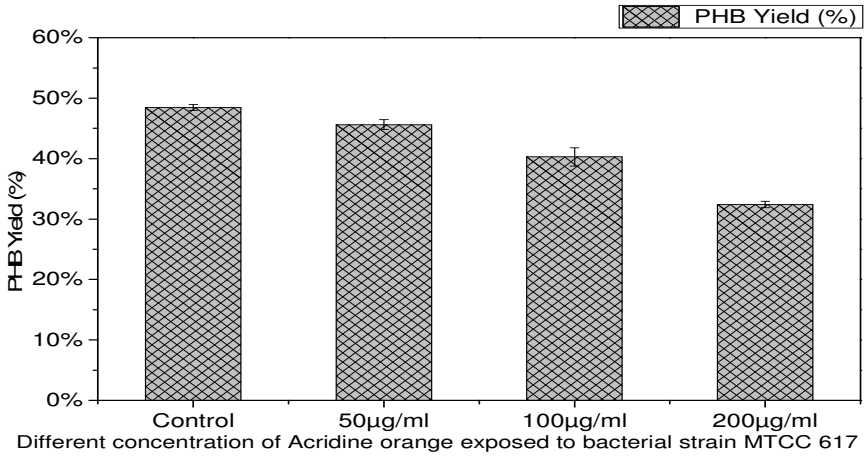


Figure 6.2.68: PHB Yield (%) of MTCC 617 strain exposed to Acridine orange mutagen

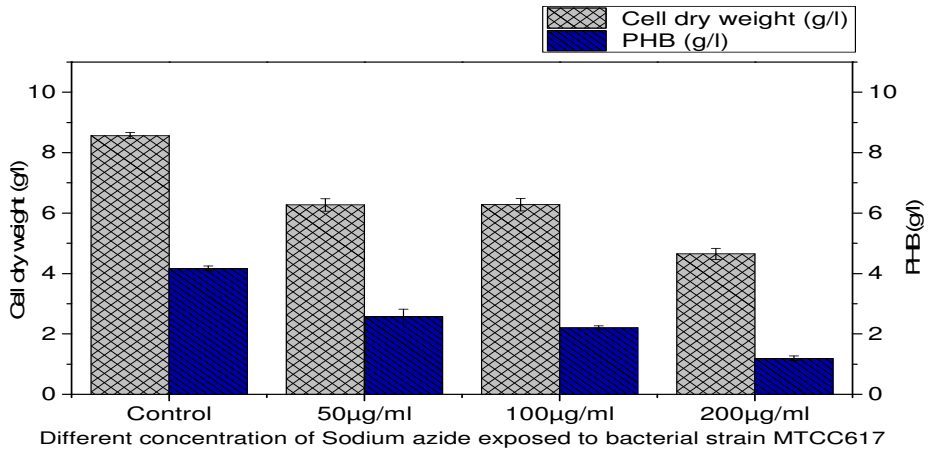


Figure 6.2.69: Cell dry weight (g/l) and PHB (g/l) of MTCC 617 strain exposed to Sodium azide mutagen

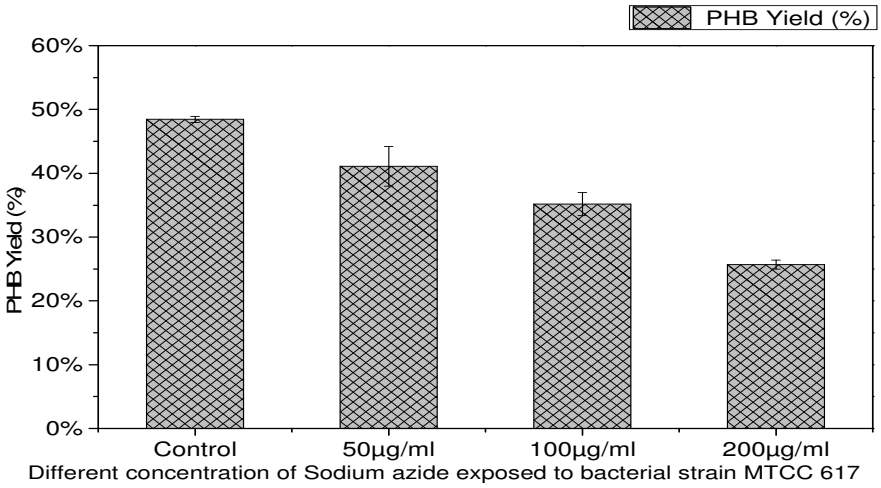


Figure 6.2.70: PHB Yield (%) of MTCC 617 strain exposed to Sodium azide mutagen

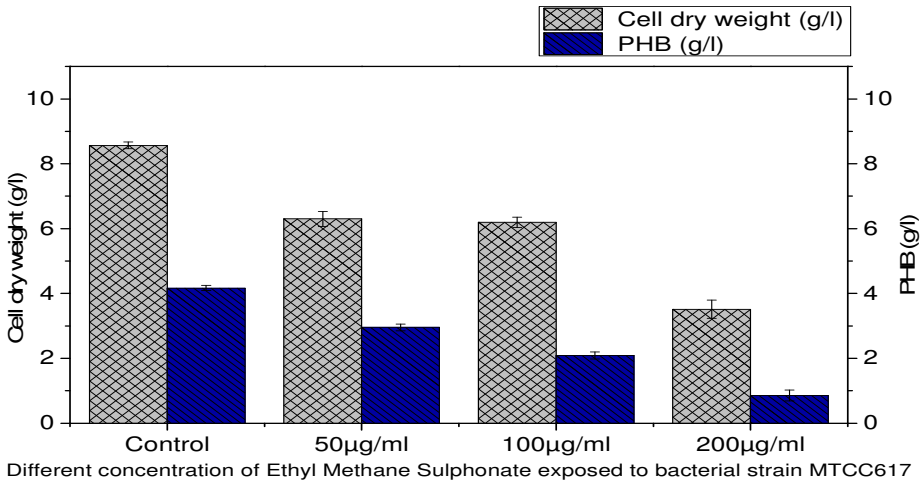


Figure 6.2.71: Cell dry weight (g/l) and PHB (g/l) of MTCC 617 strain exposed to EMS mutagen

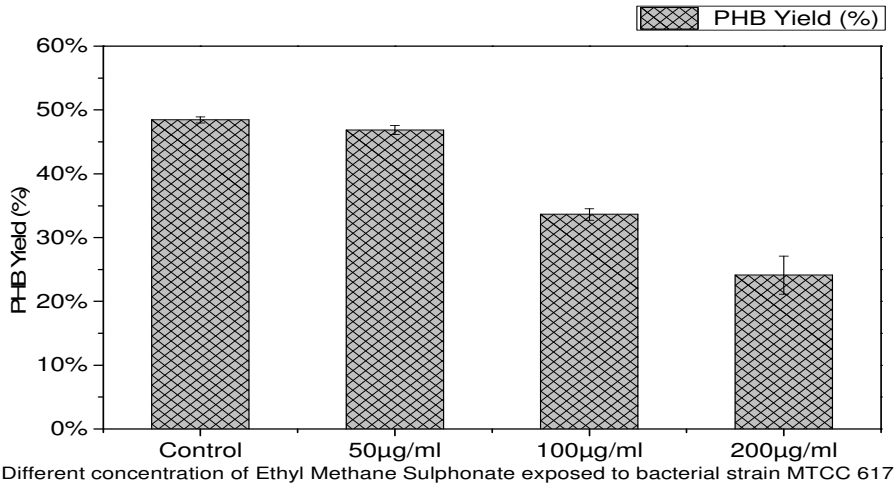


Figure 6.2.72: PHB Yield (%) of bacterial isolate MTCC 617 exposed to EMS mutagen

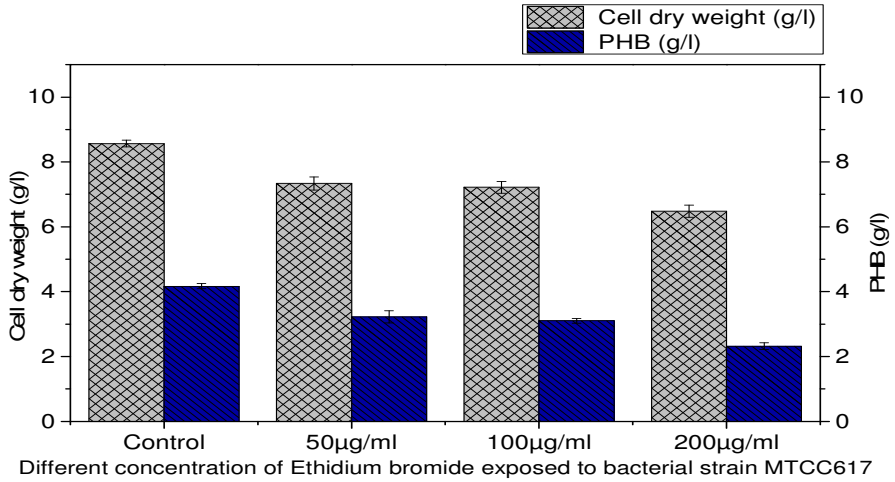


Figure 6.2.73: Cell dry weight (g/l) and PHB (g/l) of MTCC 617 strain exposed to EtBr mutagen

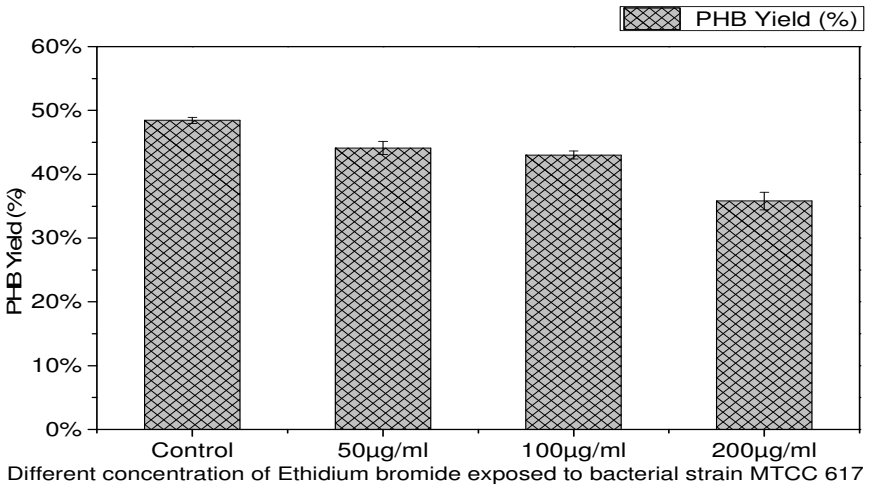


Figure 6.2.74: PHB Yield (%) of MTCC 617 strain exposed to EtBr mutagen

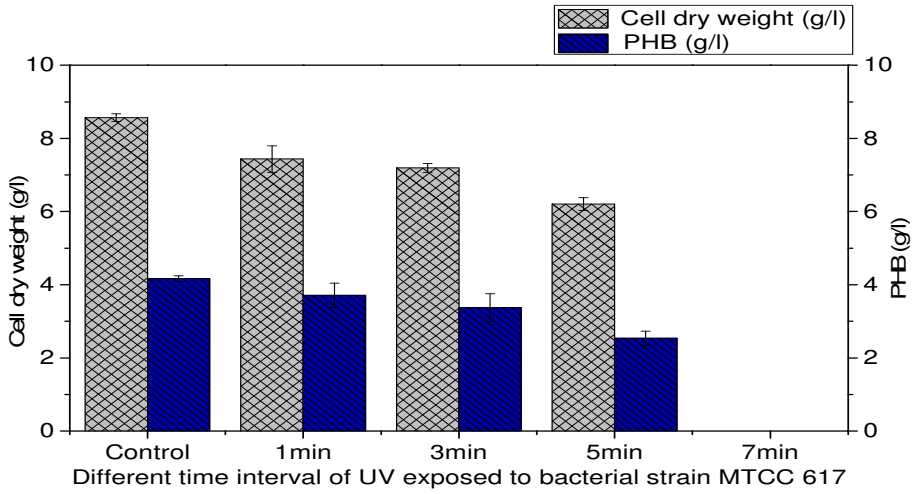


Figure 6.2.75: Cell dry weight (g/l) and PHB (g/l) of MTCC 617 strain exposed to UV radiation mutagen

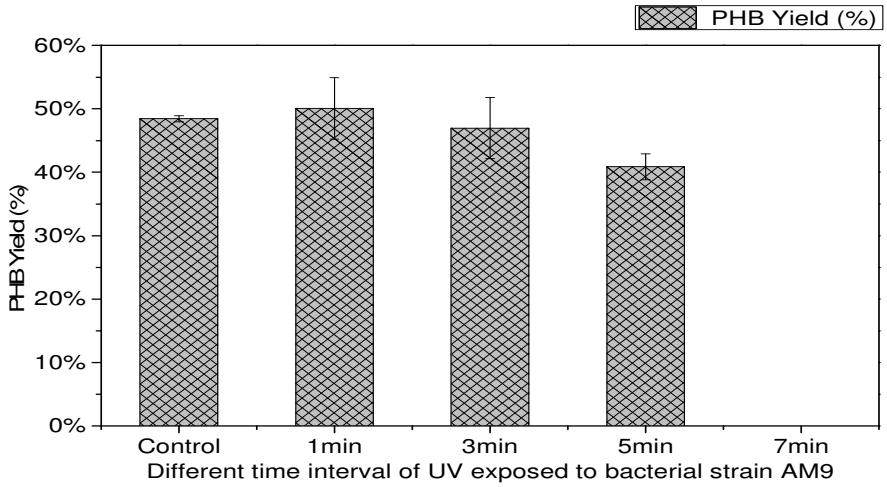


Figure 6.2.76: PHB Yield (%) of MTCC 617 strain exposed to UV radiation mutagen

6.2.3.8a (i): Observation of Mutagenesis Studies using different Mutagenic agents in isolate MTCC 617

Mutagenic investigations were carried out with different agents on the isolate MTCC 617. Isolate MTCC 617 initial PHB production was 48.44%, which was observed maximum. Negative effect was seen in the PHB yield when exposed to different concentration of Acridine orange. In terms of cell dry weight, maximum cell mass was observed in case of control (8.566 g/l) as compared to other exposed concentrations. Cell mass was reduced in case of 50 µg/ml (7.25 g/l) and 100 µg/ml 6.416 g/l and cdw in 200 µg/ml was 5.66 g/l. Maximum PHB accumulation was 4.166 g/l in control. The other PHB accumulation was 3.346 g/l (50 µg/ml l), 2.583 g/l (100 µg/ml) and 1.836 g/l (200 µg/ml). The highest PHB yield was observed in control i.e. 48.44%. The other exposed concentration yield of PHB was

45.62% in 50 µg/ml, 40.28% in 100 µg/ml and 32.43% in 200 µg/ml. From the overall observation, it was found that in terms of PHB yield and production, control was the maximum producer of PHB.

Sodium azide treatment at different concentration investigated that maximum PHB yield was observed in control. Negative effect was seen in the PHB yield when exposed to different concentration of sodium azide. In terms of cell dry weight, maximum cell mass was observed in case of control (8.566 g/l) as compared to other exposed concentrations. Cell mass was reduced in case of 50 µg/ml (6.273 g/l), 100 µg/ml (6.283 g/l) and 200 µg/ml (4.65 g/l). Maximum PHB accumulation was 4.166 g/l in control. The other PHB accumulation was 2.58 g/l (50 µg/ml), 2.205 g/l (100 µg/ml) and 1.193 g/l (200 µg/ml). The highest PHB yield was observed in control i.e. 48.44%. The other exposed bacterial strain yield PHB of 41.10% in 50 µg/ml, 35.16% in 100 µg/ml and 25.67% in 200 µg/ml. From the overall observation, it was found that in terms of PHB yield and production, control was the maximum producer of PHB.

In Ethyl methane sulphonate (EMS) treatment control had the highest PHB yield level of 48.44%. In terms of cell dry weight, maximum cell mass was observed in case of control (8.566 g/l) as compared to other exposed concentrations of EMS. Cell mass was reduced in case of 50 µg/ml (6.3 g/l), 100 µg/ml (6.19 g/l) and 200 µg/ml (3.513 g/l). PHB accumulation was 4.166 g/l (control), 2.9511 g/l (50 µg/ml), 2.086 g/l (100 µg/ml) and 0.855 g/l (200 µg/ml). The PHB yield of other exposed strains was 47% in 50 µg/ml, 34% in 100 µg/ml and 24% in 200 µg/ml.

Ethidium bromide treated bacterial isolate MTCC 617 at different concentration shows the maximum cdw was observed in control i.e. 8.566 g/l. It was also analyzed that cdw at 50 µg/ml was 7.336 g/l, 100 µg/ml was 7.216 g/l and 200 µg/ml was 6.48 g/l. It was observed that in control, PHB accumulation was 4.166 g/l, which was maximum as compared to the mutagenic affected strains. The accumulation of PHB was 3.223 g/l in 50 µg/ml, 3.099 g/l in 100 µg/ml and 2.319 g/l in 200 µg/ml of EtBr exposure. The PHB yield was highest in control i.e. 48.44%. The other yield percentage was 44.11% in 50 µg/ml, 43.01% in 100 µg/ml and 35.80% in 200 µg/ml. The overall analysis shows that maximum PHB accumulated in control i.e. 4.166 g/l, which shows that in terms of PHB production, control was better among the other mutated strains.

UV treatment at 1min time interval yields PHB production level of 50.08%. In terms of cell dry weight, maximum cell mass was observed in control i.e. 8.566 g/l as compared to other mutagen exposed strains. Cell mass was reduced in case of 1 min UV exposure i.e. (7.433 g/l), 3 min (7.193 g/l), 5 min (6.206 g/l) and no growth observed in 7 min. PHB accumulation was maximum in control i.e. 4.166 g/l. The observation shows that the PHB accumulation was 3.716 g/l (1 min), 3.376 g/l (3 min) and 2.54 g/l (5 min). The highest PHB yield was observed at 1 min UV exposure, which was 50.08%. The other exposure of mutagen, yielded PHB of 46.95% in 3 min, 40.86% in 5 min and 48.44% in control. From the overall observation, it was found that in terms of PHB yield, 1 min UV exposed strain shows a high PHB yield i.e. 50.08%. The high PHB accumulation was observed in control i.e. 4.166 g/l. The results are in coherence with Adwitiya *et al.,* 2009, Hikmet *et al.,* 2003 who have reported an increase in the PHB yield of *B. megaterium* Y6, *B. subtilis* K8, and *B. firmus* G2 and various other strains via mutation.

Table: 6.34: Comparison of mutated and non-mutated strains and further selection for optimization on the basis of PHB production (g/l) and PHB Yield (%)

Strain name		**PHB (g/l)**	**PHB Yield (%)**	**Selection of strains for further Optimization**
S-3	Wild strain with 2% glucose	2.066 g/l	40.13%	
	Mutated strain with 2% glucose	3.372 g/l	50.53%	✔
M1	Wild strain with 2% glucose	0.33 g/l	15.34%	
	Mutated strain with 2% glucose	0.665 g/l	24.99%	✔
M10	Wild strain with 2% glucose	0.343 g/l	12.69%	
	Mutated strain with 2% glucose	0.793 g/l	37.80%	✔
MD1	Wild strain with 2% glucose	0.5133g/l	32.77%	
	Mutated strain with 2% glucose	0.494 g/l	45.77%	✔
MD2	Wild strain with 2% glucose	0.9933 g/l	28.41%	✔
	Mutated strain with 2% glucose	Non-mutated	Non-mutated	
MD5	Wild strain with 2% glucose	1.137 g/l	28.69%	✔
	Mutated strain with 2% glucose	Non-mutated	Non-mutated	
AM6	Wild strain with 2% glucose	2.01 g/l	27.60%	
	Mutated strain with 2% glucose	1.593 g/l	29.92%	✔
AM9	Wild strain with 2% glucose	3.916 g/l	51.19%	✔
	Mutated strain with 2% glucose	1.44g/l	48.55%	
MTCC 453	Wild strain with 2% glucose	3.543 g/l	36.85%	
	Mutated strain	2.923 g/l	39.38%	✔
MTCC 617	Wild strain with 2% glucose	4.166 g/l	48.49%	
	Mutated strain with 2% glucose	3.716 g/l	50.08%	✔

6.2.4: Representative photographs of extracted PHB from bacterial isolates

Figure 6.2.77: Extracted from MD1 strain

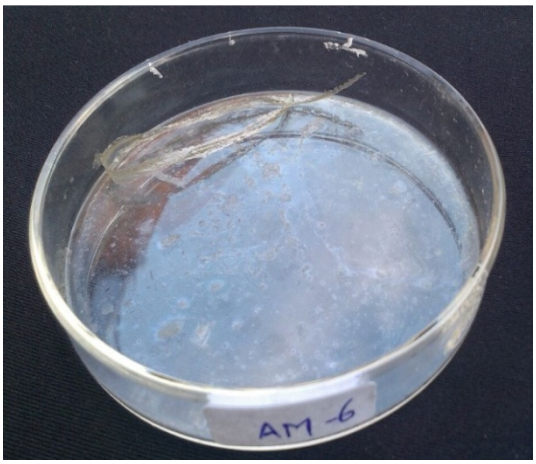


Figure 6.2.78: Extracted from AM6 strain

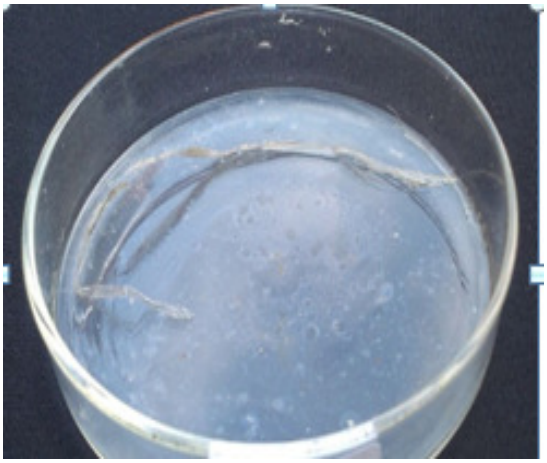


Figure 6.2.79: Extracted from AM9 strain

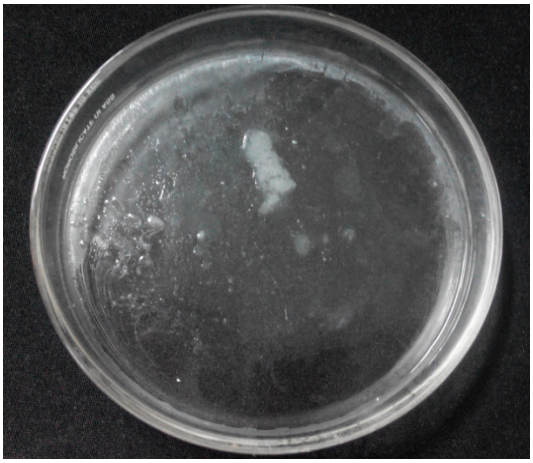


Figure 6.2.80: Extracted from MD2 strain

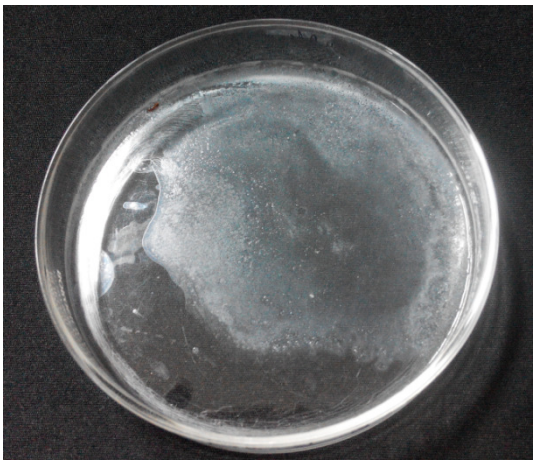


Figure 6.2.81: Extracted from MD5 strain

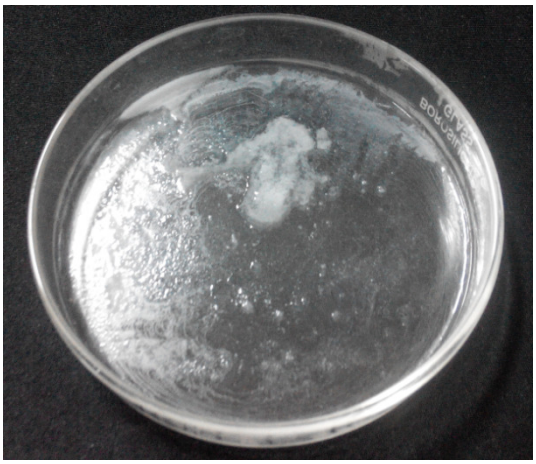


Figure 6.2.82: Extracted from MD2 strain

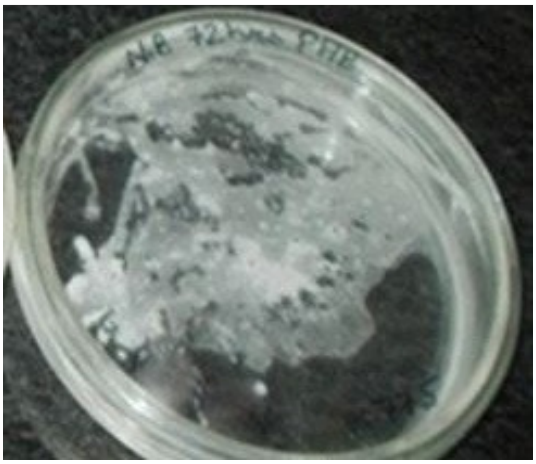


Figure 6.2.83: Extracted from S3 strain

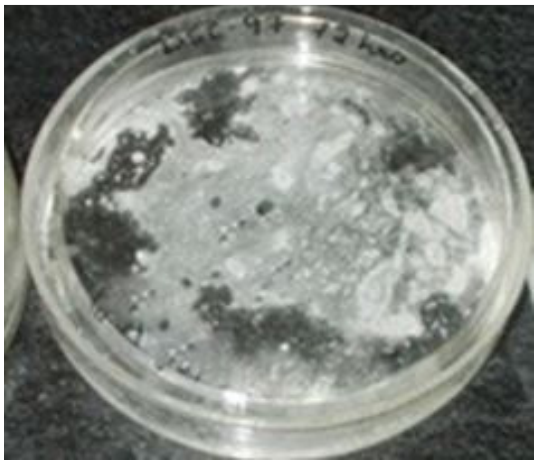


Figure 6.2.84: Extracted from M1 strain

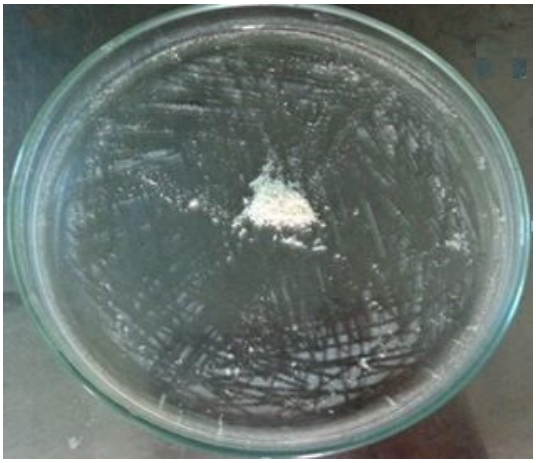


Figure 6.2.85: Powder form of PHB

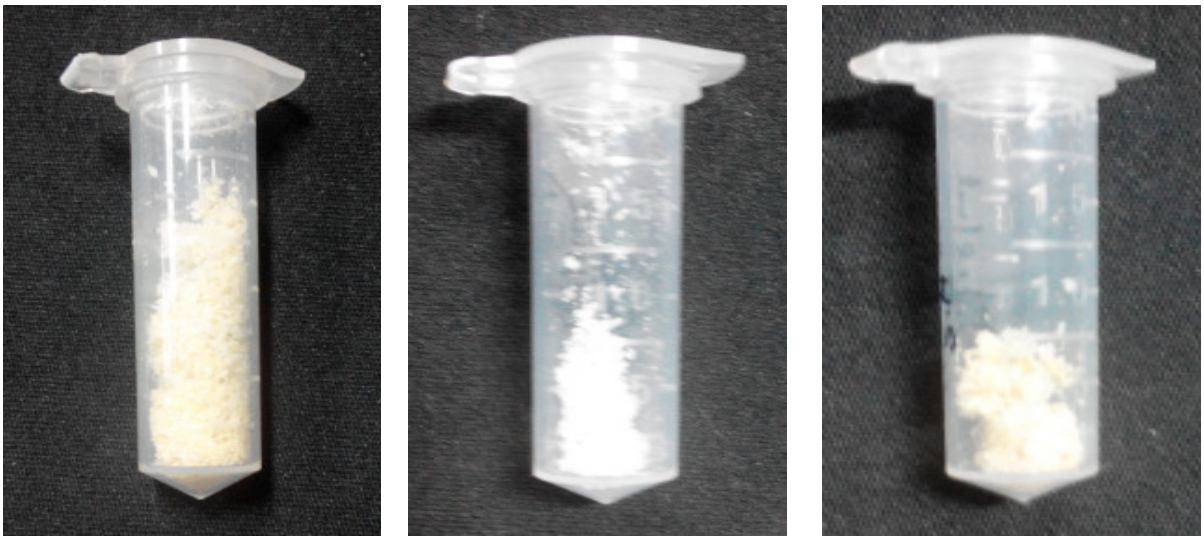


Figure 6.2.86: Representative photographs of extracted PHB in powder form

6.3: Optimization of media and large scale fermenter production of bacterial extracted PHB from improved strains and its characterization for quality

6.3.1: Results of Carbon source optimization at induction stage

Carbon Source optimization was carried out at induction stage. All of the experimental protocols were carried out as described in material and method section.

The carbon sources utilized for the current study were Glucose, Sucrose, Fructose, Maltose and Lactose with concentration variation of 1%, 2%, 3%.

The results shown under are in coherence with Wang and Lee, 1997 that nitrogen limitation and excess of carbon promotes the accumulation of PHB.

6.3.1a: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate S-3

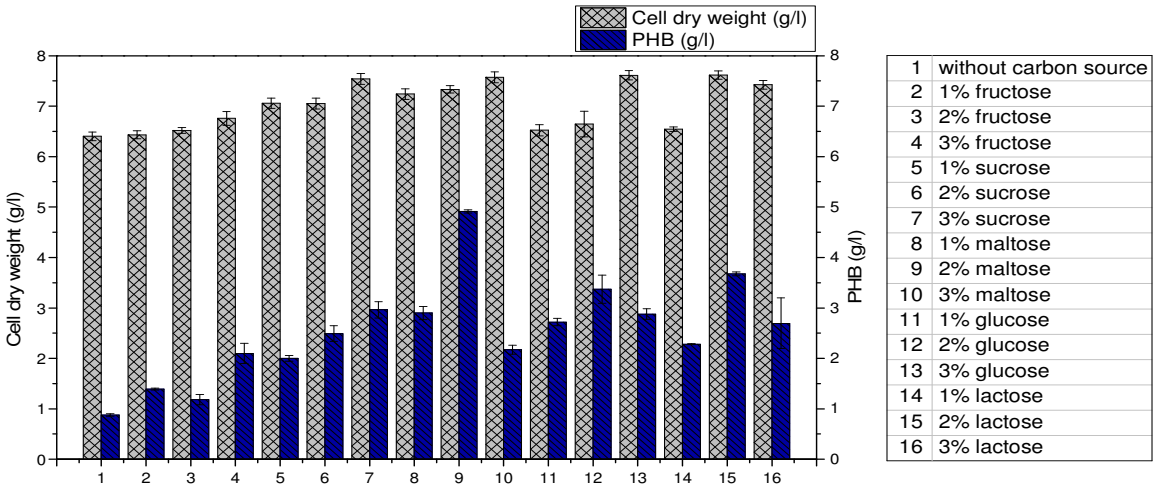


Figure 6.3.1: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate S-3

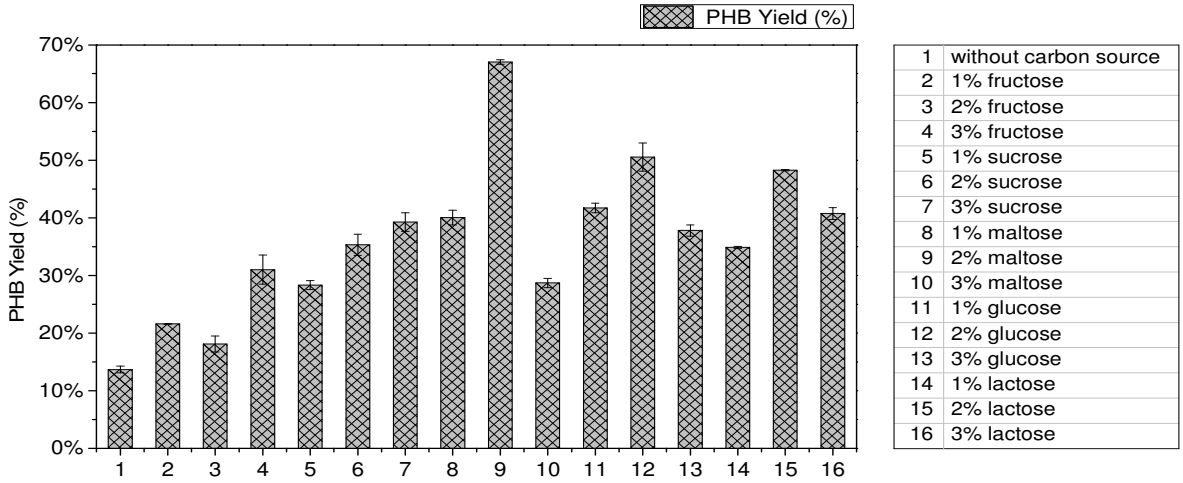


Figure 6.3.2: PHB yield (%) of bacterial isolate S-3

6.3.1.1: Observation of different carbon sources optimization in isolate S-3

The initial PHB production in S-3 was 13.70% without any glucose induction. The cell dry weight without adding any carbon source was observed 6.403 g/l with PHB production was 0.8773 g/l. In order to know the best carbon source necessary for high PHB production, optimization was carried out using different carbon sources as described in material and method section. Carbon source utilization is an important parameter for high PHB production. The carbon sources were optimized in 3 different concentrations i.e. 1%, 2% and 3% respectively. The highest observations have been described here for parameters of cdw, PHB yield % and PHB accumulation in g/l.

The initial growth curve optimization and utilization of 2% glucose leads to an increase in PHB accumulation level and observed to be 6.46 g/l of cdw, 2.588 g/l of PHB accumulation and PHB yield of 40.13%. The mutagenesis studies shows that 100µg/ml of Acridine orange treatment produced cdw of 6.64 g/l, PHB accumulation of 3.372 g/l and PHB yield of 50.53%.

It was observed that by inducing 2% lactose, cdw was 7.616 g/l, PHB production was 3.675 g/l and yield was 48.27%. The induction of 2% maltose, produced PHB yield of 67.03%, PHB accumulation was 4.912 g/l and cell dry weight of 7.33 g/l. 3% sucrose produced PHB yield of 39.27%, PHB was 2.963 g/l and cell dry weight of 7.543 g/l. High cell growth and further high PHB production are two important steps to be followed for achieving high PHB yield. From the overall analysis it was investigated that 2% maltose induction was the best optimized condition for achieving high PHB (g/l) with 67.03%.

6.3.1b: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate M1

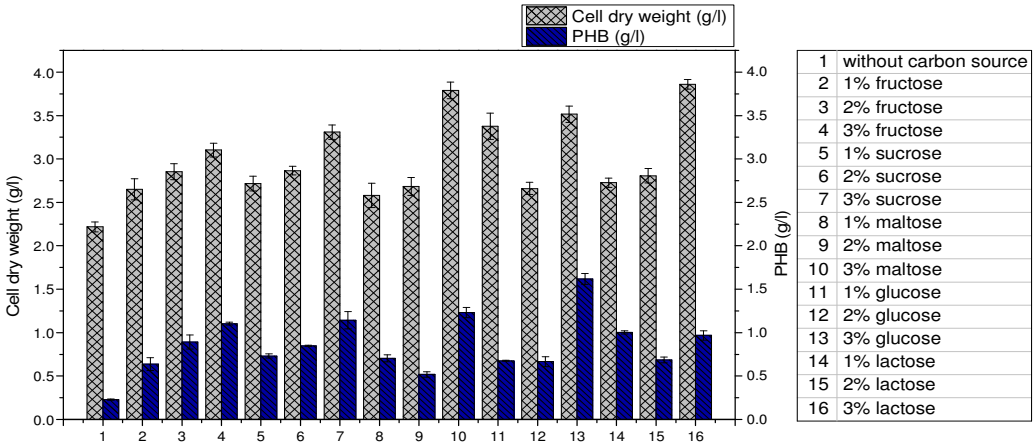


Figure 6.3.3: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate M1

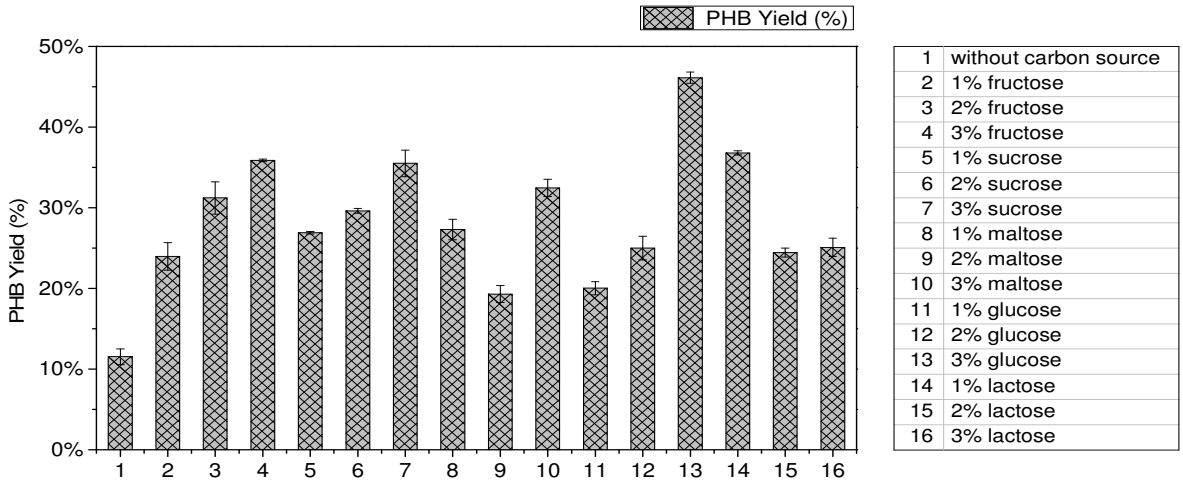


Figure 6.3.4: PHB Yield (%) of bacterial isolate M1

6.3.1.2: Observation of different carbon sources optimization in isolate M1

The initial PHB production in M1 was 11.52%. The cell dry weight without adding any carbon source was observed 2.218 g/l with PHB production was 0.228 g/l. In order to know the best carbon source necessary for high PHB production, optimization was carried out as described in material and method section. The carbon sources were optimized in 3 different concentrations i.e. 1%, 2% and 3% respectively.

The initial growth curve optimization and utilization of 2% glucose leads to an increase in PHB accumulation level and observed to be 2.15 g/l of cdw, 0.33 g/l of PHB accumulation and PHB yield of 15.34%. The mutagenesis studies shows that by UV 7 min exposure cdw was 2.66 g/l, PHB accumulation was 0.665 g/l and PHB yield was 24.99%.

It was observed that PHB yield was increased from 11.52% to 46.11% by inducing 3% glucose as carbon source. The cdw was increase from 2.218 g/l to 3.516 g/l, whereas PHB production was increased from 0.228 g/l to 1.618 g/l. 1% lactose induction produced cell dry weight of 2.726 g/l, PHB g/l was 1.003 g/l and PHB yield was 36.79%. The induction of 3% fructose, produced PHB yield of 35.85%, PHB was 1.103 g/l and cell dry weight of 3.103 g/l. 1% sucrose induction yielded cell dry weight of 2.716 g/l, PHB production was 0.7307 g/l and PHB Yield was observed to be 26.90%. 3% sucrose induction yielded cell dry weight of 3.31 g/l, PHB production was 1.1431 g/l and PHB Yield was observed to be 35.49%. High cell growth and high PHB production are the two important steps to be followed to achieve high PHB yield. From the overall analysis it was investigated that 3% glucose induction was the best optimized condition for achieving high PHB (g/l) and PHB yield for the M1 isolate

6.3.1c: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate M10

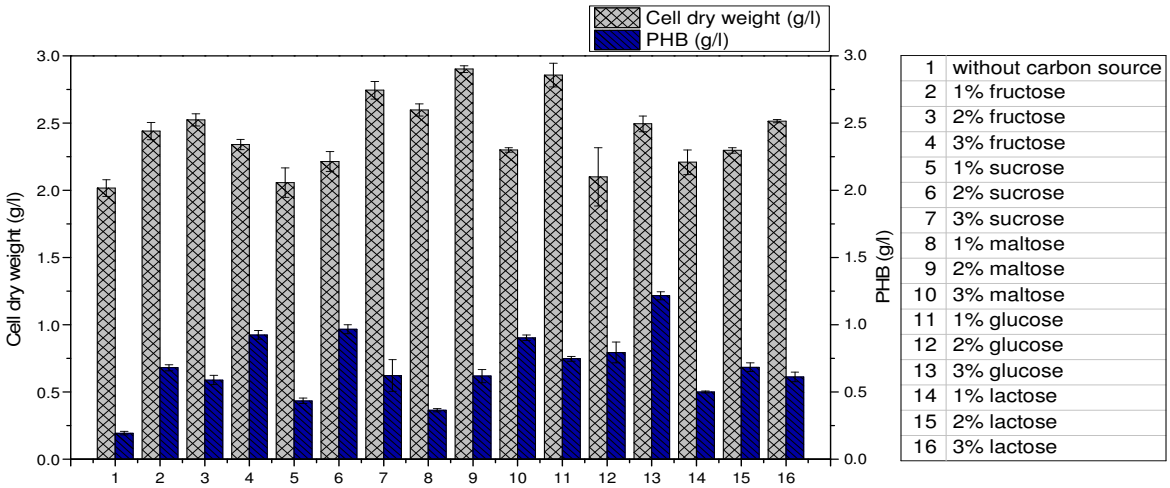


Figure 6.3.5: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate M10

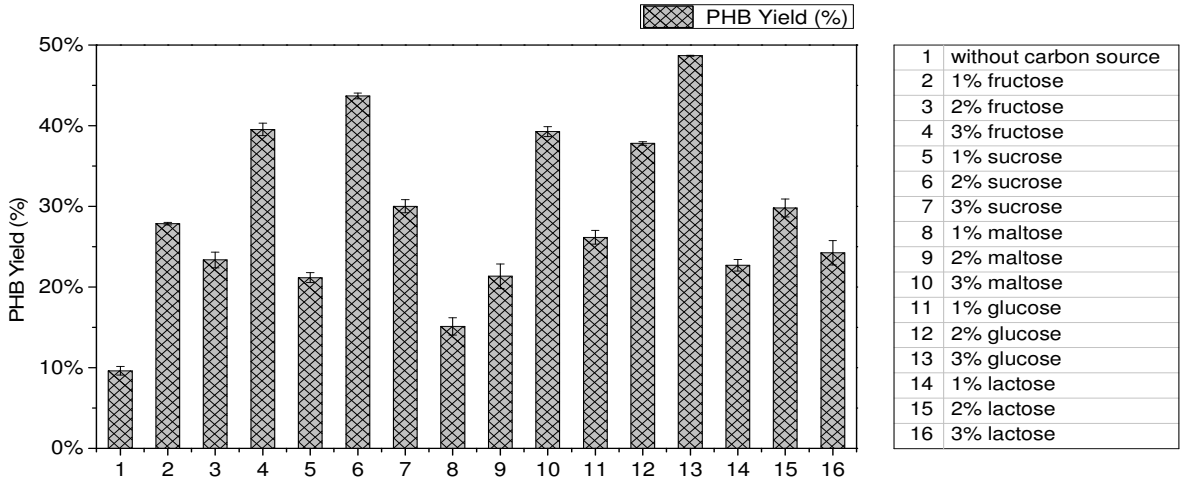


Figure 6.3.6: PHB Yield (%) of bacterial isolate M10

6.3.1.3: Observation of different carbon sources optimization in isolate M10

The initial PHB production in M10 was 9.63%. The cell dry weight without adding any carbon source was observed 2.016 g/l with PHB production was 0.1942 g/l. In order to know the best carbon source necessary for high PHB production, optimization as described in material and method section.

The initial growth curve optimization and utilization of 2% glucose leads to an increase in PHB accumulation level and was observed to be 2.7 g/l of cdw, 0.343 g/l of PHB accumulation and PHB yield of 12.69%. The mutagenesis studies shows that in UV 5 min exposure the cdw was 2.1 g/l, PHB accumulation was 0.793 g/l and PHB yield from 37.80%.

The cell dry weight was 2.493 g/l, PHB production was 1.2153 g/l and PHB yield was

48.65% by utilizing 3% lactose as inducer. It was observed that by utilizing 3% maltose, cdw was 2.3 g/l, PHB production was 0.9028 g/l and yield was 39.26%, 2% sucrose, cdw was 2.213 g/l, PHB production was 0.965 g/l and yield was 43.68%. High cell growth and high PHB production are the two important steps to be utilized to get high PHB yield. From the overall analysis it was investigated that 3% glucose induction was the best optimized condition for achieving high PHB (g/l).

6.3.1d: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate MD1

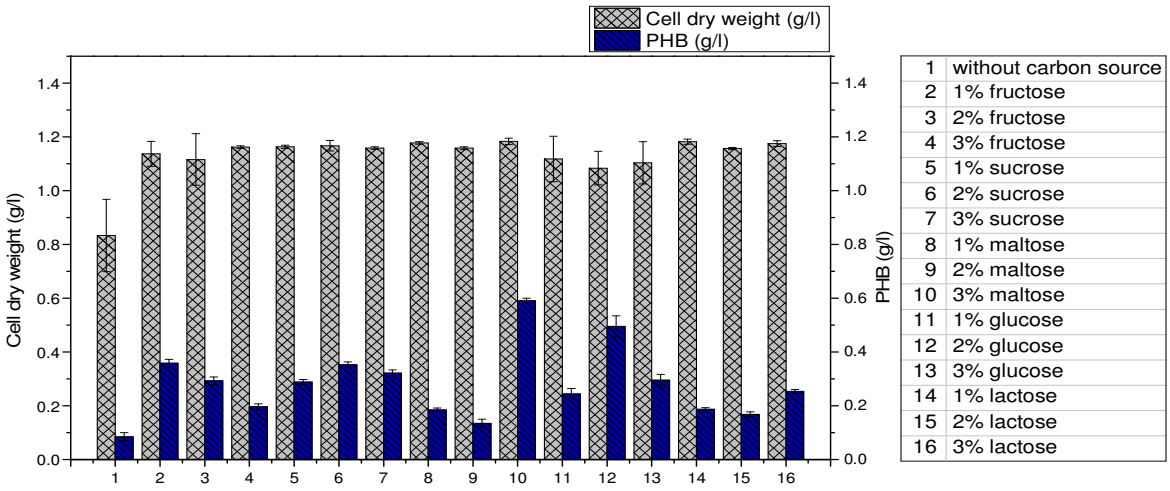


Figure 6.3.7: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate MD1

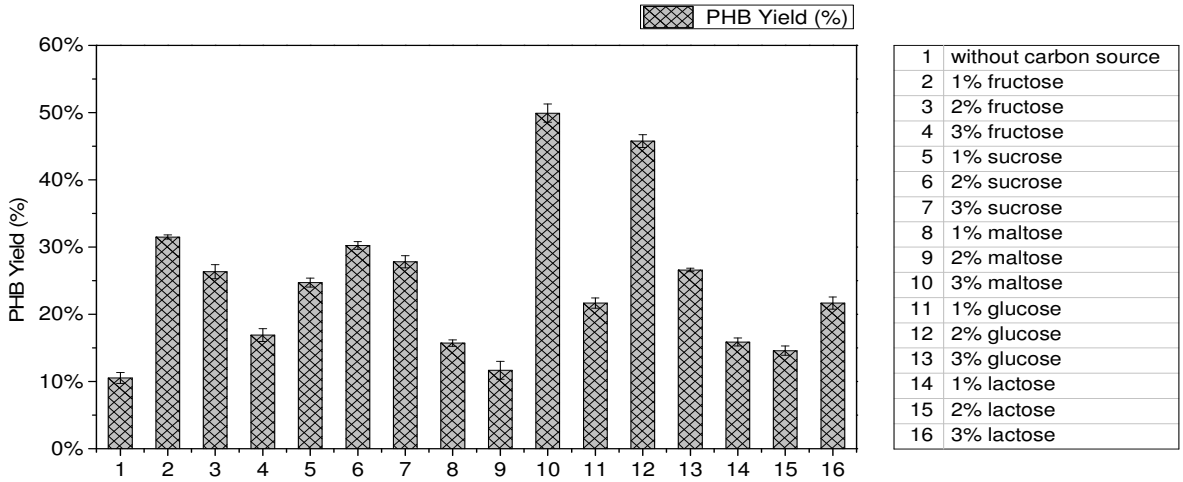


Figure 6.3.8: PHB Yield (%) of bacterial isolate MD1

6.3.1.4: Observation of different carbon sources optimization in isolate MD1

The initial PHB production in MD1 was 10.52%. The cell dry weight without adding any carbon source was observed 0.833 g/l with PHB production was 0.0849 g/l. In order to know the best carbon source necessary for high PHB production, optimization was carried out as described in material and method section.

The initial growth curve optimization and utilization of 2% glucose leads to an increase in PHB accumulation level and was observed to be 0.5133 g/l of cdw, 1.56 g/l of PHB accumulation and PHB yield of 32.77%. The mutagenesis studies shows that in 100 µg/ml sodium azide treatment, the cdw was 1.083 g/l, PHB accumulation was 0.494 g/l and PHB yield from 45.77%.

Highest cell dry weight was increased from 1.08 g/l to 1.183 g/l, PHB production from 0.4947 g/l to 0.59 g/l and PHB yield from 45.77% to 49.89% by utilizing 3% maltose. It was observed that by utilizing 1% fructose, cdw was 1.136 g/l, PHB production was 0.3578 g/l and yield was 31.48%. The induction of 2% sucrose, produced PHB yield of 30.34%, PHB was 0.3527 g/l and cell dry weight of 1.166 g/l. From the overall analysis it was investigated that 3% maltose induction was the best optimized condition for achieving high PHB (g/l).

6.3.1e: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate MD2

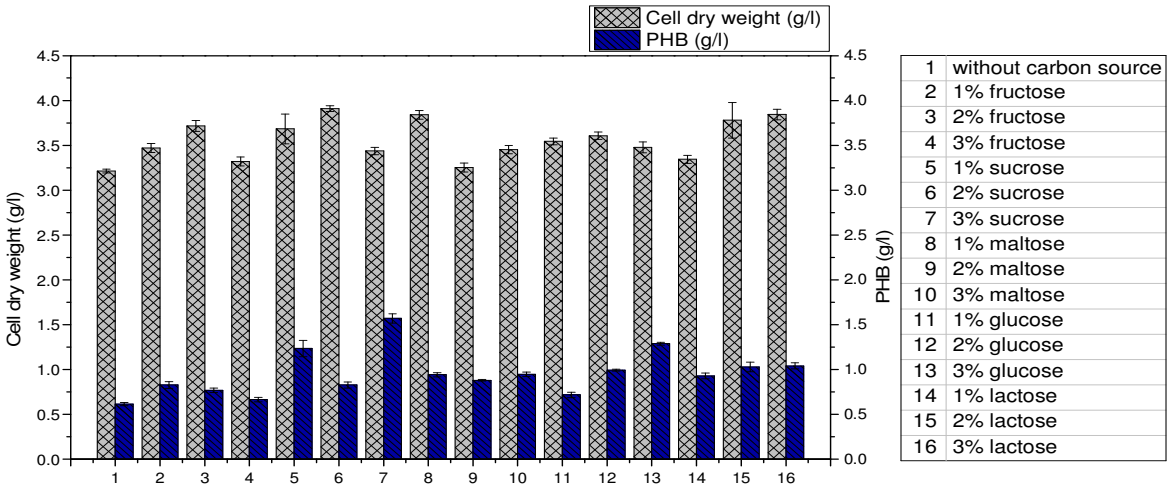


Figure 6.3.9: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate MD2

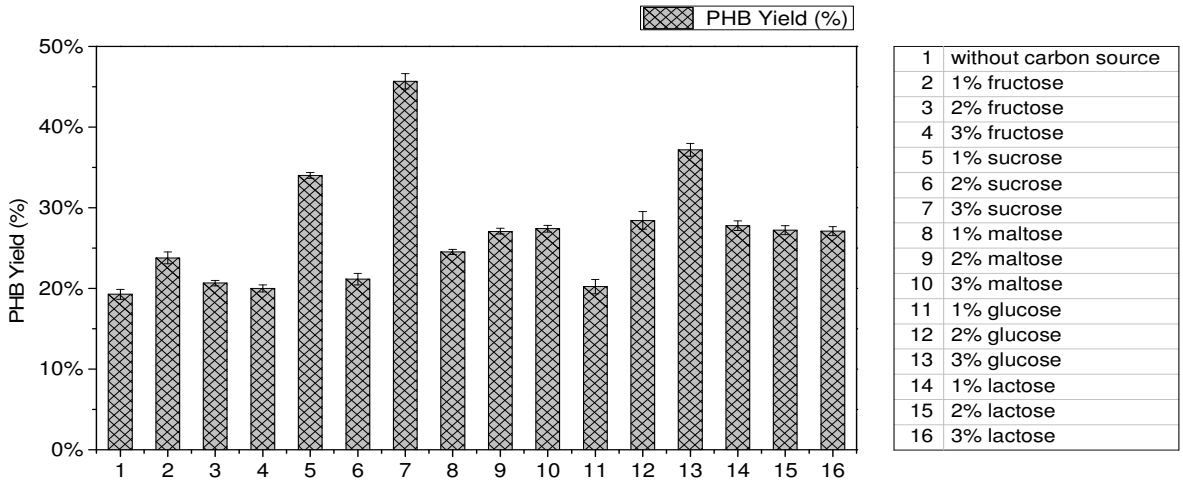


Figure 6.3.10: PHB Yield (%) of bacterial isolate MD2

6.3.1.5: Observation of different carbon sources optimization in isolate MD2

The initial PHB production in MD2 was 19.28%. The cell dry weight without adding any carbon source was observed 3.213 g/l with PHB production was 0.613 g/l. In order to know the best carbon source necessary for high PHB production, optimization was carried out as described in material and method section. It was observed that by inducing 3% sucrose, cdw was 3.436 g/l, PHB production was 1.569 g/l and yield was 45.67%. The utilization of 3% glucose, produced PHB yield of 37.16%, PHB was 1.288 g/l and cell dry weight of 3.476 g/l. The utilization of 1% sucrose, produced PHB yield of 34%, PHB was 1.234 g/l and cell dry weight of 3.683 g/l. From the overall analysis it was investigated that 3% sucrose induction was the best optimized condition for achieving high PHB (g/l).

6.3.1f: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate MD5

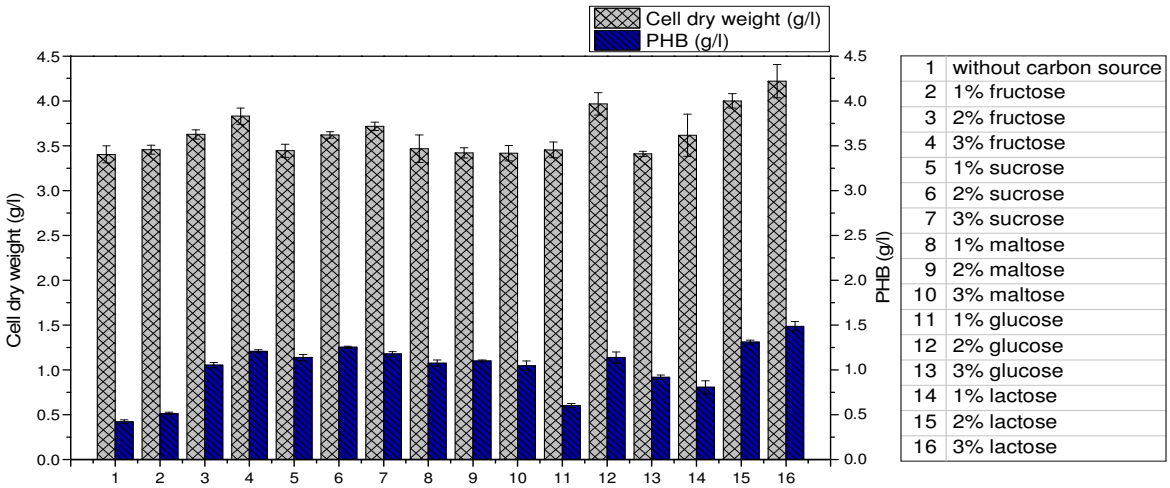


Figure 6.3.11: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate MD5

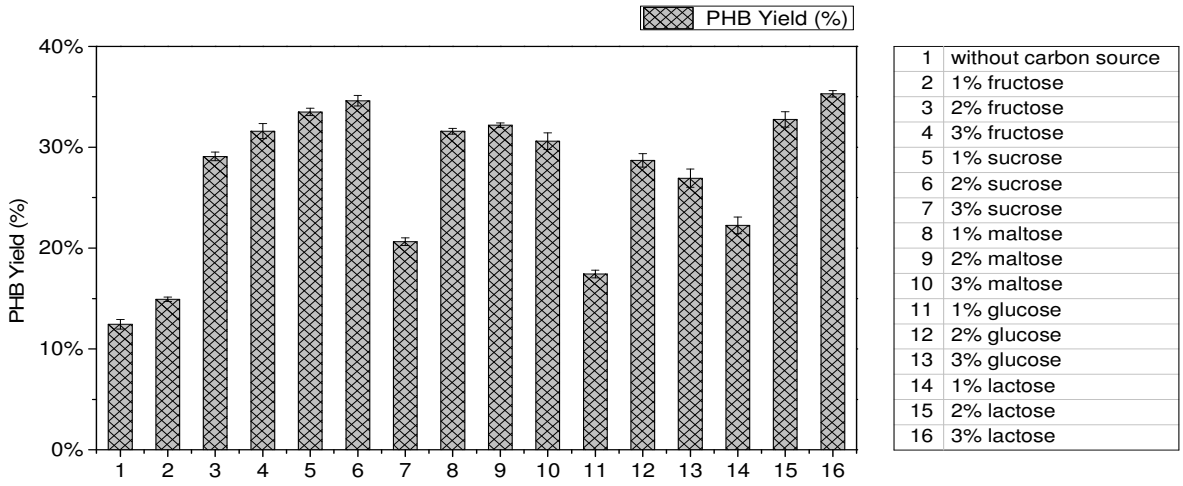


Figure 6.3.12: PHB Yield (%) of bacterial isolate MD5

6.3.1.6: Observation of different carbon sources optimization in isolate MD5

The initial PHB production in MD5 was 12.43%. The cell dry weight without adding any carbon source was observed 3.403 g/l with PHB production was 0.423 g/l. In order to know the best carbon source necessary for high PHB production, optimization was carried out as described in material and method section. It was observed that by utilizing 3% lactose, cdw was 4.22 g/l, PHB production was 1.487 g/l and yield was 35.28%. The utilization of 3% glucose, produced PHB yield of 26.92%, PHB accumulation was 0.917 g/l and cell dry weight of 3.41 g/l. 2% glucose utilization yielded PHB of 28.69%, PHB accumulation was 1.1373 g/l and cell dry weight of 3.96 g/l. 2% sucrose utilization yielded PHB of 34.61%, PHB accumulation was 1.252 g/l and cell dry weight of 3.62 g/l. From the overall analysis it was investigated that 3% lactose induction was the best optimized condition for achieving high PHB (g/l).

6.3.1g: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate AM6

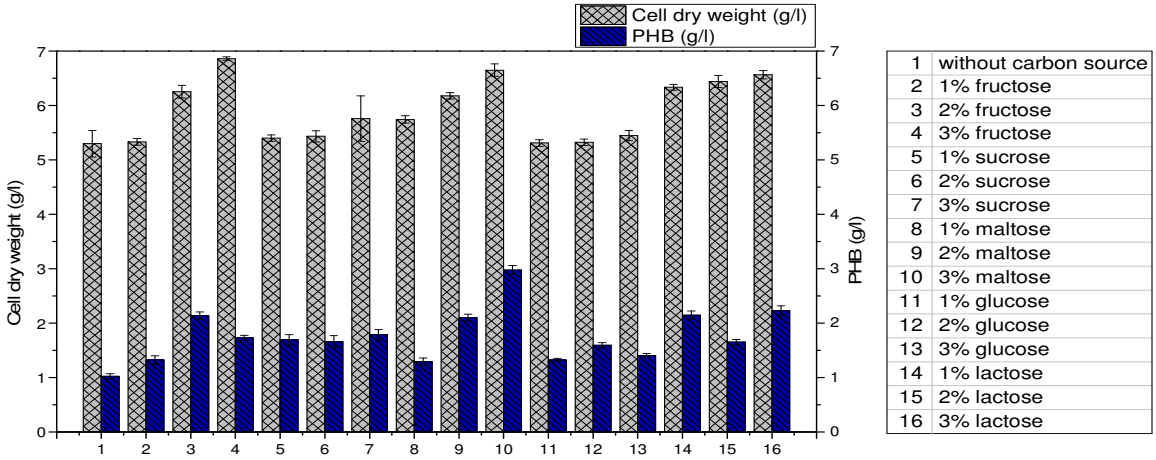


Figure 6.3.13: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate AM6

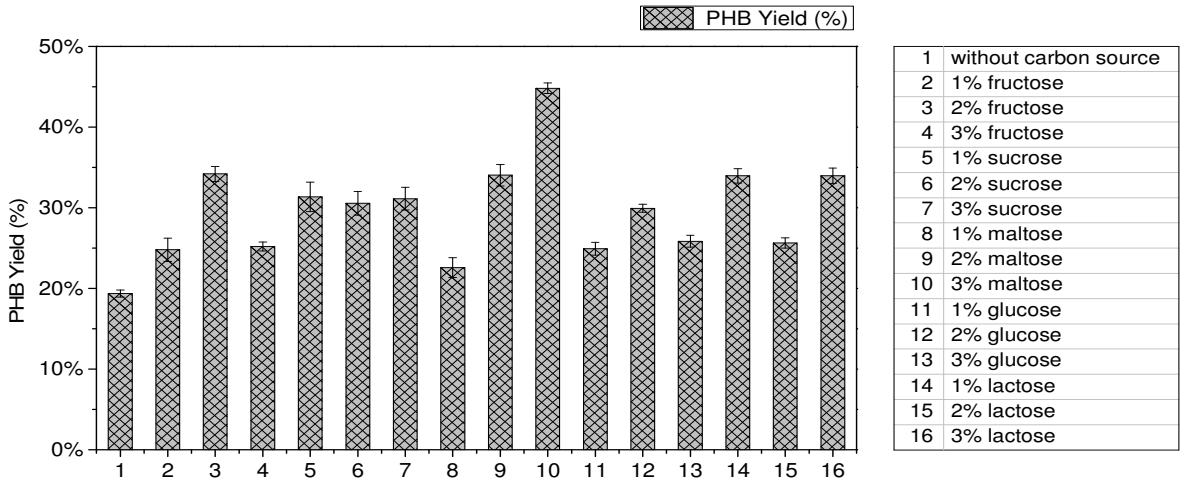


Figure 6.3.14: PHB Yield (%) of bacterial isolate AM6

6.3.1.7: Observation of different carbon sources optimization in isolate AM6

The initial PHB production in AM6 was 19.35%. The cell dry weight without adding any carbon source was observed 5.3 g/l with PHB production was 1.023 g/l. In order to know the best carbon source necessary for high PHB production, optimization was carried out as described in material and method section.

The initial growth curve optimization and utilization of 2% glucose leads to an increase in PHB accumulation level and was observed to be 7.3 g/l of cdw, 2.016 g/l of PHB accumulation and PHB yield of 27.60%. The mutagenesis studies shows that in 100 µg/ml acridine orange, the cdw was 5.323 g/l, PHB accumulation was 1.593 g/l and PHB yield from 29.92%.

It was observed that PHB yield was increased from 19.35% to 44.80% by utilizing 3% maltose as carbon source. PHB production was increased from 1.593 g/l to 2.979 g/l. 3% lactose utilization produced cell dry weight of 6.566 g/l, PHB g/l was 2.229 g/l and PHB yield was 33.94%. The utilization of 1% lactose, produced PHB yield of 33.95%, PHB was 2.149 g/l and cell dry weight of 6.336 g/l. High cell growth and high PHB production are the two important points to be noted to get high PHB yield. From the analysis, it was concluded that 3% maltose was the best optimized carbon induction source for high PHB production.

6.3.1h: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate AM9

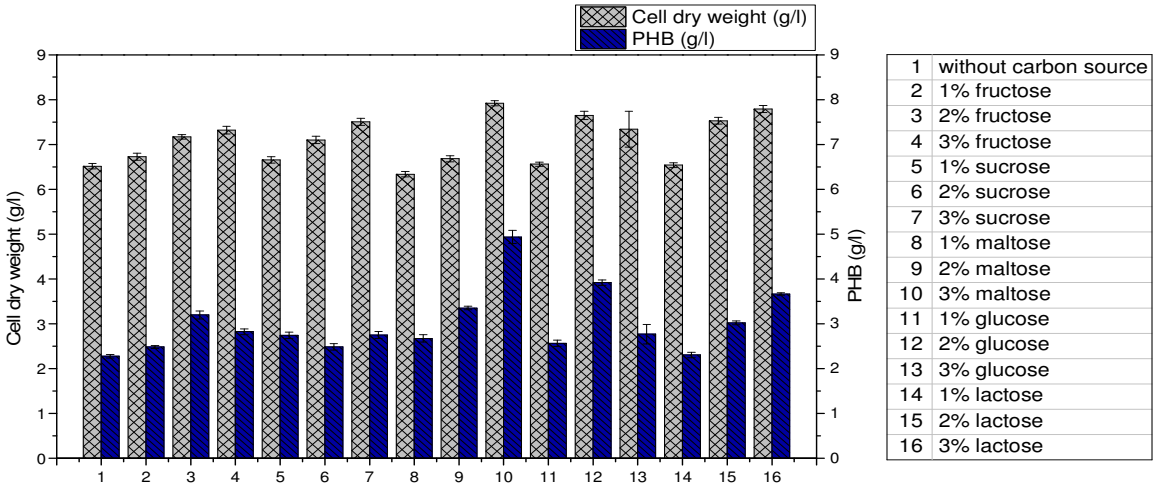


Figure 6.3.15: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate AM9

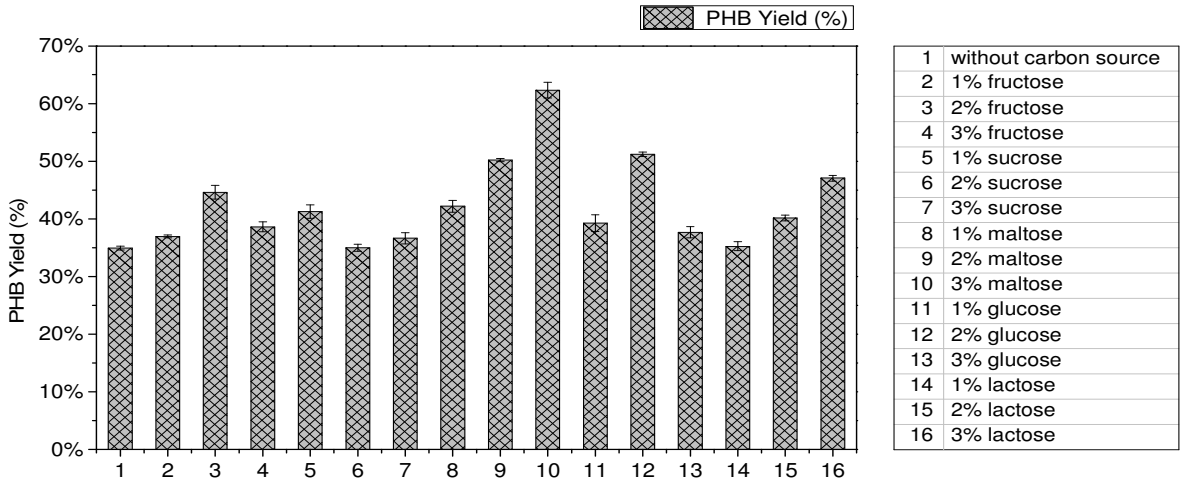


Figure 6.3.16: PHB Yield (%) of bacterial isolate AM9

6.3.1.8: Observation of different carbon sources optimization in isolate AM9

The initial PHB production in AM9 was 34.94%. The cell dry weight without adding any carbon source was observed 6.516 g/l with PHB production was 2.276 g/l. In order to know the best carbon source necessary for high PHB production, optimization was carried out as described in material and method section.

It was observed that PHB yield was increased from 34.94% to 62.34% by utilizing inducing agent 3% sucrose as carbon source. The cdw was increase from 6.51 g/l to 7.92 g/l, whereas PHB production was increased from 2.276 g/l to 4.938 g/l. 2% glucose induction produced cell dry weight of 7.64 g/l, PHB g/l was 3.916 g/l and PHB yield was 51.19%. The induction

of 2% sucrose, produced PHB yield of 50.20%, PHB was 3.353 g/l and cell dry weight of 6.68 g/l. High cell growth and high PHB production are the two important points to be noted to get high PHB yield. From the overall analysis it was investigated that 3% sucrose induction was the best optimized condition for achieving high PHB (g/l).

6.3.1i: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate MTCC 453

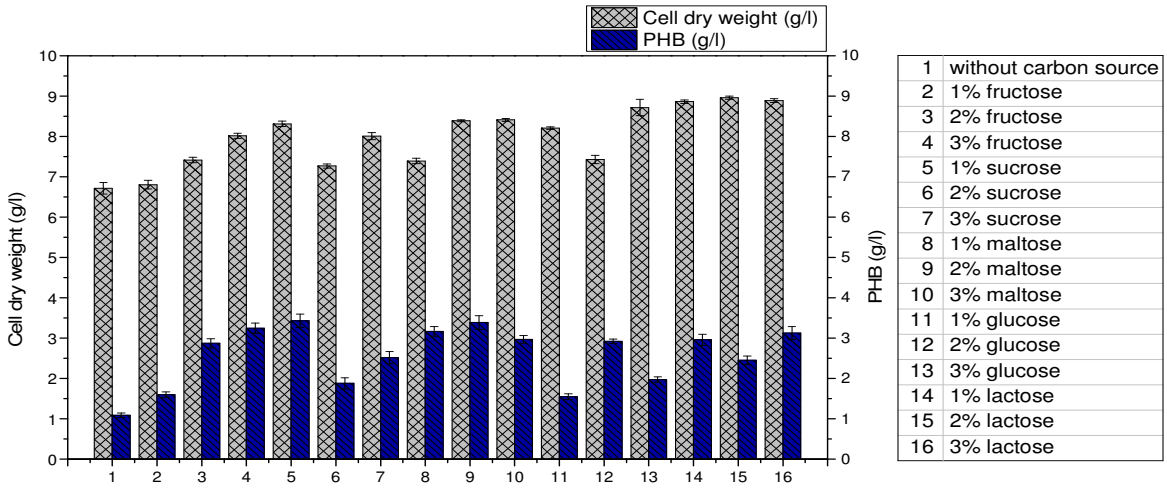


Figure 6.3.17: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate MTCC 453

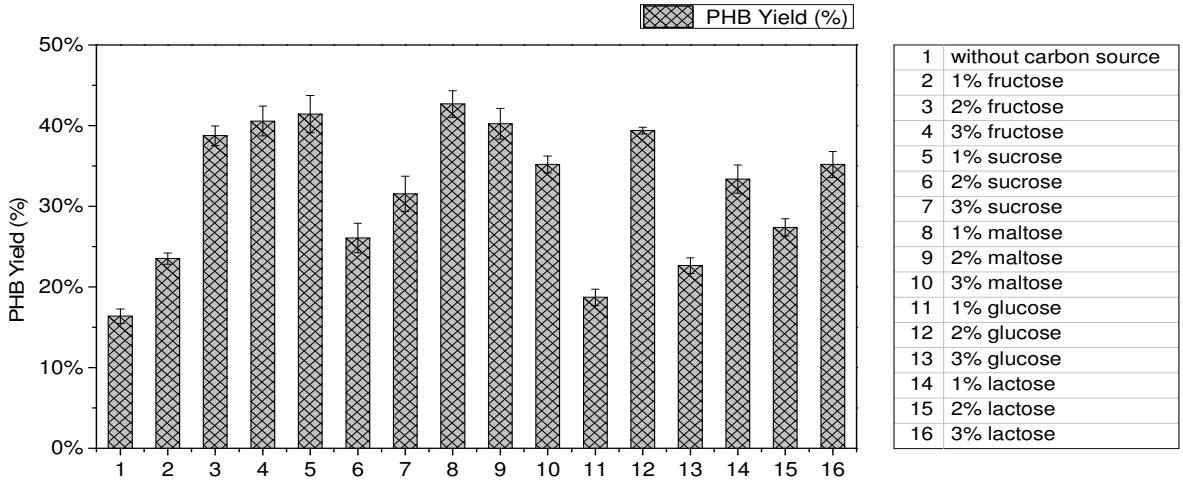


Figure 6.3.18: PHB Yield (%) of bacterial isolate MTCC 453

6.3.1.9: Observation of different carbon sources optimization in isolate MTCC 453

The initial PHB production in MTCC 453 was 16.38%. The cell dry weight without adding any carbon source was observed 6.713 g/l with PHB production was 1.088 g/l. In order to know the best carbon source necessary for high PHB production, optimization was carried out as described in material and method section.

The initial growth curve optimization and utilization of 2% glucose leads to an increase in PHB accumulation level and was observed to be 9.616 g/l of cdw, 3.548 g/l of PHB accumulation and PHB yield of 36.85%. The mutagenesis studies shows that in 50 µg/ml of sodium azide treatment, the cdw was 7.423 g/l, PHB accumulation was 2.923 g/l and PHB yield from 39.38%.

It was observed that by utilizing 1% sucrose, cdw was 8.31 g/l, PHB production was 3.429 g/l and yield was 41.42%. The induction of 2% maltose, produced PHB yield of 40.23%, PHB was 3.4384 g/l and cell dry weight of 8.386 g/l. 1% maltose induction yielded PHB of 42.68%, PHB was 3.165 g/l and cell dry weight of 7.39 g/l. High cell growth and high PHB production are the two important points to be noted to get high PHB yield. From the overall analysis it was demonstrated that 1% maltose induction was the best optimized condition for achieving high PHB (g/l).

6.3.1j: Graphical representation of Cell dry weight (g/l), PHB (g/l) and PHB yield (%) of bacterial isolate MTCC 617

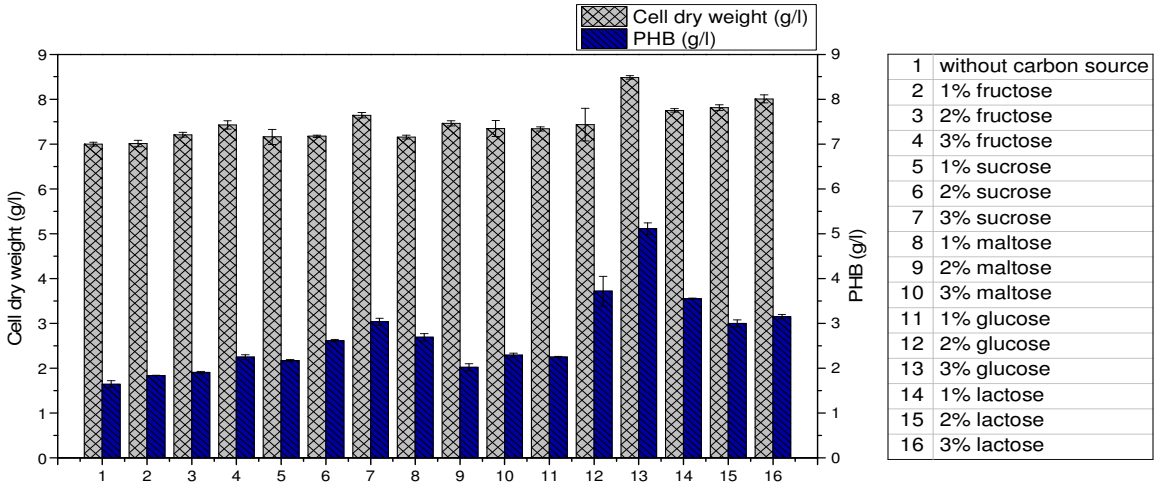


Figure 6.3.19: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate MTCC 617

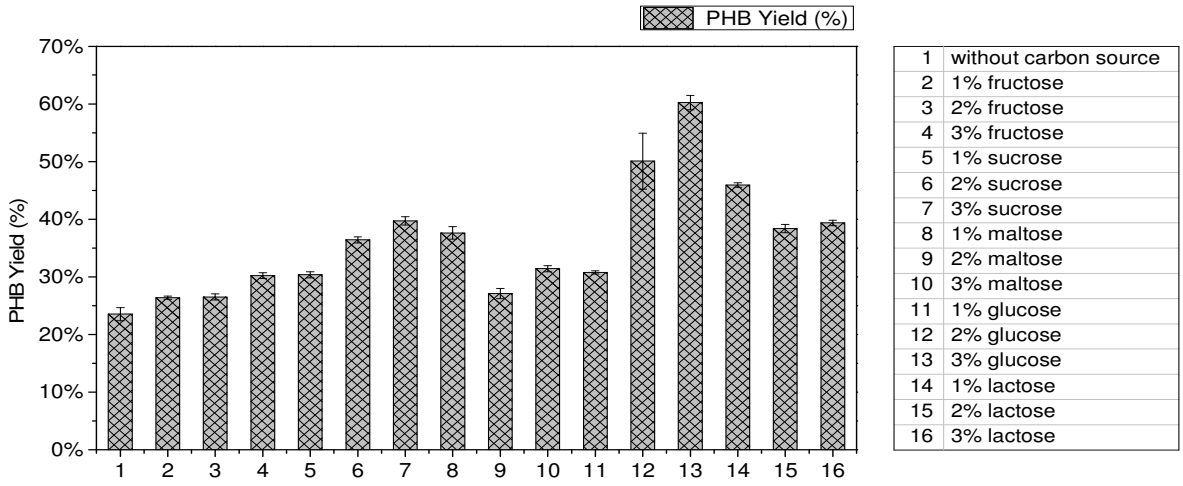


Figure 6.3.20: PHB Yield (%) of bacterial isolate MTCC 617

6.3.1.10: Observation of different carbon sources optimization in isolate MTCC 617

The initial PHB production in MTCC617 was 24%. The cell dry weight without adding any carbon source was observed 7 g/l and PHB production was 1.646 g/l. In order to know the best carbon source necessary for high PHB production, optimization was carried out as described in material and method section. It was observed that maximum PHB yield was observed by utilizing 3% glucose source.

The initial growth curve optimization and utilization of 2% glucose leads to an increase in PHB accumulation level and was observed to be 8.7 g/l of cdw, 4.166 g/l of PHB accumulation and PHB yield of 48.44%. The mutagenesis studies shows that in UV 1min exposure, the cdw was 7.43 g/l, PHB accumulation was 3.716 g/l and PHB yield from 50.08%.

The induction utilization of 3% glucose, produced PHB yield of 60%, PHB accumulation was 5.11 g/l and cell dry weight of 8.483 g/l. From the overall analysis it was investigated that 3% glucose induction was the best optimized condition for achieving high PHB (g/l).

6.3.2: Results of Nitrogen source optimization added at media preparation stage

Nitrogen Source optimization was carried out with addition of different nitrogen sources in mineral medium for normal agriculture soil isolates and DSC-97 for halophiles isolated from Saltren Sambhar Lake sites as described in material and method section. The cultures were induced further with the best carbon source concentration obtained during carbon source optimization stage and similar methodology was followed.

The different nitrogen sources taken for optimization were peptone, yeast extract, ammonium carbonate, ammonium sulphate and ammonium chloride.

6.3.2(i): Graphical representation of Nitrogen optimization studies in different bacterial strains

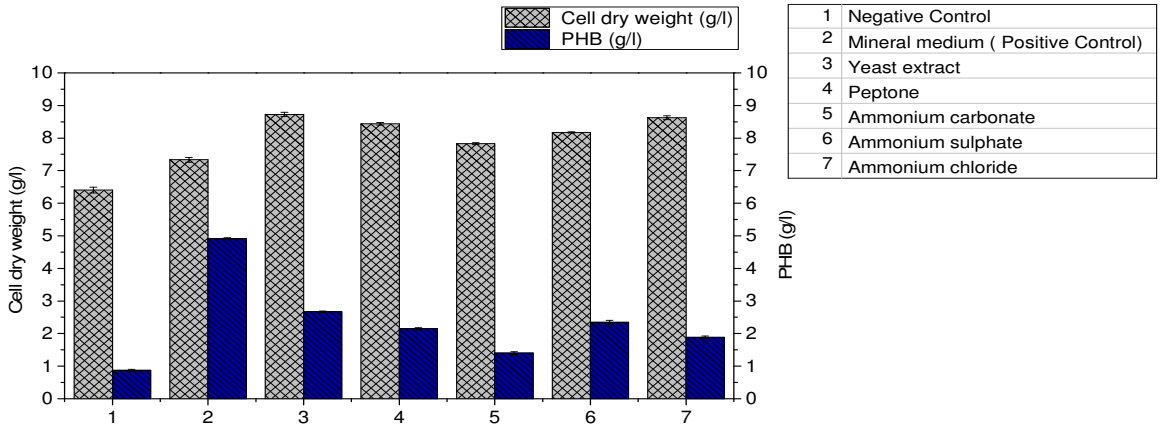


Figure 6.3.21: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate S-3

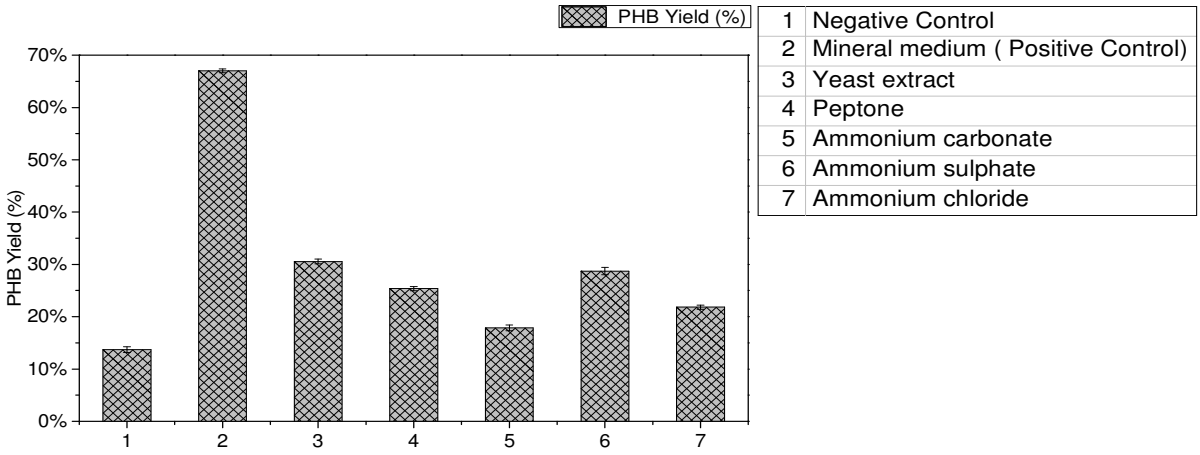


Figure 6.3.22: PHB yield (%) of bacterial isolate S-3

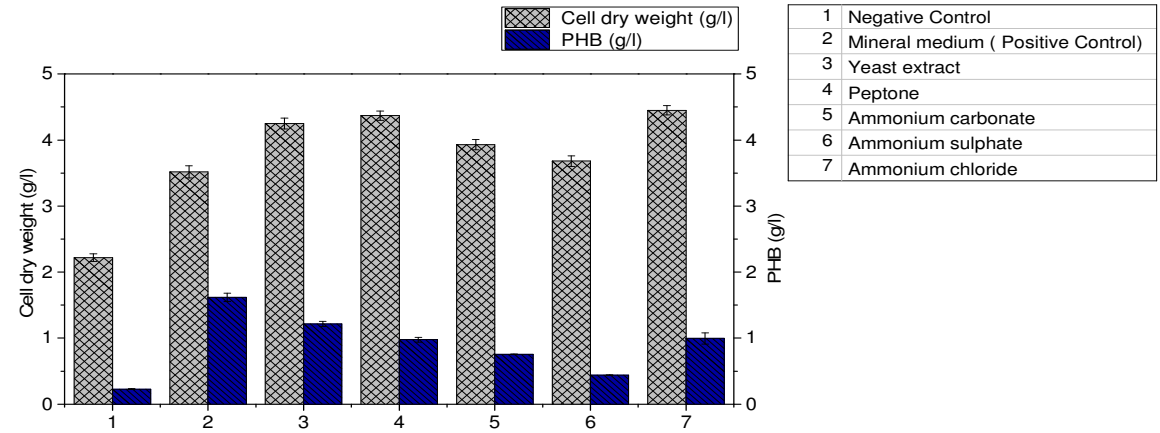


Figure 6.3.23: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate M1

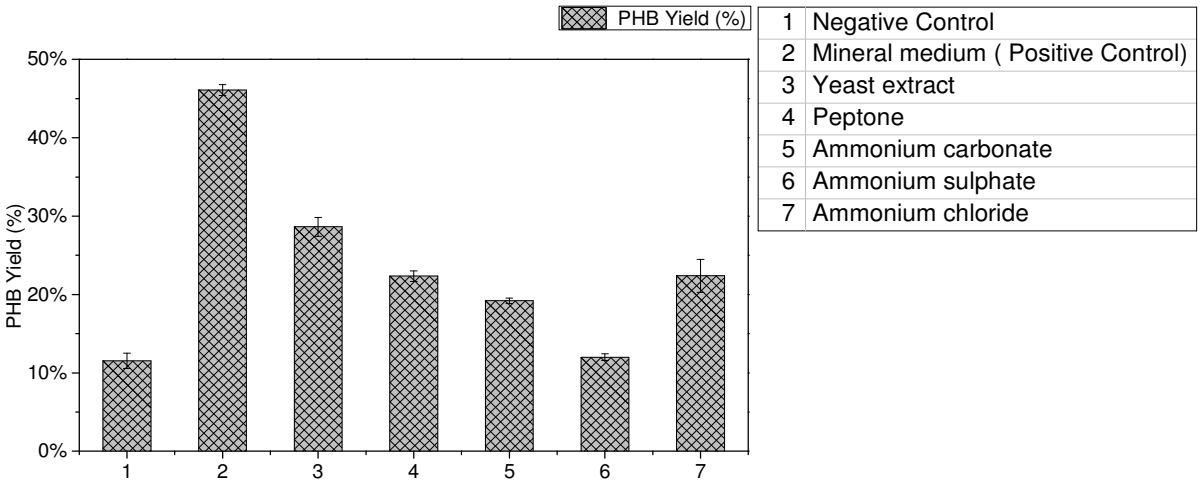


Figure 6.3.24: PHB yield (%) of bacterial isolate M1

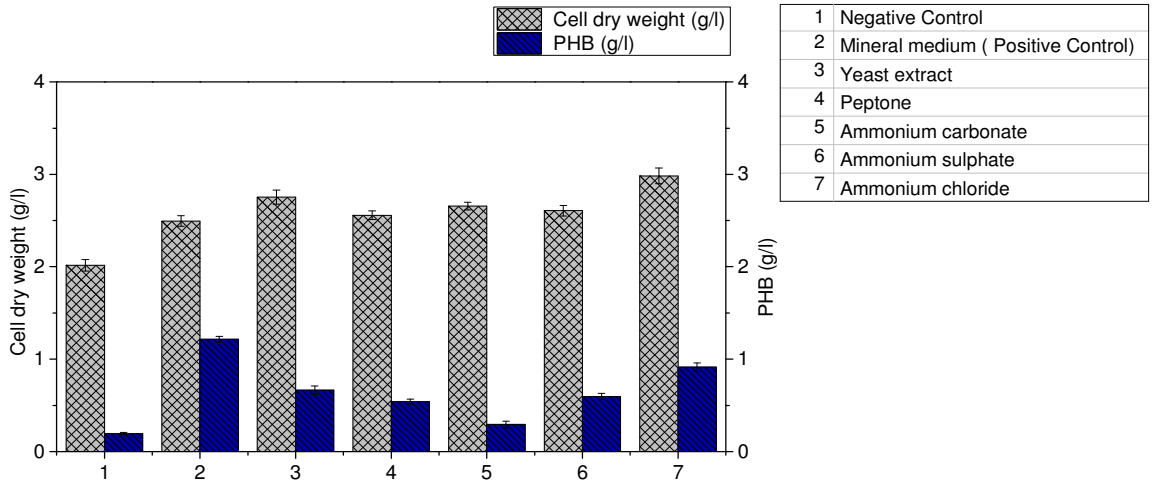


Figure 6.3.25: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate M10

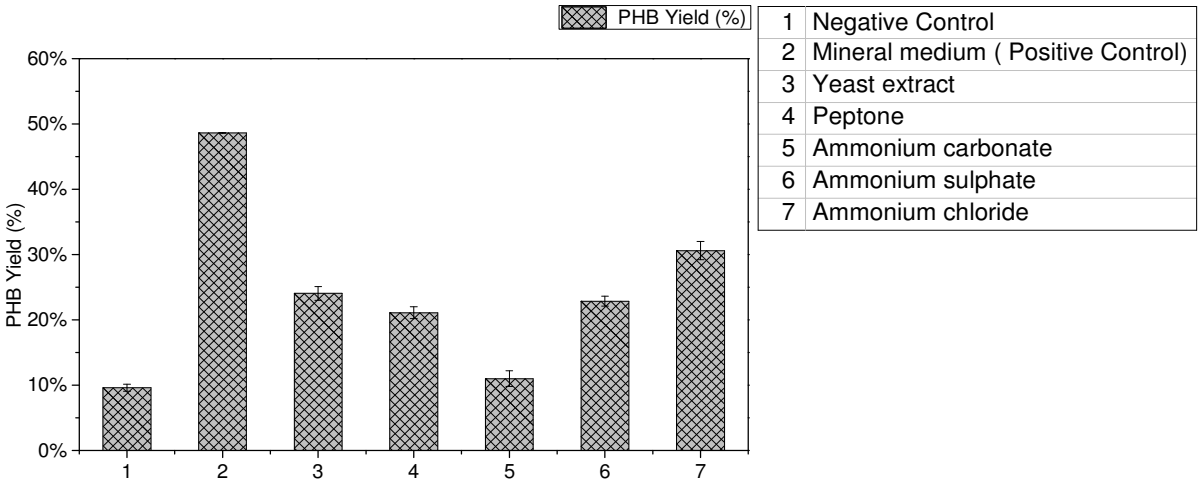


Figure 6.3.26: PHB yield (%) of bacterial isolate M10

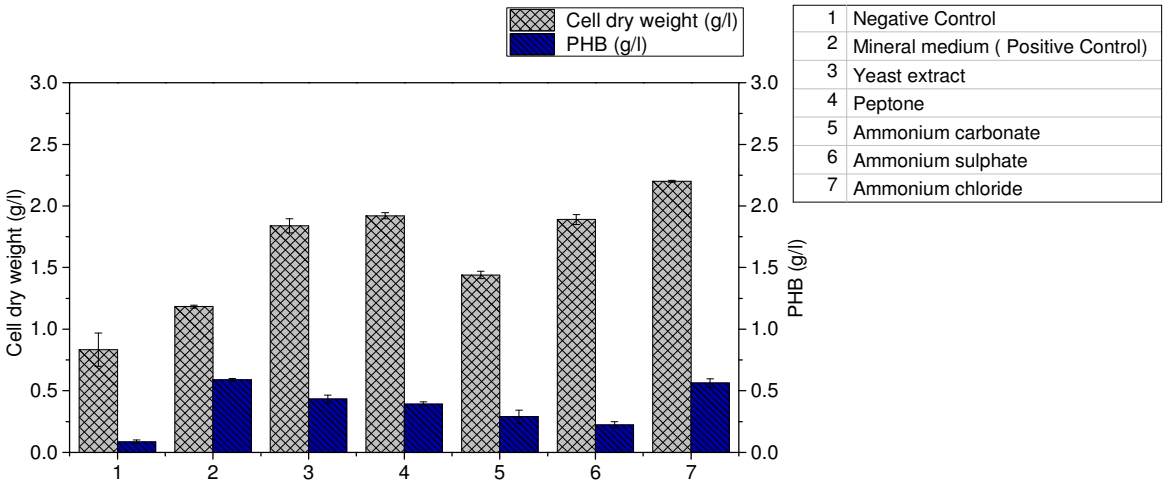


Figure 6.3.27: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate MD1

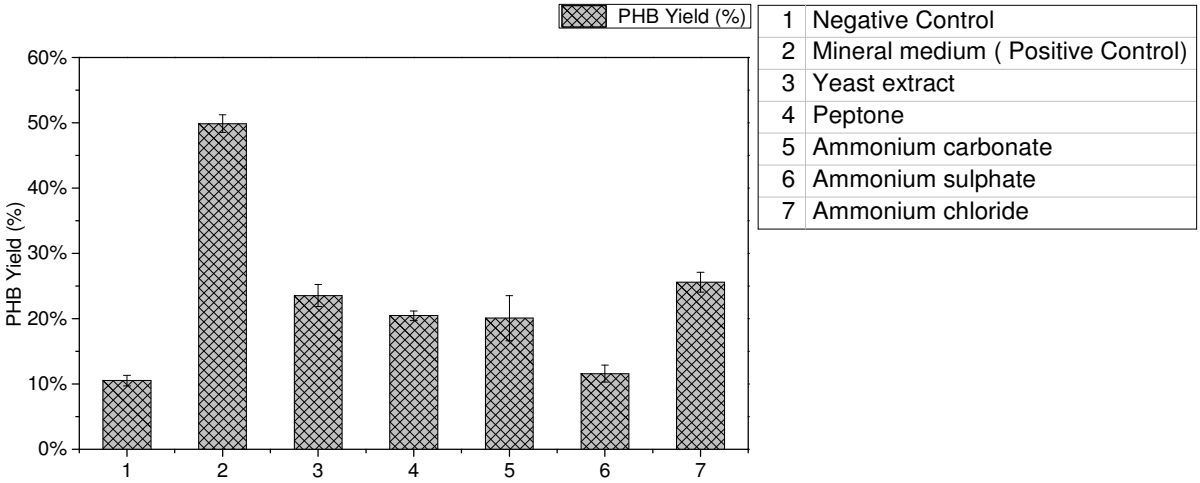


Figure 6.3.28: PHB yield (%) of bacterial isolate MD1

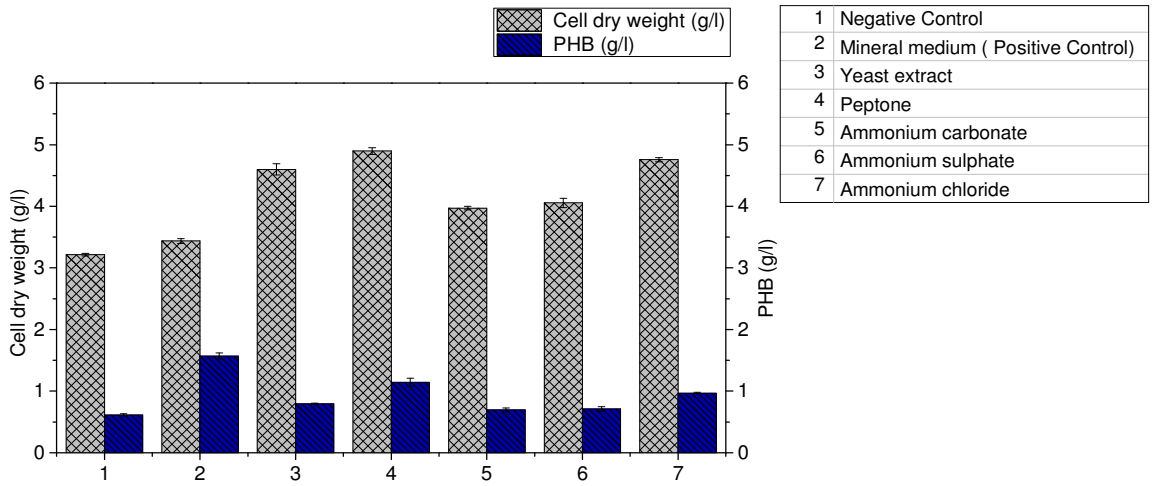


Figure 6.3.29: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate MD2

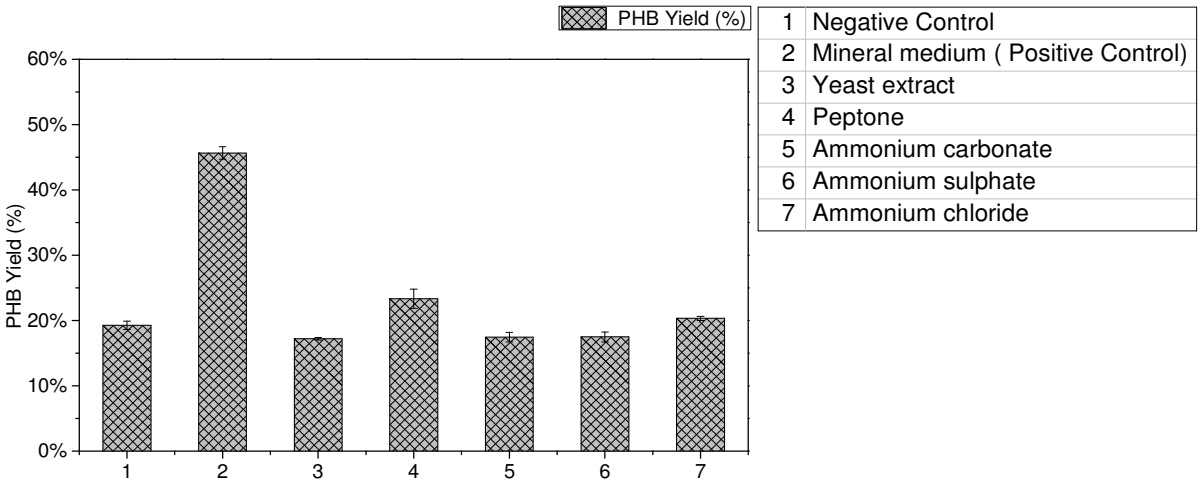


Figure 6.3.30: PHB yield (%) of bacterial isolate MD2

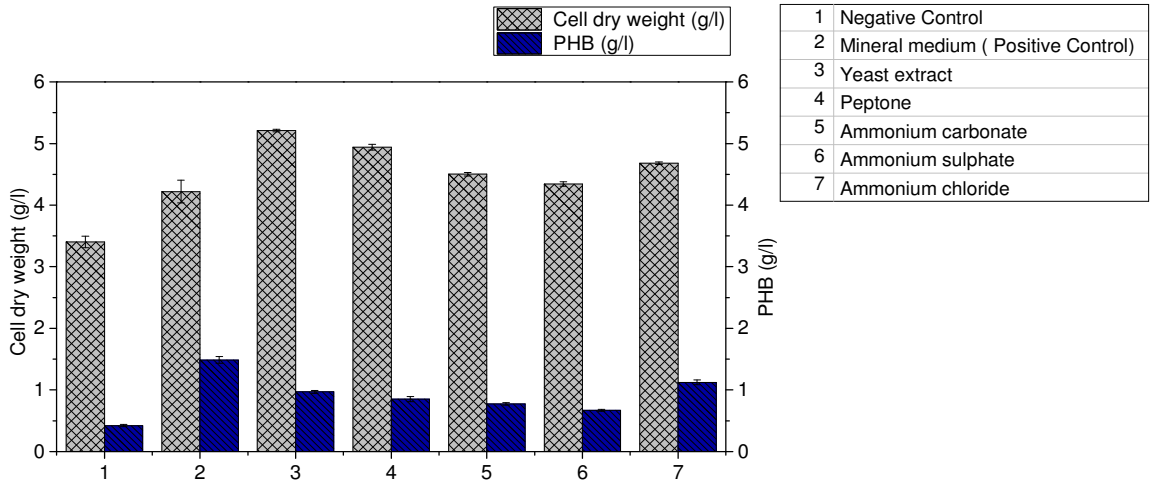


Figure 6.3.31: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate MD5

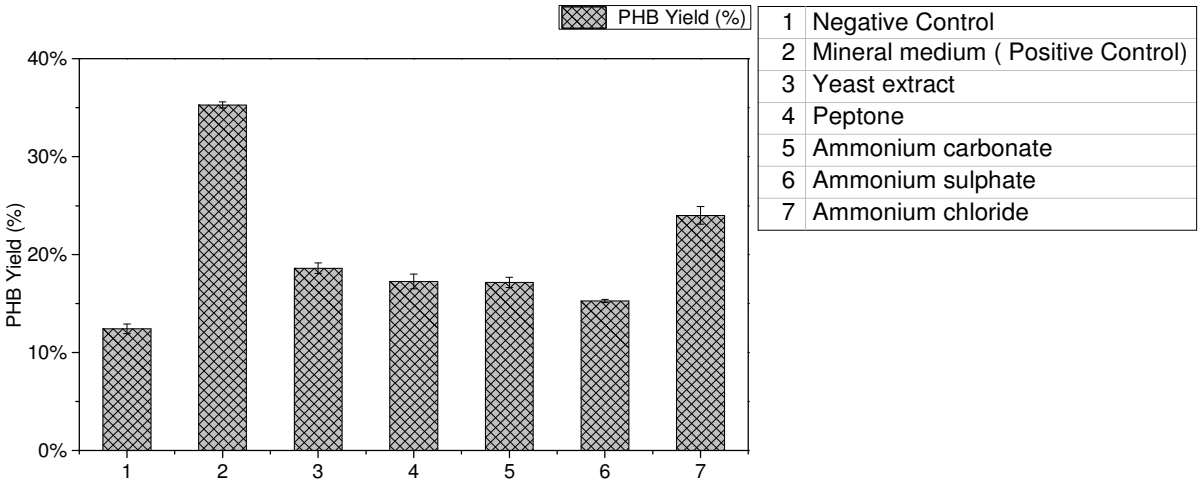


Figure 6.3.32: PHB yield (%) of bacterial isolate MD5

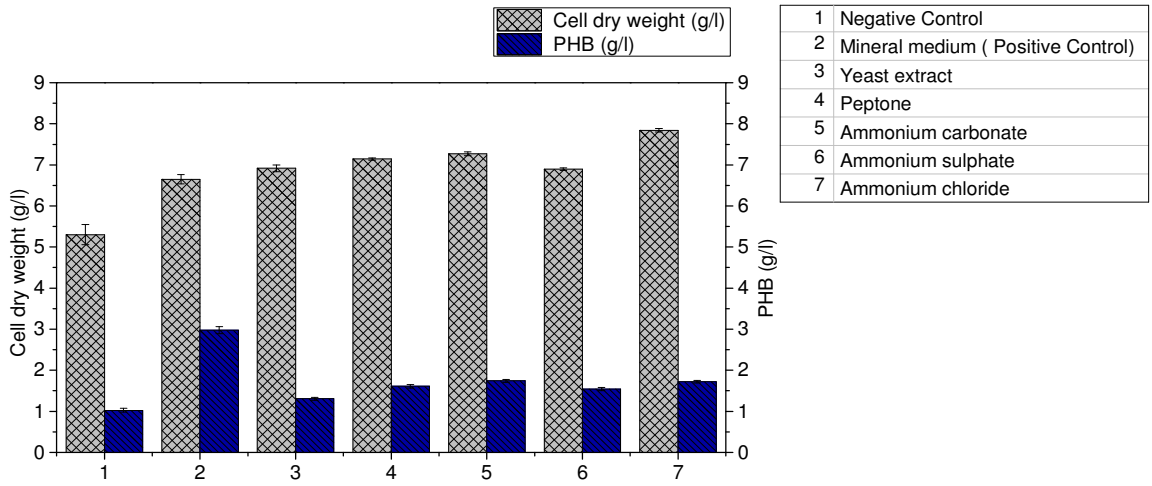


Figure 6.3.33: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate AM6

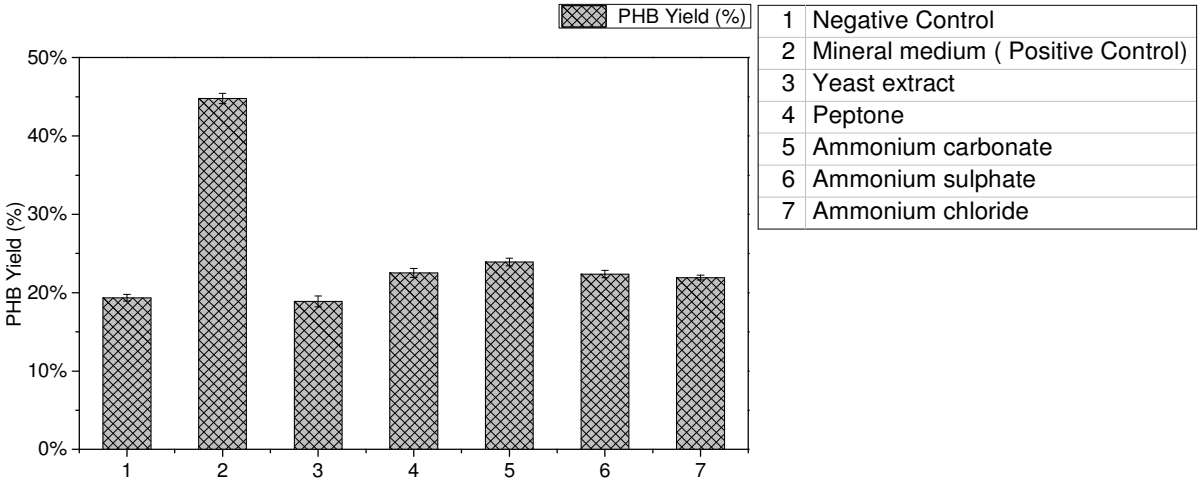


Figure 6.3.34: PHB yield (%) of bacterial isolate AM6

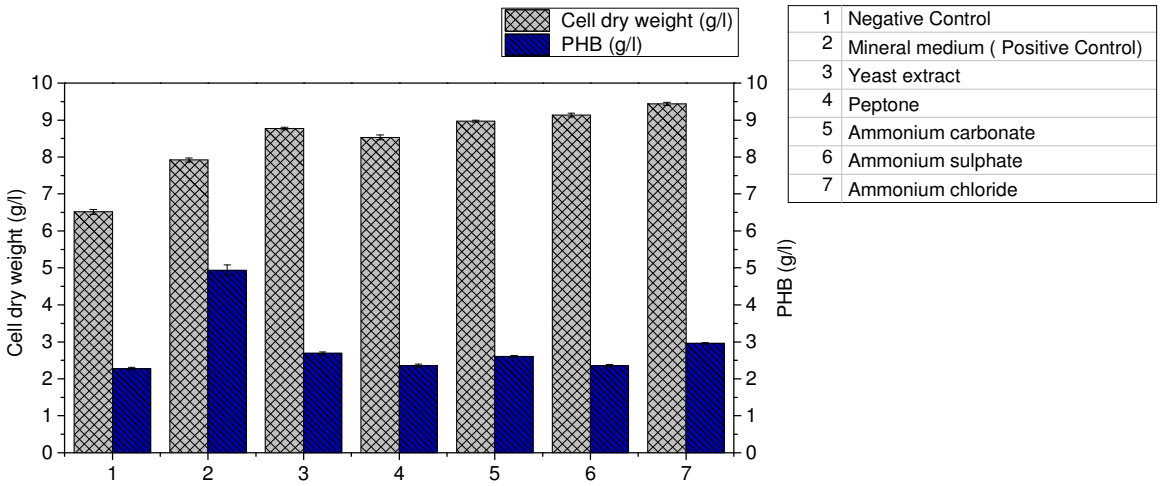


Figure 6.3.35: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate AM9

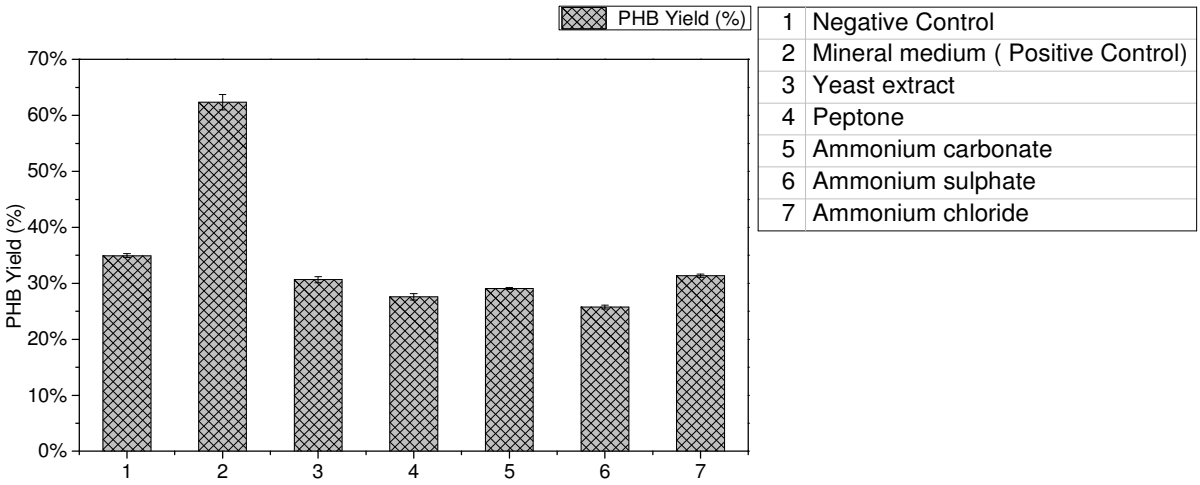


Figure 6.3.36: PHB yield (%) of bacterial isolate AM9

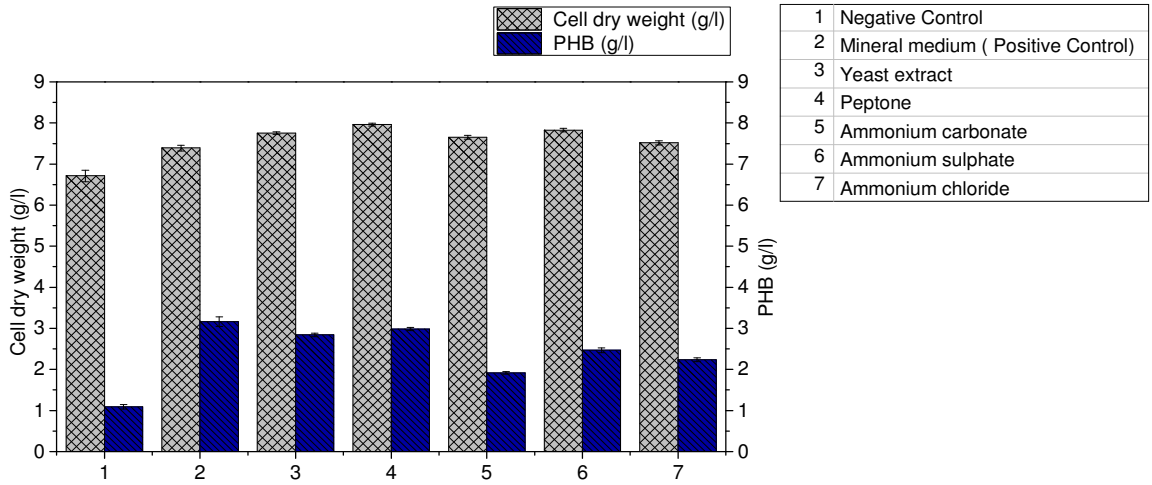


Figure 6.3.37: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate MTCC 453

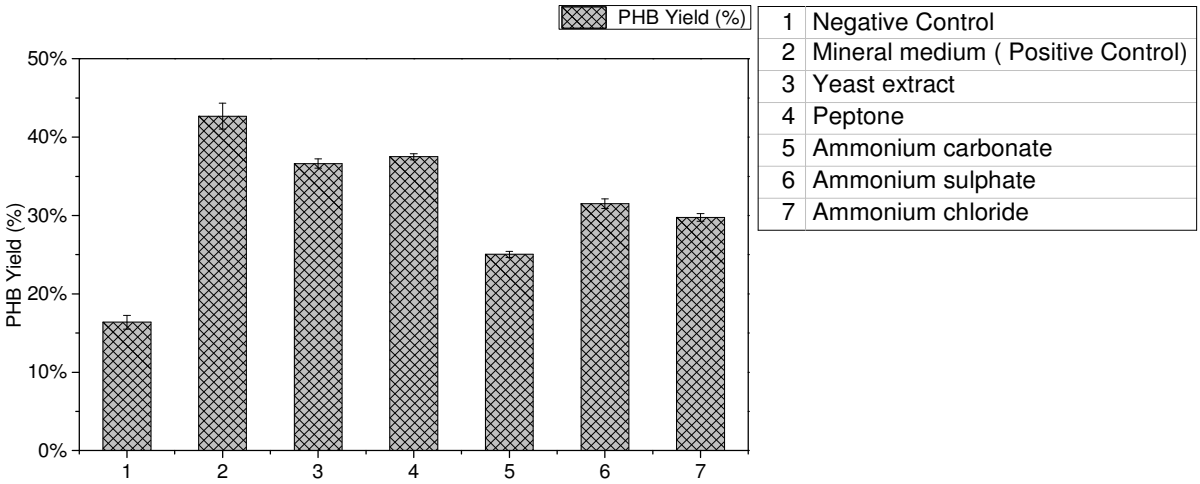


Figure 6.3.38: PHB yield (%) of bacterial isolate MTCC 453

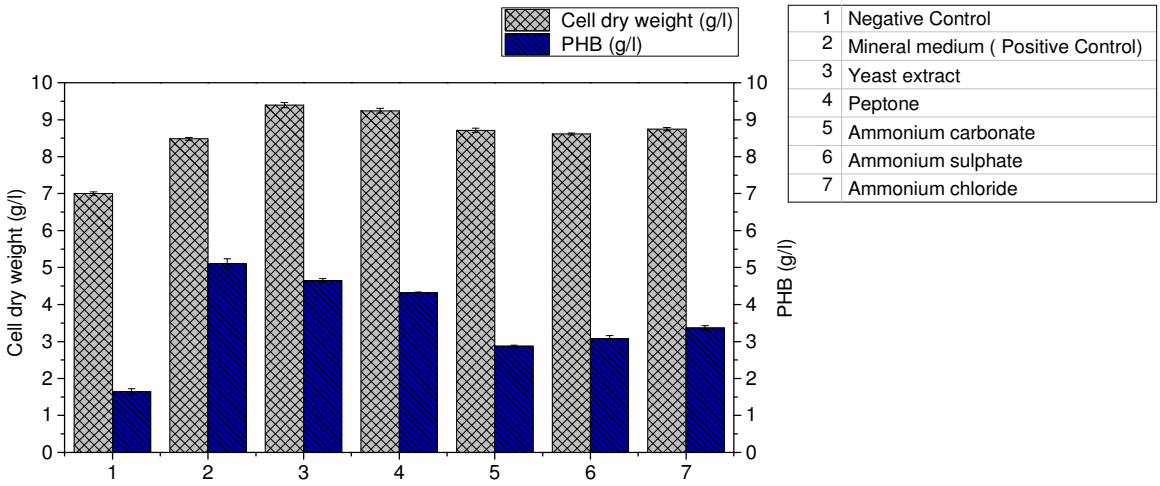


Figure 6.3.39: Cell dry weight (g/l) and PHB (g/l) of bacterial isolate MTCC 617

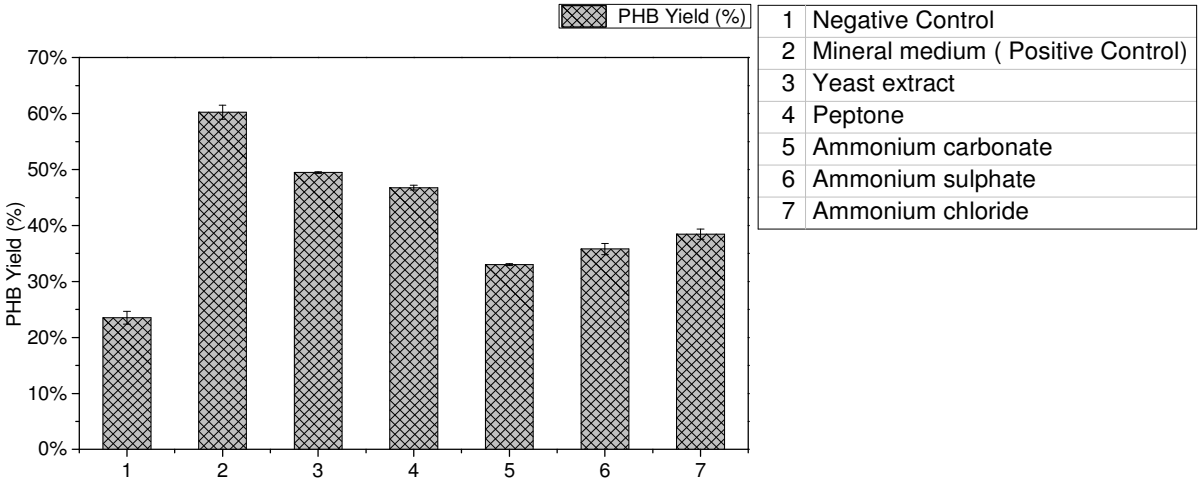


Figure 6.3.40: PHB yield (%) of bacterial isolate MTCC 617

6.3.2.1: Observation of nitrogen source optimization in biphasic culture condition

The biphasic culture protocol for determination and optimization of best nitrogen source was carried out as described in material and method section.

It was observed that in S-3 bacterial isolate, negative control (without any carbon and nitrogen source, basic mineral media), that cdw was 6.403 g/l, PHB accumulation was 0.8773 g/l and PHB yield was 13.70%. Best carbon source optimized condition (2% maltose) in mineral medium was taken as positive control. It was observed in this condition that cell mass was increased to 7.33 g/l, PHB accumulation was 4.912 g/l and PHB yield was elevated to 67.03%. Supplementation of different nitrogen sources shows that yeast extract, cdw was 8.726 g/l, PHB production was 2.67 g/l and yield was 30.56% which was the highest value of PHB production achieved. In case of ammonium carbonate, cdw of 7.826 g/l, PHB production

was 1.4 g/l and yield was 17.86% and was the lowest value achieved.

It was observed that in M1 bacterial isolate, negative control (without any carbon and nitrogen source, basic mineral media), that cdw was 2.218 g/l, PHB accumulation was 0.228 g/l and PHB yield was 11.52%. Best carbon source optimized condition (3% glucose) in mineral medium was taken as positive control. It was observed in this condition that cell mass was increased to 3.516 g/l, PHB accumulation was 1.618 g/l and PHB yield was elevate to 46.11%. Supplementation of different nitrogen sources shows that yeast extract, cdw was 4.251 g/l, PHB production was 1.216 g/l and yield was 28.63% which was the highest value of PHB production achieved. In case of ammonium sulphate, cdw of 3.68 g/l, PHB production was 0.4406 g/l and yield was 11.98% and was the lowest value achieved.

It was observed that in M10 bacterial isolate, negative control (without any carbon and nitrogen source, basic mineral media), that cdw was 2.0166 g/l, PHB accumulation was 0.1942 g/l and PHB yield was 9.63%. Best carbon source optimized condition (3% glucose) in mineral medium was taken as positive control. It was observed in this condition that cell mass was increased to 2.493 g/l, PHB accumulation was 1.215 g/l and PHB yield was elevated to 48.65%. Supplementation of different nitrogen sources shows that ammonium chloride, cdw was 2.983 g/l, PHB production was 0.913 g/l and yield was 30.62% which was the highest value of PHB production achieved. In case of ammonium carbonate, cdw of 2.656 g/l, PHB production was 0.293 g/l and yield was 11.02% and was the lowest value achieved.

It was observed that in MD1 bacterial isolate, negative control (without any carbon and nitrogen source, basic mineral media), that cdw was 0.833 g/l, PHB accumulation was 0.084 g/l and PHB yield was 10.52%. Best carbon source optimized condition (3% maltose) in mineral medium was taken as positive control. It was observed in this condition that cell mass was increased to 1.183 g/l, PHB accumulation was 0.59 g/l and PHB yield was elevated to 49.89%. Supplementation of different nitrogen sources shows that ammonium chloride, cdw was 2.2 g/l, PHB production was 0.563 g/l and yield was 25.60% which was the highest value of PHB production achieved. In case of ammonium sulphate, cdw of 1.89 g/l, PHB production was 0.223 g/l and yield was 11.60% and was the lowest value achieved.

It was observed that in MD2 bacterial isolate, negative control (without any carbon and nitrogen source, basic mineral media), that cdw was 3.213 g/l, PHB accumulation was 0.6135 g/l and PHB yield was 19.28%. Best carbon source optimized condition (3% sucrose) in mineral medium was taken as positive control. It was observed in this condition that cell mass was increased to 3.436 g/l, PHB accumulation was 1.569 g/l and PHB yield was elevated to 45.67%. Supplementation of different nitrogen sources shows that peptone, cdw was 4.896 g/l, PHB production was 1.143 g/l and yield was 23.35% which was the highest value of PHB production achieved. In case of yeast extract, cdw of 4.6 g/l, PHB production was 0.7926 g/l and yield was 17.23% and was the lowest value achieved.

It was observed that in MD5 bacterial isolate, negative control (without any carbon and nitrogen source, basic mineral media), that cdw was 3.403 g/l, PHB accumulation was 0.423 g/l and PHB yield was 12.43%. Best carbon source optimized condition (3% lactose) in mineral medium was taken as positive control. It was observed in this condition that cell mass was increased to 4.22 g/l, PHB accumulation was 1.487 g/l and PHB yield was elevated to 35.28%. Supplementation of different nitrogen sources shows that ammonium chloride, cdw was 4.68 g/l, PHB production was 1.123 g/l and yield was 24% which was the highest value of PHB production achieved. In case of ammonium sulphate, cdw of 4.8343 g/l, PHB production was 0.67 g/l and yield was 15.27% and was the lowest value achieved.

It was observed that in AM6 bacterial isolate, negative control (without any carbon and nitrogen source, basic mineral media), that cdw was 5.3 g/l, PHB accumulation was 1.023 g/l and PHB yield was 19.35%. Best carbon source optimized condition (3% maltose) in mineral medium was taken as positive control. It was observed in this condition that cell mass was increased to 6.65 g/l, PHB accumulation was 2.976 g/l and PHB yield was elevated to 44.80%. Supplementation of different nitrogen sources shows that ammonium carbonate, cdw was 7.27 g/l, PHB production was 1.74 g/l and yield was 23.93% which was the highest value of PHB production achieved. In case of yeast extract, cdw of 6.916 g/l, PHB production was 1.306 g/l and yield was 18.90% and was the lowest value achieved.

It was observed in AM9 negative control (without any carbon and nitrogen source, basic mineral media), that cdw was 6.51 g/l, PHB accumulation was 2.276 g/l and PHB yield was 34.94%. Best carbon source optimized condition (3% Sucrose) in mineral medium was taken

as positive control. It was observed in this condition that cell mass was increased to 7.92 g/l, PHB accumulation was 4.938 g/l and PHB yield was elevated to 62.34%. Supplementation of different nitrogen sources shows that ammonium chloride cdw was 9.44 g/l, PHB production was 2.96 g/l and yield was 31.35% which was the highest value of PHB production achieved. In case of ammonium sulphate, cdw of 9.133 g/l, PHB production was 2.353 g/l and yield was 25.76% and was the lowest value achieved.

It was observed that in MTCC 453 bacterial isolate, negative control (without any carbon and nitrogen source, basic mineral media), that cdw was 6.713 g/l, PHB accumulation was 1.088 g/l and PHB yield was 16.38%. Best carbon source optimized condition (1% maltose) in mineral medium was taken as positive control. It was observed in this condition that cell mass was increased to 7.39 g/l, PHB accumulation was 3.165 g/l and PHB yield was elevated to 42.68%. Supplementation of different nitrogen sources shows that peptone, cdw was 7.96 g/l, PHB production was 2.985 g/l and yield was 37.50% which was the highest value of PHB production achieved. In case of ammonium chloride, cdw of 7.516 g/l, PHB production was 2.236 g/l and yield was 29.75% and was the lowest value achieved.

It was observed that in MTCC 617 bacterial isolate, negative control (without any carbon and nitrogen source, basic mineral media), that cdw was 7 g/l, PHB accumulation was 1.646 g/l and PHB yield was 24%. Best carbon source optimized condition (3% glucose) in mineral medium was taken as positive control. It was observed in this condition that cell mass was increased to 8.483 g/l, PHB accumulation was 5.11 g/l and PHB yield was elevated to 60%. Supplementation of different nitrogen sources shows that yeast extract, cdw was 9.396 g/l, PHB production was 4.65 g/l and yield was 49% which was the highest value of PHB production achieved. In case of ammonium carbonate, cdw of 8.713 g/l, PHB production was 2.876 g/l and yield was 33% and was the lowest value achieved.

The above analysis shows that supplementation of nitrogen source with optimized carbon source, promotes cell biomass growth and decreases the PHB production level as compared to the positive control, which only utilizes the carbon source. It has already been reported in literature and demonstrated earlier that in the condition of presence of carbon along with the nitrogen source, the major part of the energy is utilized in the production of biomass. Hence, in this condition, where both carbon and nitrogen sources are present, PHB production is

reduced while the biomass production elevates. The results are in coherence with Wang and Lee, 1997.

6.3.3: Large Scale growth in fermenter for high PHB production

Large scale growth was carried out in 3 litre fermenter, working volume 2.75 litre with the mutated strain S-3 as described in material and method section. Mutated strain S-3 was fermented at optimized concentration of carbon i.e. 2% maltose, 2 M salt concentration, 37^{o}C temperature, incubation time 96 hrs, agitation speed of 120 rpm and 7.2 pH was used for carrying out large scale fermentation. Initially at lab scale studies 7.33 g/l cdw was obtained along with PHB accumulation of 4.912 g/l. In large scale fermenter the cdw values were increased to 7.63 g/l with PHB accumulation of 5.263 g/l. The PHB yield was analyzed to be 68.95%. The increase in values reflected better conditions of growth available in a fermenter with better aeration, mixing along with larger volume of media. All of these parameters could have contributed towards higher yield of PHB.

6.3.4: Statistical analysis of highest PHB producing strains by using ANOVA SPSS 16.0 tool

It was analyzed from the above results that S-3strain of Majha Region and AM9 strain of Sambhar Lake Region was the highest PHB producers at different set of parameters. These parameters were statistically analyzed by ANOVA SPSS 16.0 tool.

6.3.4.1: Statistical analysis of high PHB producer S-3 strain

The parameters were as follow:

In S-3 strain, PHB yield was 13.70% without the supplementation of carbon source. The strategies followed to increase the PHB yield are described below. It was analyzed that with the supplementation of 2% glucose, induction of PHB production occurs and the yield increases to 40.13%. It was observed that mutagenesis by acridine orange at 100µg/ml under 2% glucose induction increased the PHB production to 50.53%. Further, optimization of mutated strain by carbon source with 2% maltose, increased the PHB yield to 67.03%.

Table 6.35: Comparison of different optimization parameters in S-3 strain for high PHB yield using one way ANOVA

S-3 strain	**Mean± Standard deviation**	**P value≥0.05**
Wild strain without carbon source	13.7033± 0.69945	0.00
Wild strain with 2%glucose	50.7000±3.15753	0.00
Mutated strain at 100µg/ml A.O with 2% glucose	40.1267±3.9359	0.00
Mutated strain with 2% maltose	67.0333± 20.36594	0.00

Table 6.36: Statistical analysis of multiple comparison of S-3 strain in different optimized parameters for PHB Yield using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

S-3 strain		**Mean Difference**	**P value≥ 0.05**
A	B	(A-B)	
Wild strain without carbon source	Wild strain with 2% glucose	-36.996*	.00
	Mutated strain at 100 µg/ml A.O with 2% glucose	-26.423*	.00
	Mutated strain with 2% maltose	-53.330*	.00
Wild strain with 2% glucose	Wild strain without carbon source	36.996*	.00
	Mutated strain at 100 µg/ml A.O with 2% glucose	10.573*	.00
	Mutated strain with 2% maltose	-16.333*	.00
Mutated strain at 100 µg/ml A.O with 2% glucose	Wild strain without carbon source	26.423*	.00
	Wild strain with 2% glucose	-10.573*	.00
	Mutated strain with 2% maltose	-26.906*	.00
Mutated strain with 2% maltose	Wild strain without carbon source	53.330*	.00
	Wild strain with 2% glucose	16.333*	.00
	Mutated strain at 100 µg/ml A.O with 2% glucose	26.906*	.00

The analysis shows that the values with superscript * were statistically significant (p≥0.05).

6.3.4.2: Statistical analysis of high PHB producer AM9 strain

The parameters were as follow:

In AM9 strain, PHB yield was 34.94% without the supplementation of carbon source. The strategies followed to increase the PHB yield are described below. It was analyzed that with the supplementation of 2% glucose, induction of PHB production occurs and the yield increases to 51.19%. Further, optimization of AM9 strain by carbon source with 3% sucrose, increased the PHB yield to 62.34%.

Table 6.37: Comparison of different optimization parameters in AM9 strain for high PHB yield using one way ANOVA

AM9 strain	**Mean± Standard deviation**	**P value≥0.05**
AM9 strain without carbon source	34.9367± 0.42852	0.00
AM9 strain with 2%glucose	51.1867±0.46694	0.00
AM9 strain with 3% sucrose	62.3433± 1.68791	0.00

Table 6.38: Statistical analysis of multiple comparison of AM9 strain in different optimized parameters for PHB Yield using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

S-3 strain		**Mean Difference**	**P value≥0.05**
A	B	(A-B)	
AM9 strain without carbon source	AM9 strain with 2% glucose	-16.25000*	.00
	AM9 strain with 3% sucrose	-27.40667*	.00
AM9 strain with 2% glucose	AM9 strain without carbon source	16.25000*	.00
	AM9 strain with 3% sucrose	-11.15667*	.00
Mutated strain at 100 µg/ml A.O	AM9 strain without carbon source	27.40667*	.00
	AM9 strain with 2% glucose	-11.15667*	.00

The analysis shows that the values with superscript * were statistically significant (p≥0.05).

6.3.5: Characterization for PHB quality

6.3.5.1: FTIR analysis of extracted PHB from bacterial isolates

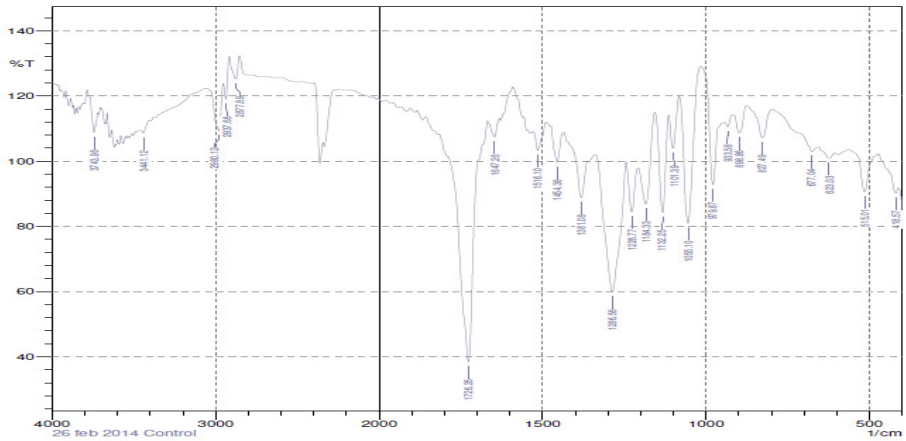


Figure 6.3.41: FTIR analysis of Pure PHB (Sigma-Aldrich)

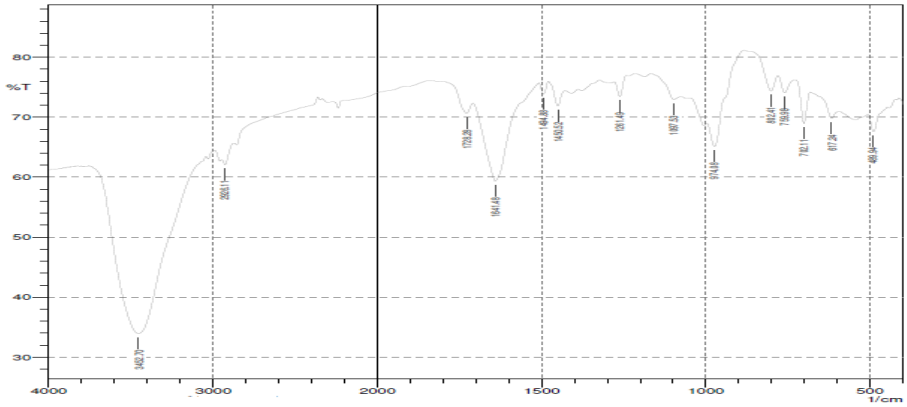


Figure 6.3.42: FTIR analysis of PHB extracted from 2% maltose induction in S-3 strain

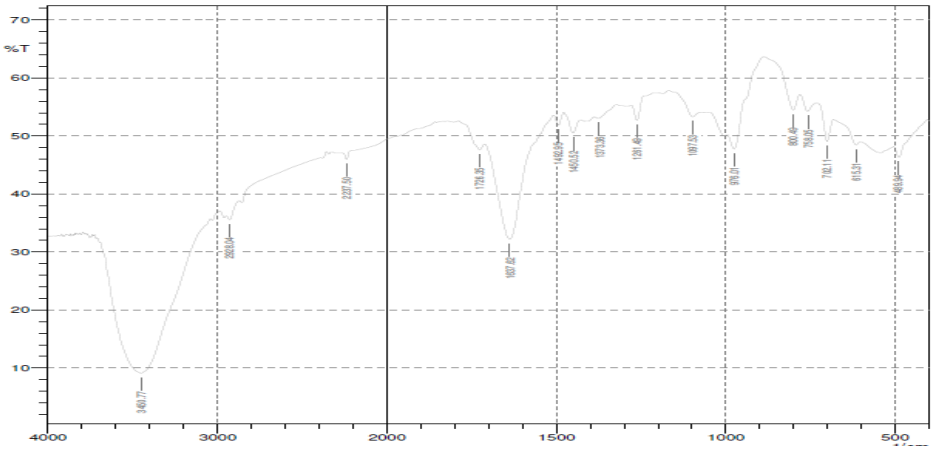


Figure 6.3.43: FTIR analysis of PHB extracted from 3% sucrose induction in AM9 strain

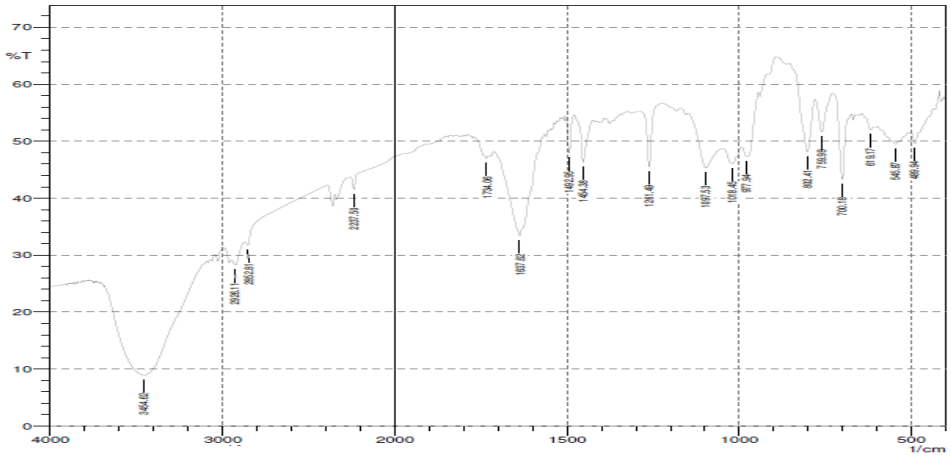


Figure 6.3.44: FTIR analysis of PHB extracted from 1% sucrose induction in MTCC 453 strain

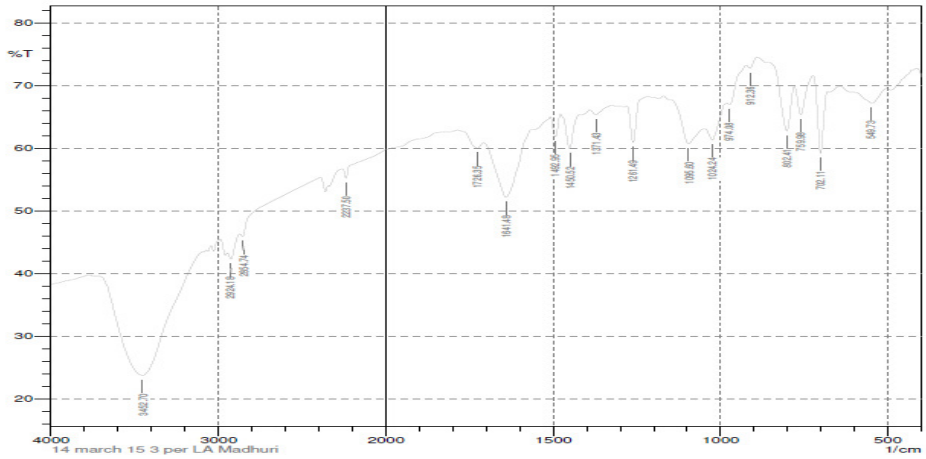


Figure 6.3.45: FTIR analysis of PHB extracted from 3% glucose induction in MTCC 617 strain

6.3.5.1(i): Quantitative analysis of extracted PHB by FTIR analysis

IR study was carried out on standard PHB (Sigma Aldrich) and extracted PHB samples produced from bacterial isolates. The peak values observed for the PHB of bacterial isolate samples was near to the peak value of pure PHB (1726.35 cm^{-1} for C=O, 1226.77 cm^{-1} for C-O stretch, 1454.38 cm^{-1} for C–H bending vibration in CH3 group and 3441cm^{-1} for OH stretch) and are coming in the range of IR spectra defined for specific bonds. So the data indicates that the extracted compound is PHB.

Table 6.39: FTIR analysis of pure PHB and extracted PHB from bacterial isolates

Bacterial strain	C-O stretch functional group	C-H bend vibration in CH_3 group functional group	C=O stretch functional group	OH stretch functional group
Pure PHB	1226.77 cm^{-1}	1454.38 cm^{-1}	1726.35 cm^{-1}	3441 cm^{-1}
S-3	1261.49 cm^{-1}	1450.52 cm^{-1}	1728.28 cm^{-1}	3452.70 cm^{-1}
AM9	1261.49 cm^{-1}	1450.52 cm^{-1}	1726.35 cm^{-1}	3450.77 cm^{-1}
MTCC 453	1261.49 cm^{-1}	1454.38 cm^{-1}	1734.06 cm^{-1}	3454.62 cm^{-1}
MTCC 617	1261.49 cm^{-1}	1450.52 cm^{-1}	1726.35 cm^{-1}	3452.70 cm^{-1}

6.3.5.2 Electron-spray Ionization High Resolution Mass Spectroscopy

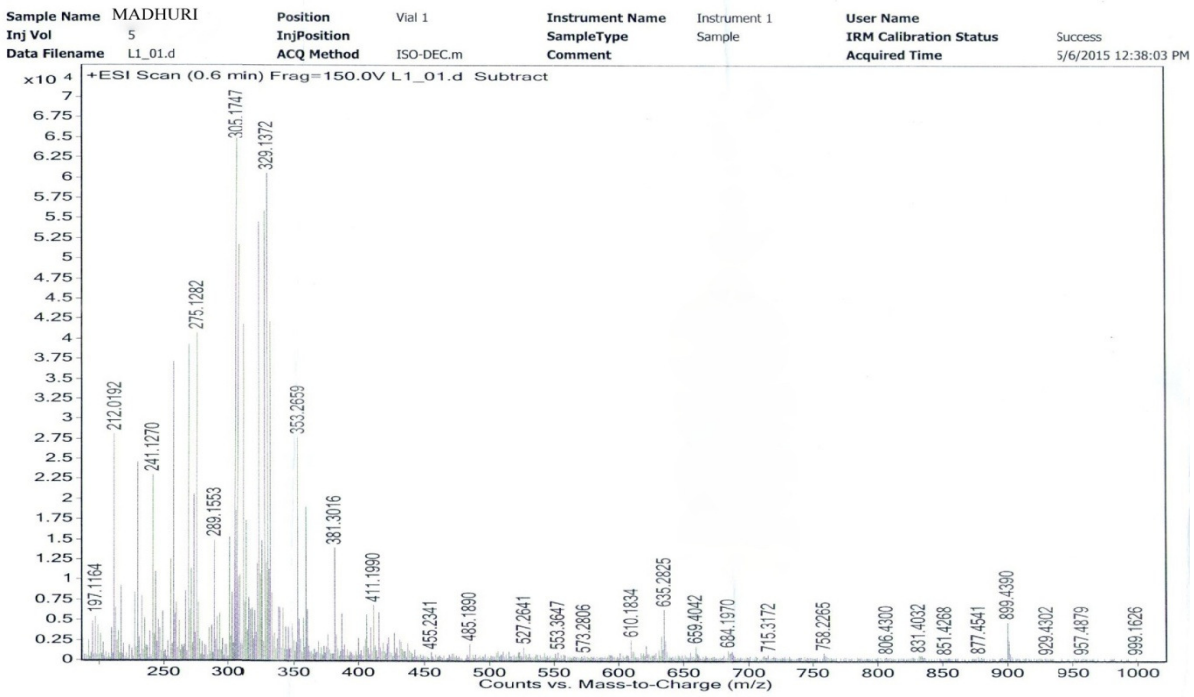


Figure 6.3.46: ESI-HRMS analysis graph of Standard PHB procured from Sigma Aldrich USA

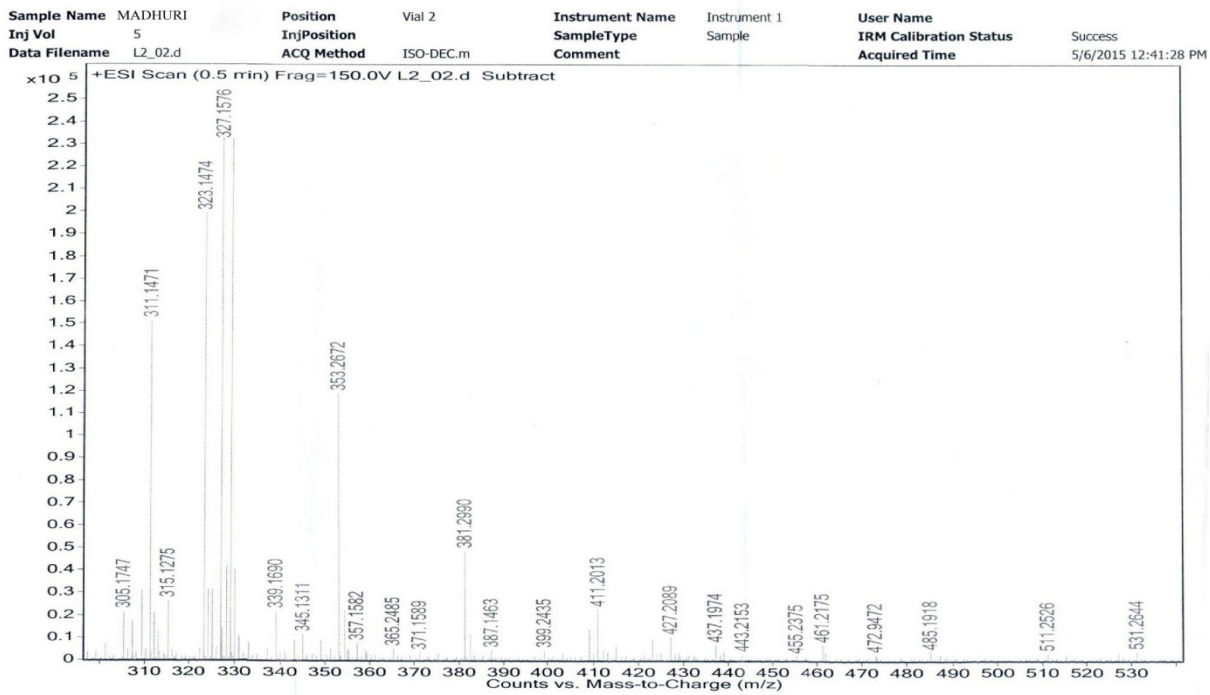


Figure 6.3.47: ESI-HRMS analysis graph of PHB samples produced from S-3 species

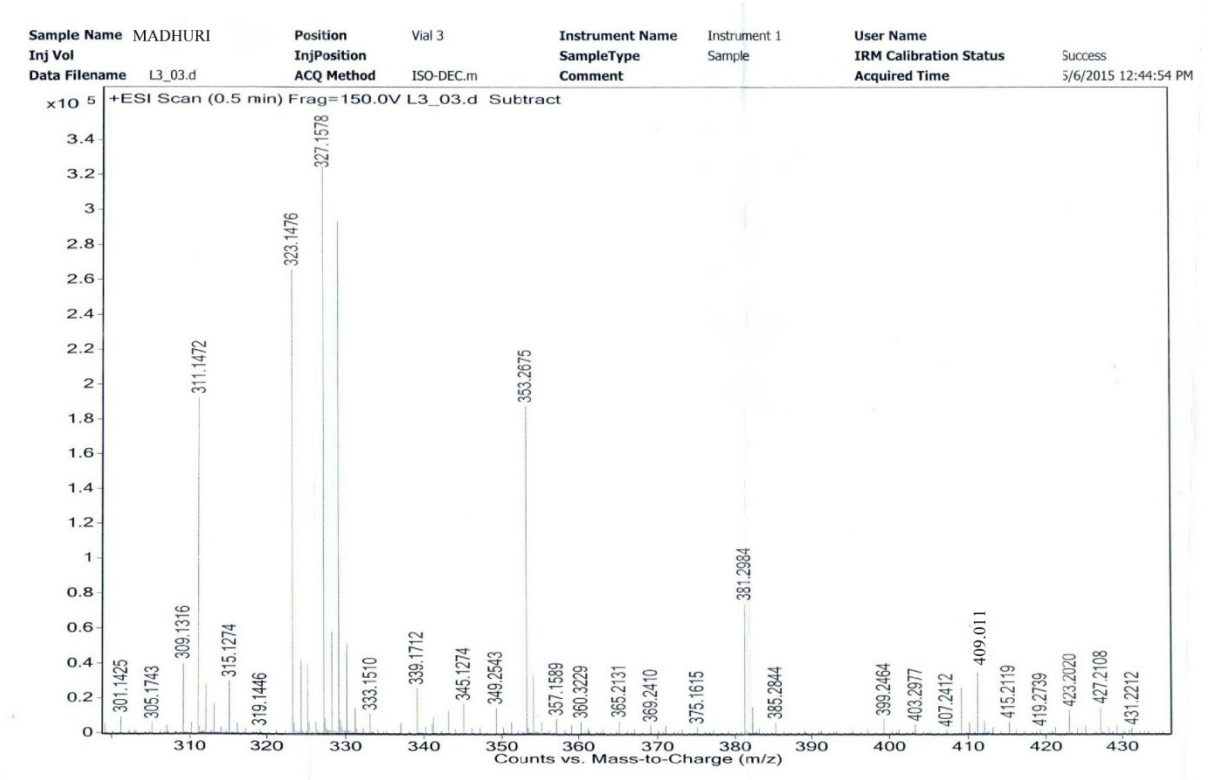


Figure 6.3.48: ESI-HRMS analysis graph of PHB samples produced from AM9 species

6.3.5.2(i): Result analysis of ESI-HRMS data

ESI-HRMS analyzes the compound through electrospray formation and further analysis through mass analyzer. Liquid containing the analyte of interest is dispersed through electrospray, into fine aerosol. These fine droplets formed further decrease in size and produce similar sized droplets. Under the conditions of analysis these further produce uniform charged large ions. These ions are further detected by the mass analyzer.

The analysis of the sample PHB graphs when compared with the standard PHB graph suggests fragmentation patterns. These fragmentation pattern suggest a difference of 28 m/z in control (325m/z - 353m/z - 381m/z - 409m/z) in all major peaks, such difference is also clearly visible in case of PHB samples 353m/z - 381m/z - 409m/z - 437m/z. This fragmentation clearly suggests the correlation between Control and PHB samples. The species that is forming peaks of 28 m/z could be $CHCH_3$, The fragmentation of PHB also produces $CHCH_3$ species. Samples of PHB are also producing many smaller peaks which could be due to impurities that could have come during purification protocol from the reagents used or from the lysed bacterial pellets.

6.3.6: 16s rRNA characterization

The efficient PHB producers were sent to Yaazh Xenomics, Madurai for its identification and molecular characterization. Below table represents the genus and species of bacterial isolates with their Accession numbers allotted by GENBANK NCBI.

Table 6.40: Identification of bacterial isolates with their Accession numbers

S.no.	Strain designation	Identity	Acession no. (Genbank)	% similarity index
1.	S3	*Thalassobacillus devorans*	KR133276	99%similar with EU029643.1
2.	M1	*Bacillus flexus*	KJ939621	99%similar with KF956683.1
3.	M10	*Oceanobacillus kimchii*	KR606391	100%similar withNR_117544.1
4.	MD1	*Aeromonas punctata*	KT75522	99%similar with JN986500.1
5.	MD2	*Halobacillus trueperi*	KT75520	100% similar with KF535157.1
6.	MD5	*Bacillus circulans*	KT75521	99%similar with AB680477.1
7.	AM6	*Halomonas desiderata*	KT006268	99%similar with EU305583.1
8.	AM9	*Oceanobacillus iheyensis*	KR136170	99% similar with EU624422.1

6.3.7: Screening of PHB production by Isolated Bacteria through Sophisticated Instrument Transmission Electron Microscopy

6.3.7(i): Photographs of PHB granules inside bacterial isolates analyzed by Transmission Electron Microscopy

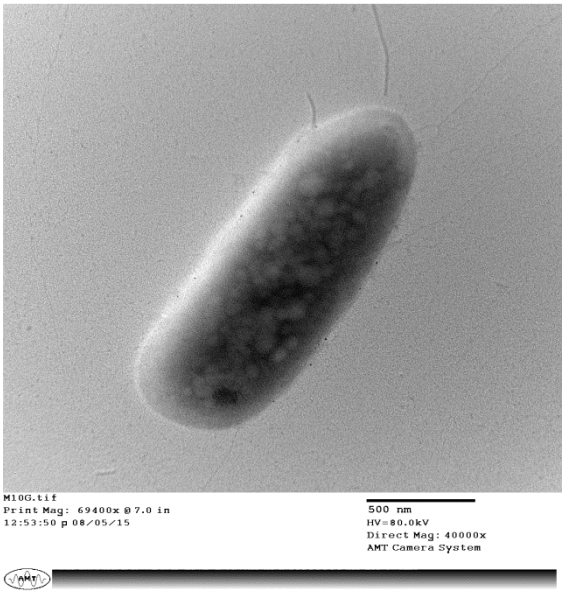


Figure 6.3.49: AM9 strain

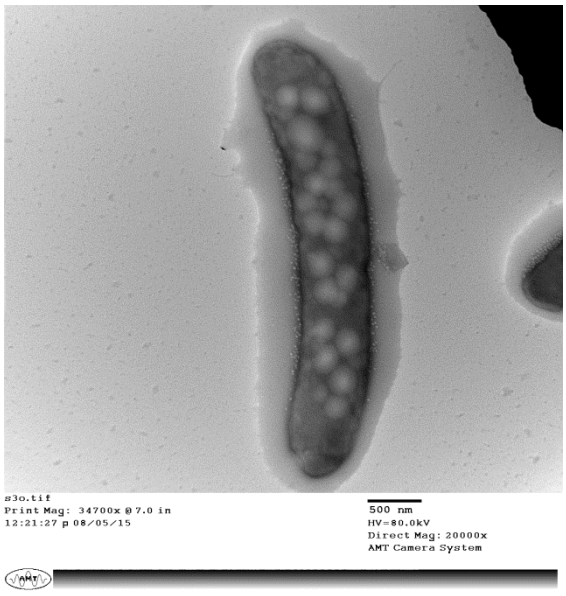


Figure 6.3.50: S3 strain

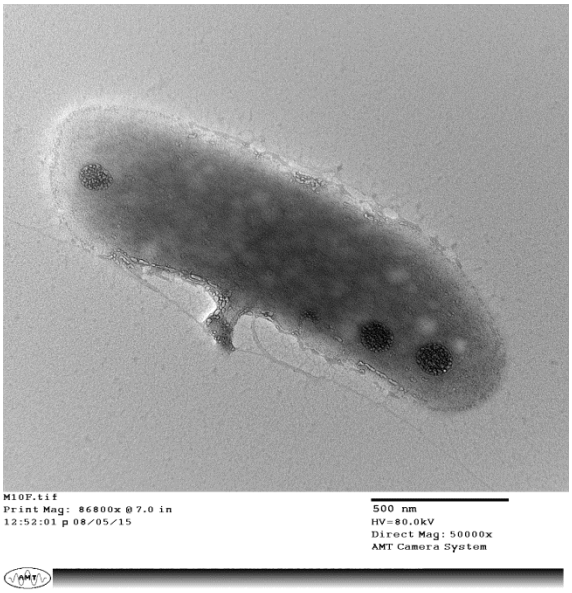


Figure 6.3.51: M1strain

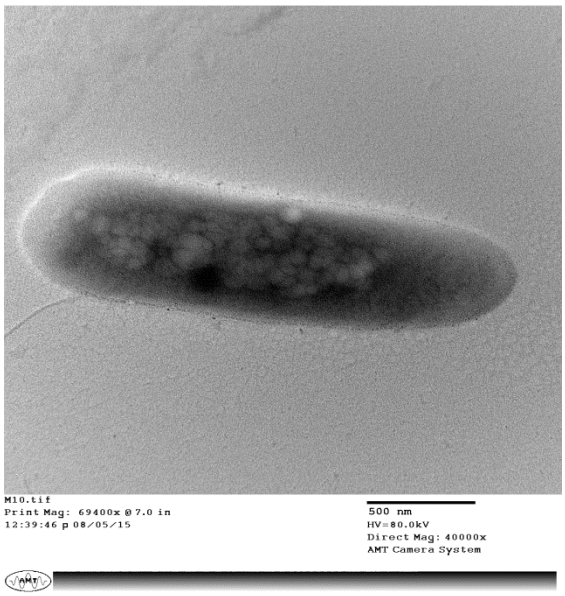


Figure 6.3.52:M10 strain

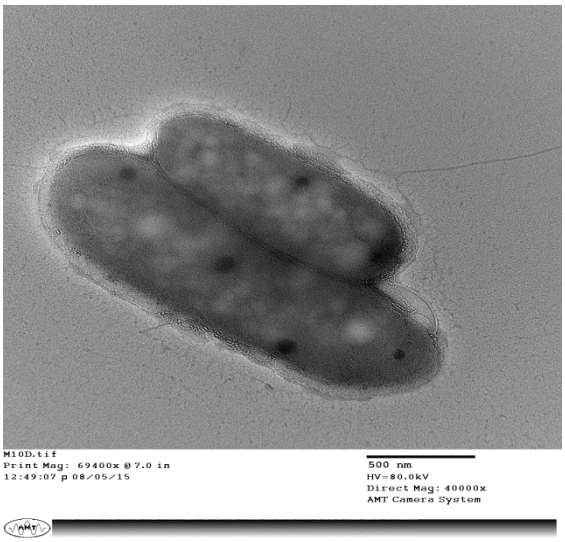


Figure 6.3.53:MD5 strain

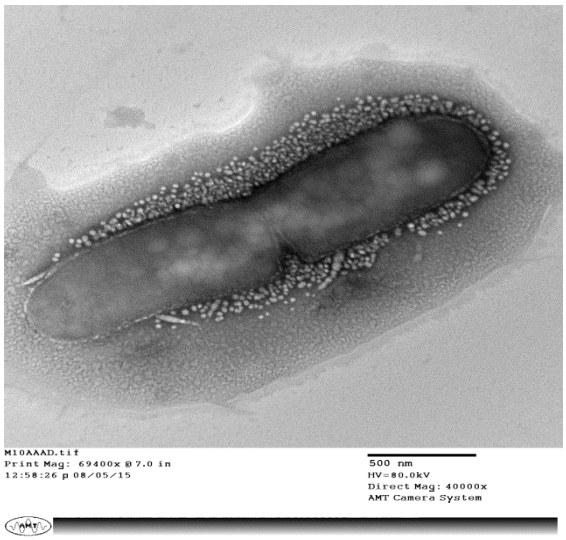


Figure 6.3.54:MD2 strain

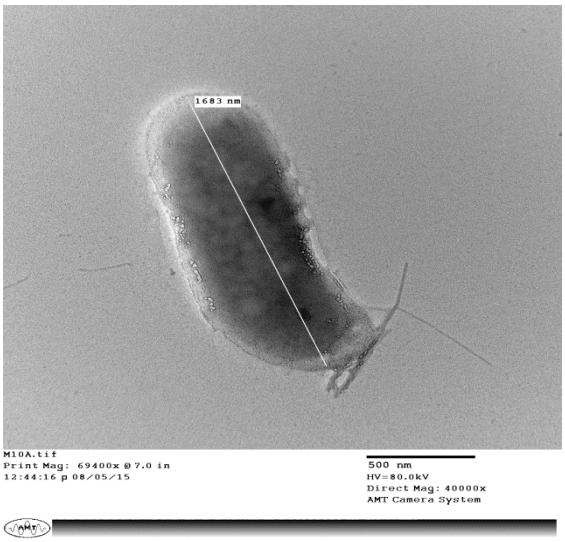


Figure 6.3.55: AM6 strain

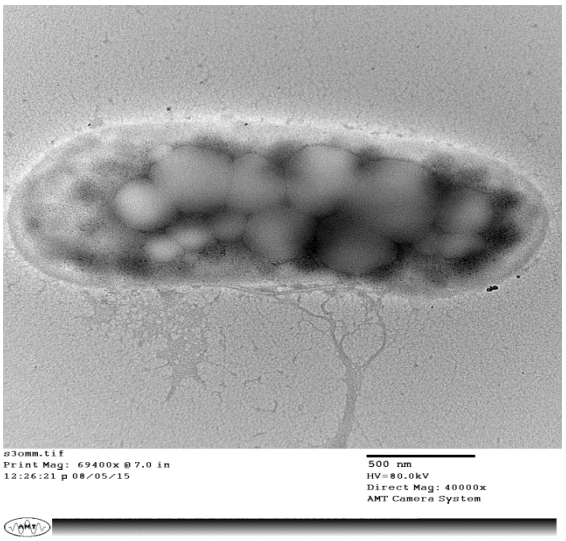


Figure 6.3.56: MD1 strain

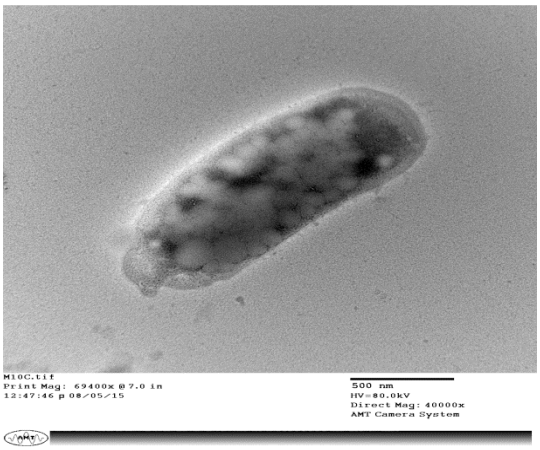


Figure 6.3.57: MTCC 617 strain

6.4: Exploration of pure form of PHB for production of nanocomposite biomaterial through mixing methods and its characterization through various nanomaterial characterization techniques

Pure form of PHB (Goodfellow Chemical Supplier, UK) was used as standard PHB model for preparation of nanocomposite films. The basic requirement for production of nanocomposite biomaterial from PHB is that the materials used should be of highest quality standards so that the standard changes in enhancement of property could be observed hence available best grades of PHB were procured and utilized for the procedures. During the experimental extraction of PHB chances of contamination are high so as to avoid unnecessary purification steps pure form of PHB material has been used for nanocomposite biomaterial preparation.

Two different types of nanocomposite films were prepared utilizing PHB as the matrix material and nanoclays as given below

1. Zeolite nanoclay
2. Organically surface modified Montmorillonite nanoclay with Dimethyl- Dialkyl amine

6.4.1: Solvent casting method: Blending was carried out by mixing 1 to 10 wt% of Zeolite nanoclay with PHB matrix material. The general strategy for the preparation was carried out as described in material and method section. The nanocomposite of Organically surface modified Montmorillonite nanoclay with Dimethyl- Dialkyl amine was also prepared 1 to 10 wt% with PHB polymer as matrix material as described in material and method section.

6.4.1(i): Photographs of prepared films of PHB and nanoclays

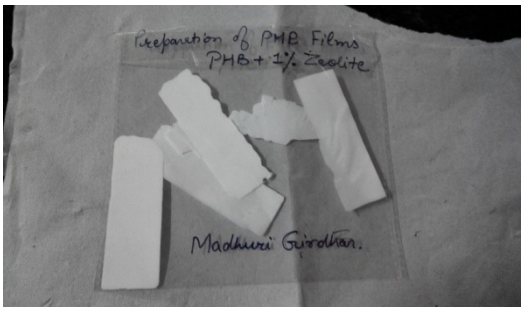


Figure 6.4.1: PHB+1% Zeolite

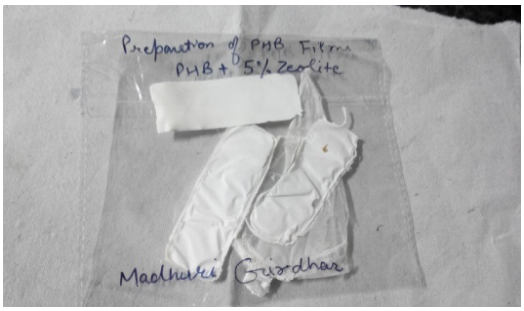


Figure 6.4.2: PHB+5% Zeolite

Figure 6.4.3: PHB+1%OMMT

Figure 6.4.4: PHB+5%OMMT

6.4.2: Analysis and Characterization of Prepared films through Sophisticated Instruments

The films prepared were analyzed for their morphological, physical and mechanical characters at various Sophisticated Instruments Analytical Facilities. Field- Emission Scanning electron microscopy (FE-SEM), Differential Scanning Calorimetry and Thermogravimetric Analysis etc were carried out at Smitha Labs, Department of Textile technology, IIT Delhi.

6.4.2.1: Field- Emission Scanning electron microscopy (FE-SEM)

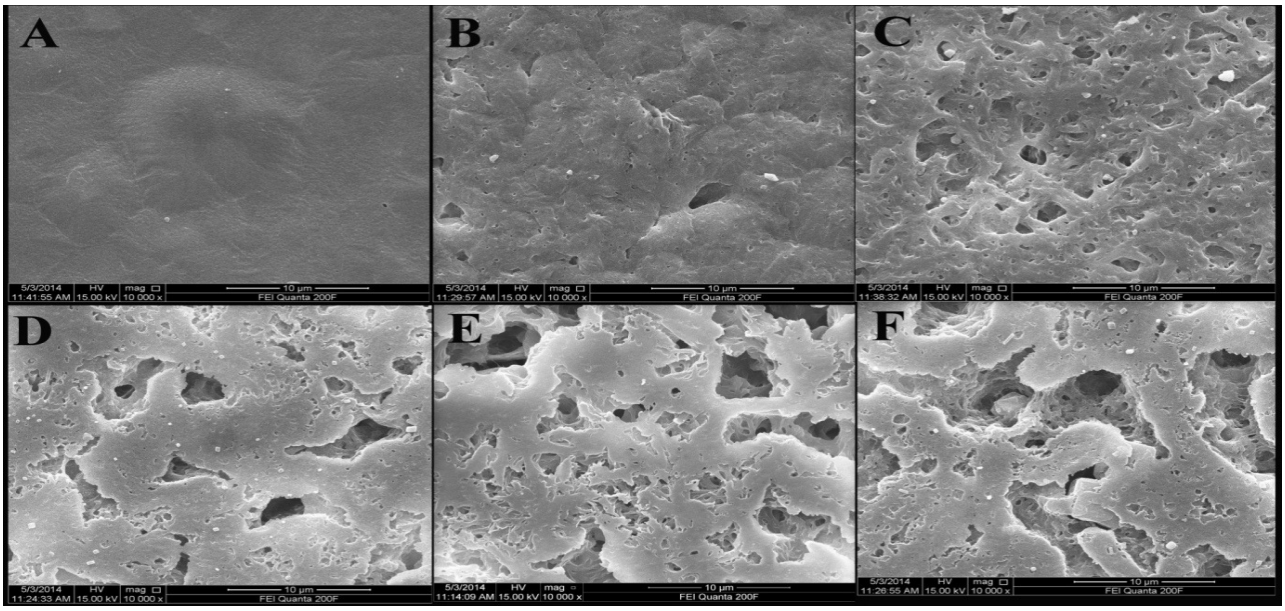


Figure 6.4.5: FESEM images of pure PHB and PHB + Zeolite nanocomposites films at different concentration (A) Control pure PHB (B) 1wt% Zeolite + PHB matrix (C) 3wt% Zeolite + PHB matrix (D) 5wt% Zeolite + PHB matrix (E) 7wt% Zeolite + PHB matrix (F) 10wt% Zeolite + PHB matrix

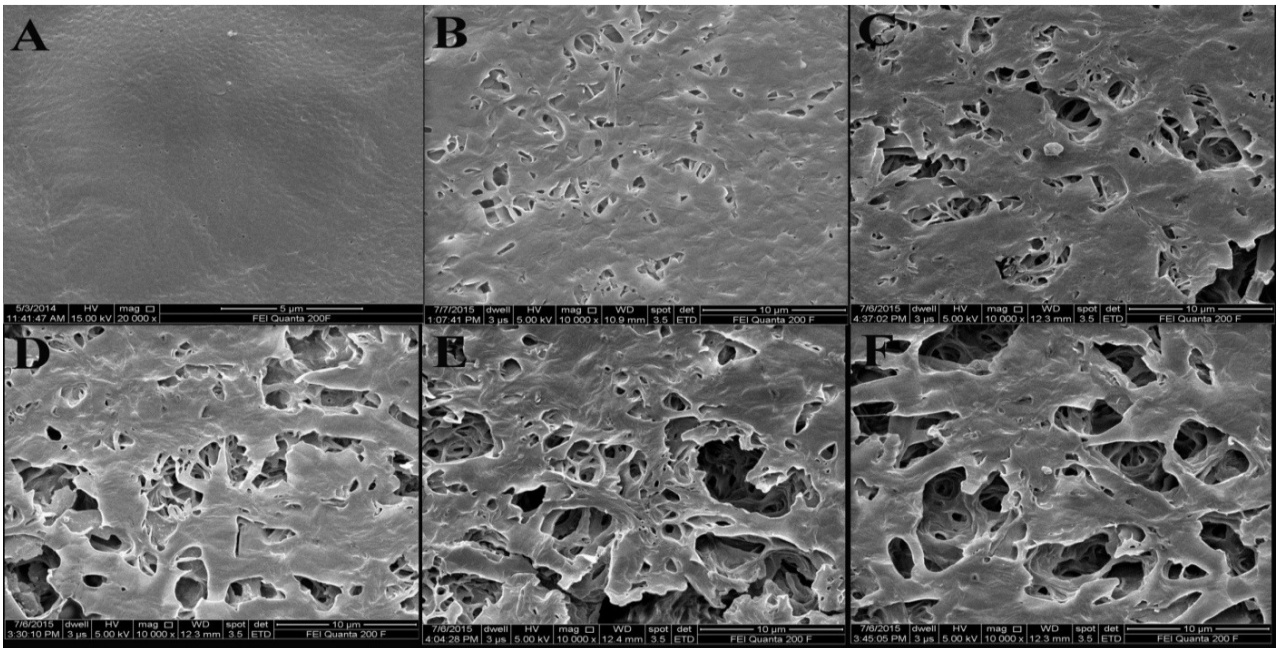


Figure 6.4.6: FESEM images of pure PHB and PHB + OMMT (dimethyl dialkyl amine organomodified MMT) nanocomposites at different concentration (A) Control pure PHB (B) 1wt% OMMT + PHB matrix (C) 3wt% OMMT + PHB matrix (D) 5wt% OMMT + PHB matrix (E) 7wt% OMMT + PHB matrix (F) 10wt% OMMT + PHB matrix

6.4.2.1(i): Observation of FESEM analysis

FESEM images of the PHB/Zeolite and PHB/OMMT blends with different concentration are depicted in Figure 6.4.5 and Figure 6.4.6. The small granules present on the matrix surface are nanoclays, whereas the bright areas indicate the matrix polymers. Morphological analysis of nanocomposites of different Zeolite and OMMT concentrations show a mixed morphology with the coexistence of exfoliated and intercalated clay patterns within the blend matrix. The observed morphology suggests the effective porosity effect of nanoclay within the blend matrix. Proper shear under optimized processing temperature, rpm, and retention time, along with effective higher basal spacing and interacting groups present in the nanoclays have facilitated the easy penetration of polymer macromolecules into the nanoclay. Higher level of porosity observed along with an increased level of Zeolite and OMMT raises the application of this nanocomposite for being used as scaffold in 3 D printing of human organs. Increased porosity is known to assists in homogeneous media percolation and better growth of cells growing over the scaffold material.

6.4.2.2:Thermogravimetric analysis (TGA) results

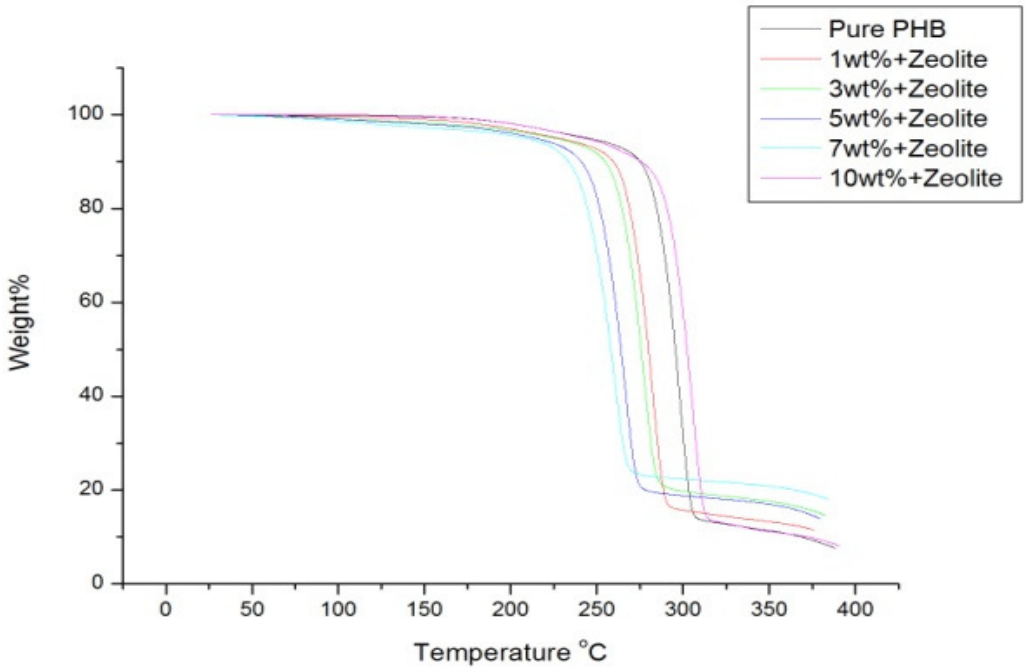


Figure 6.4.7: TGA graph represents the % degradation of loaded samples at different concentrations of nanoclays PHB+ZEOLITE

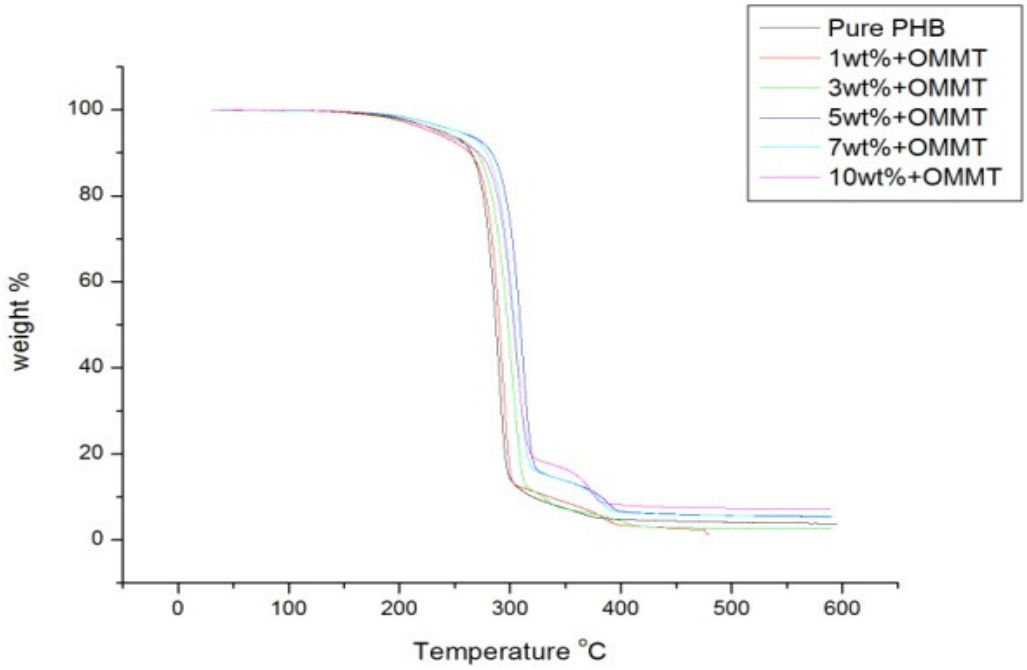


Figure 6.4.8: TGA graph represents the % degradation of loaded samples at different concentrations of nanoclays PHB+OMMT

Table 6.41: Weight percentage degradation of PHB + Zeolite blends analyzed by TGA (25^0C-400^0C)

Different concentrations of Zeolite	**Weight % degraded**	**Weight loaded (mg)**
Pure PHB	91.56%	12.47 mg
PHB+1wt% Zeolite	91.91%	14.16 mg
PHB+3wt% Zeolite	87.09%	14.33 mg
PHB+5wt% Zeolite	83.52%	10.86 mg
PHB+7wt% Zeolite	85.69%	12.14 mg
PHB+10wt% Zeolite	80.96%	12.74 mg

Table 6.42: Weight percentage degradation of PHB+OMMT blends analyzed by TGA (25^0C-600^0C)

Different concentrations of OMMT	**Weight % degraded**	**Weight loaded (mg)**
Pure PHB	97.601%	13.614 mg
PHB+1wt% OMMT	97.362%	12.543 mg
PHB+3wt% OMMT	96.173%	18.065 mg
PHB+5wt% OMMT	94.565%	13.366 mg
PHB+7wt% OMMT	94.417%	14.226 mg
PHB+10wt% OMMT	92.862%	15.65 mg

6.4.2.2(i): Analysis of results of TGA

The TGA analysis suggested that polymer/clay nanocomposites are thermally more stable than pure polymer composites. This is due to the effect of nanoclay layers which provide superior insulation and mass transport barrier against the decomposition of polymer volatile compounds under high temperatures. This occurs because clay minerals are inorganic molecules which are stable in the temperature ranges in which organic molecules of pure polymer are susceptible to volatile compound conversion. The TG thermograph shows that the weight loss of the blended films is due to degradation, which is monitored as a function of temperature.

In the PHB+Zeolite nanocomposite, as the concentration (wt%) of Zeolite in PHB matrix increases, the percentage degradation of the composite decreases as compared to control samples. The pure PHB has a degradation percentage of 91.56%, whereas the other blends

have decreasing percentages i.e. 3wt% (87.09%), 5wt% (83.52%), 7wt% (85.69%) and 10wt% (80.96%).

In the PHB+OMMT nanocomposite, as the concentration (wt%) of OMMT in PHB matrix increases, the percentage degradation of the composite decreases as compared to control samples. The pure PHB have a degradation percentage of 97.601%, whereas the other blends have decreasing percentages i.e. 1wt% (97.362%), 3wt% (96.173%), 5wt% (94.565%), 7wt% (94.417%) and 10wt% (92.862%).

The work is in coherence with Zuburtikudis *et al.,* 2008 that introduction of different wt% of nanoclay increases the degradation temperature.

The analysis shows that the addition of OMMT % in polymer matrix affects the degradation percentage of nanocomposite with increase in temperature, which implies that OMMT becomes incorporated as filler in the polymeric matrix. The incorporation of nanoclay was also found to enhance the thermal stability of the polymer.

6.4.2.3: Differential Scanning Calorimetry (DSC)

Table 6.43: Different values of melting temperature and enthalpy of melting of different blends of PHB with Zeolite, degradation temperature analyzed through TGA

Different concentrations of Zeolite	**Melting temperature values**	**Degradation temperature values**	**Processing window**	**Enthalpy of Melting ΔH_M (J/g)**
Pure PHB	169.78 ^oC	296.98 ^oC	127.26 ^oC	57.18 J/g
PHB+1wt% Zeolite	171.50 ^oC	300.38 ^oC	128.88 ^oC	74.56 J/g
PHB+3wt% Zeolite	174 ^oC	295.85 ^oC	121.85 ^oC	61.95 J/g
PHB+5wt% Zeolite	170.49 ^oC	284.71 ^oC	114.22 ^oC	59.09 J/g
PHB+7wt% Zeolite	170.13 ^oC	277.09 ^oC	106.96 ^oC	66.30 J/g
PHB+10wt% Zeolite	171.67 ^oC	266.42 ^oC	94.75 ^oC	59.38 J/g

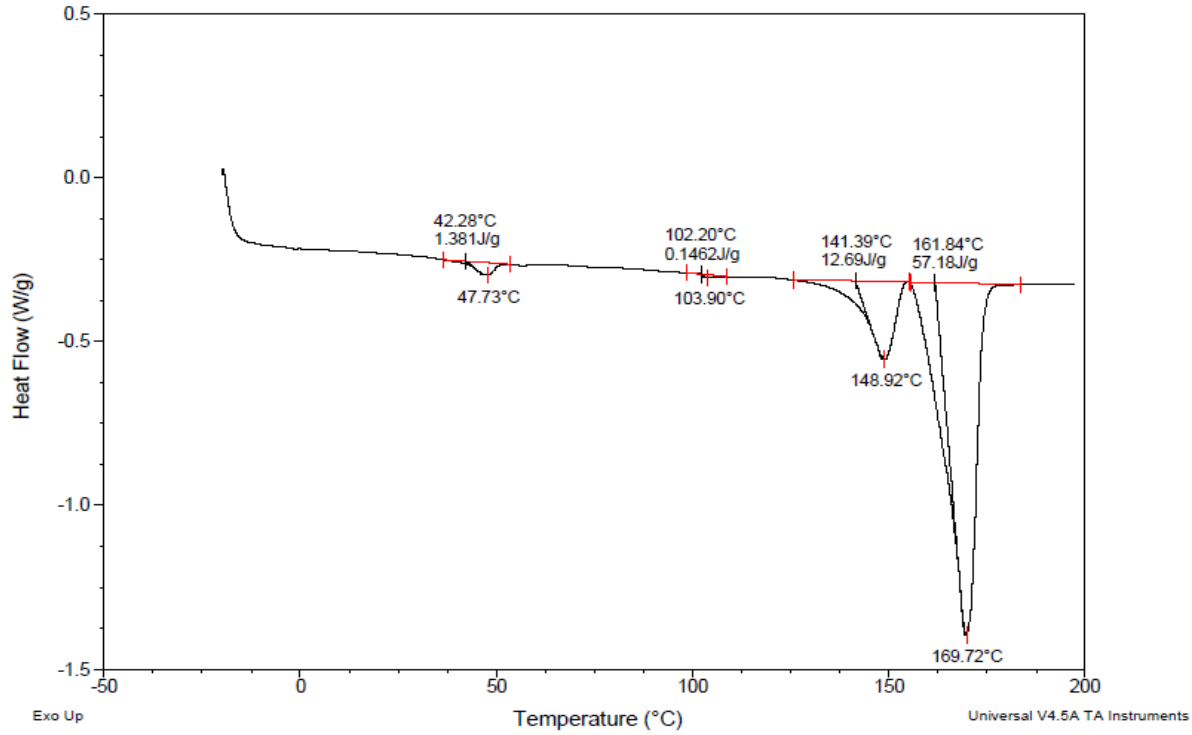


Figure 6.4.9: DSC curve of Pure PHB

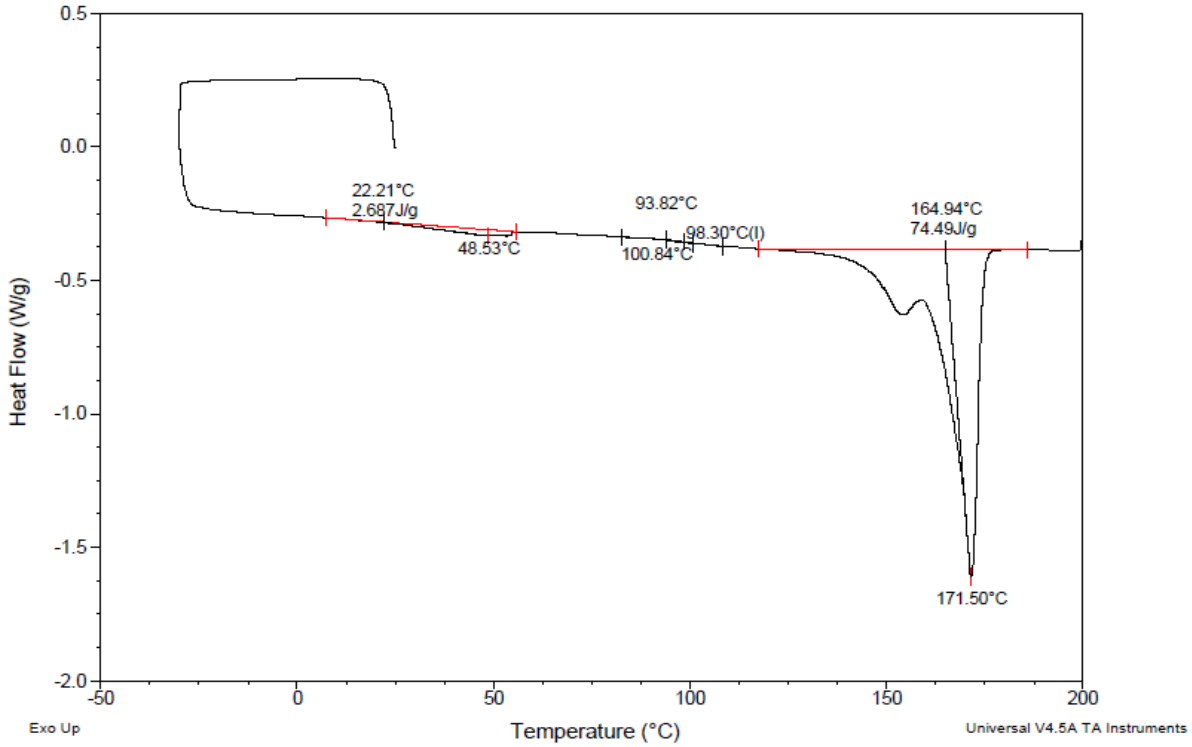


Figure 6.4.10: DSC curve of PHB+1wt% Zeolite

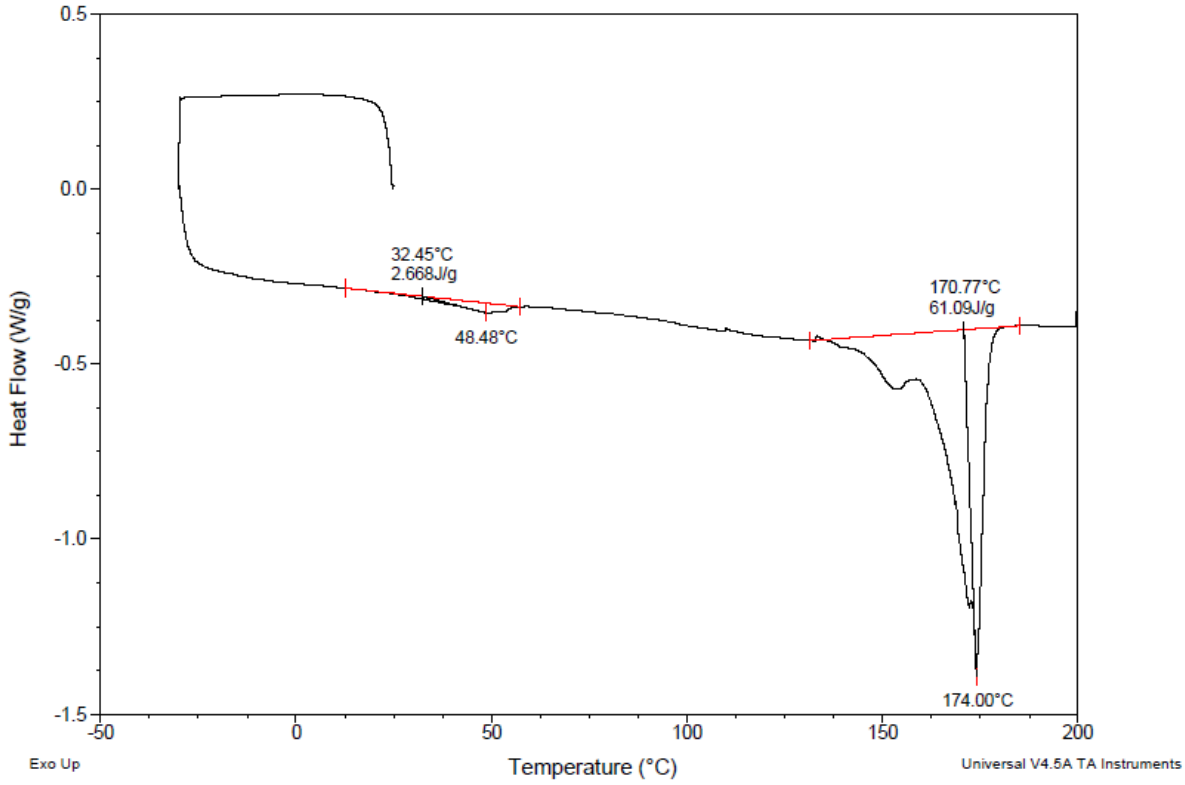


Figure 6.4.11: DSC curve of PHB+3wt% Zeolite

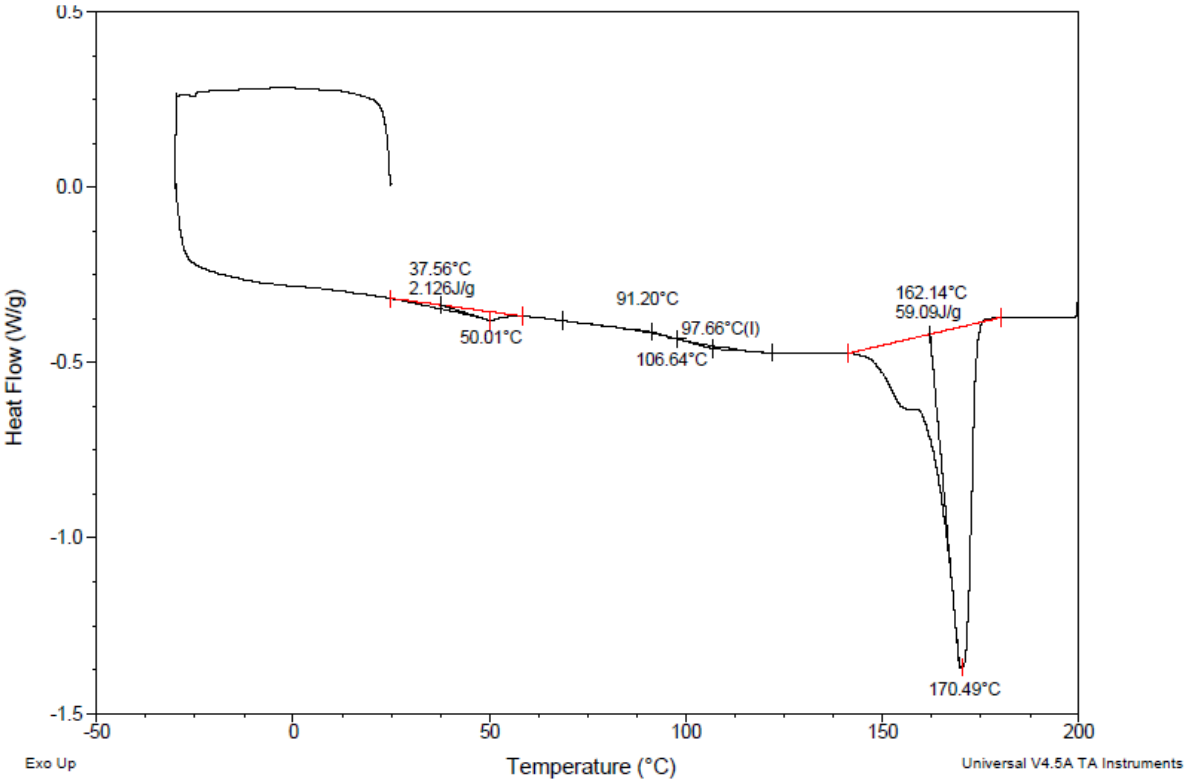


Figure 6.4.12: DSC curve of PHB+5wt% Zeolite

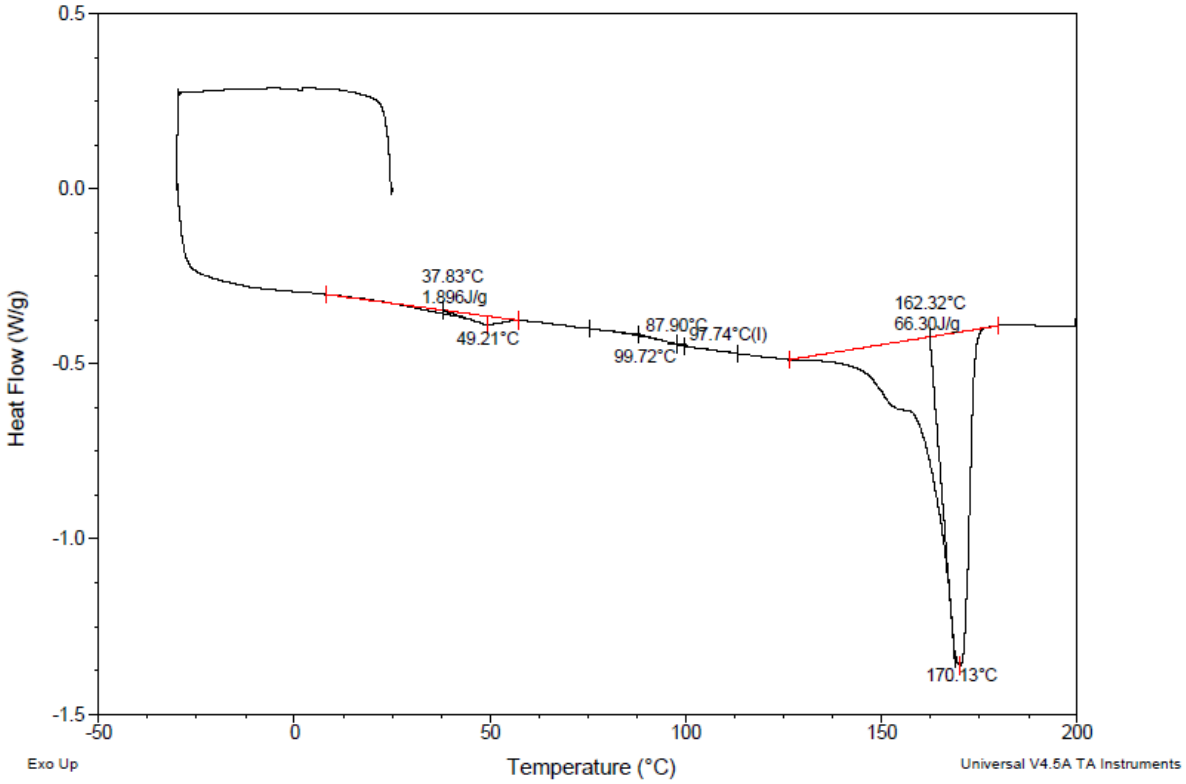


Figure 6.4.13: DSC curve of PHB+7wt% Zeolite

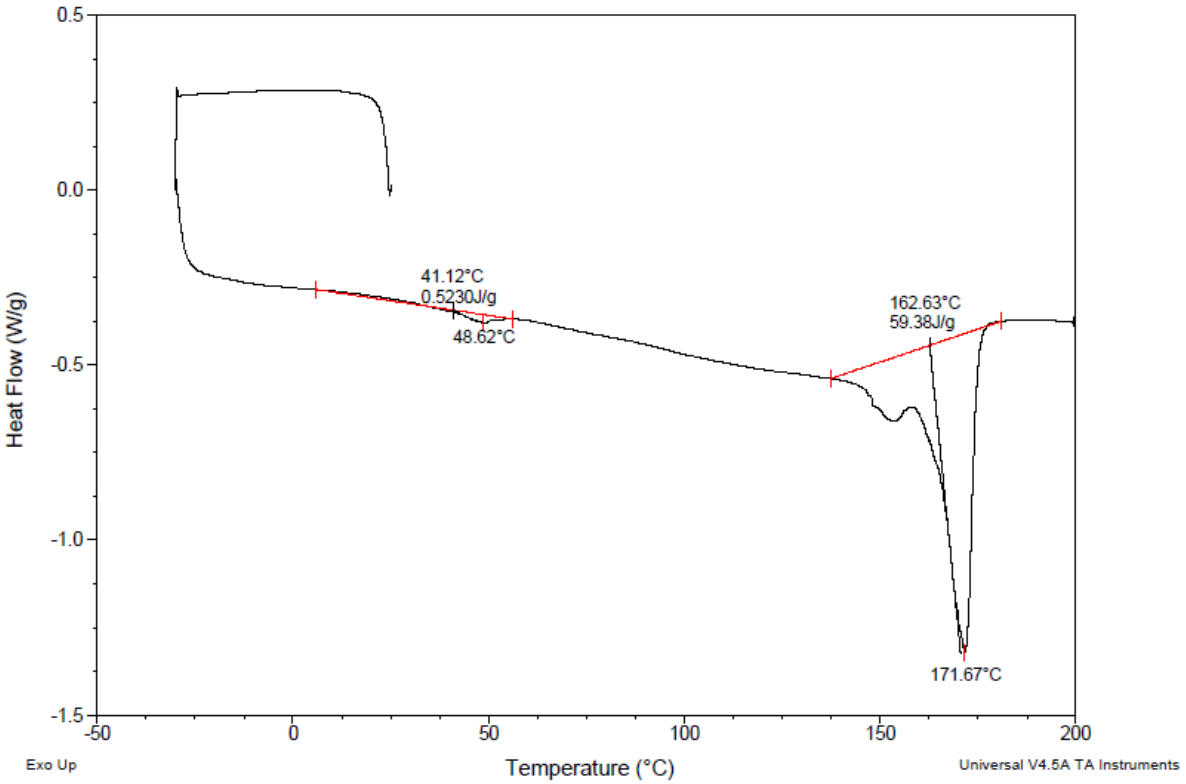


Figure 6.4.14: DSC curve of PHB+10wt% Zeolite

Table 6.44: Different values of melting temperature and enthalpy of melting of different blends of PHB with OMMT

Different concentrations of OMMT	**Melting temperature values**	**Degradation temperature values**	**Processing window**	**Enthalpy of Melting ΔH_M (J/g)**
Pure PHB	169.72 ^oC	296.98 ^oC	127.26^oC	57.18 J/g
PHB+1wt% OMMT	168.39 ^oC	301.56 ^oC	133.17 ^oC	73.38 J/g
PHB+3wt% OMMT	168.52 ^oC	289.39 ^oC	120.87 ^oC	72.64 J/g
PHB+5wt% OMMT	168.45 ^oC	304.72 ^oC	136.27 ^oC	67.99 J/g
PHB+7wt% OMMT	169.70 ^oC	304.54 ^oC	135.02 ^oC	56.64 J/g
PHB+10wt% OMMT	169.64 ^oC	304.61 ^oC	134.97 ^oC	50.25 J/g

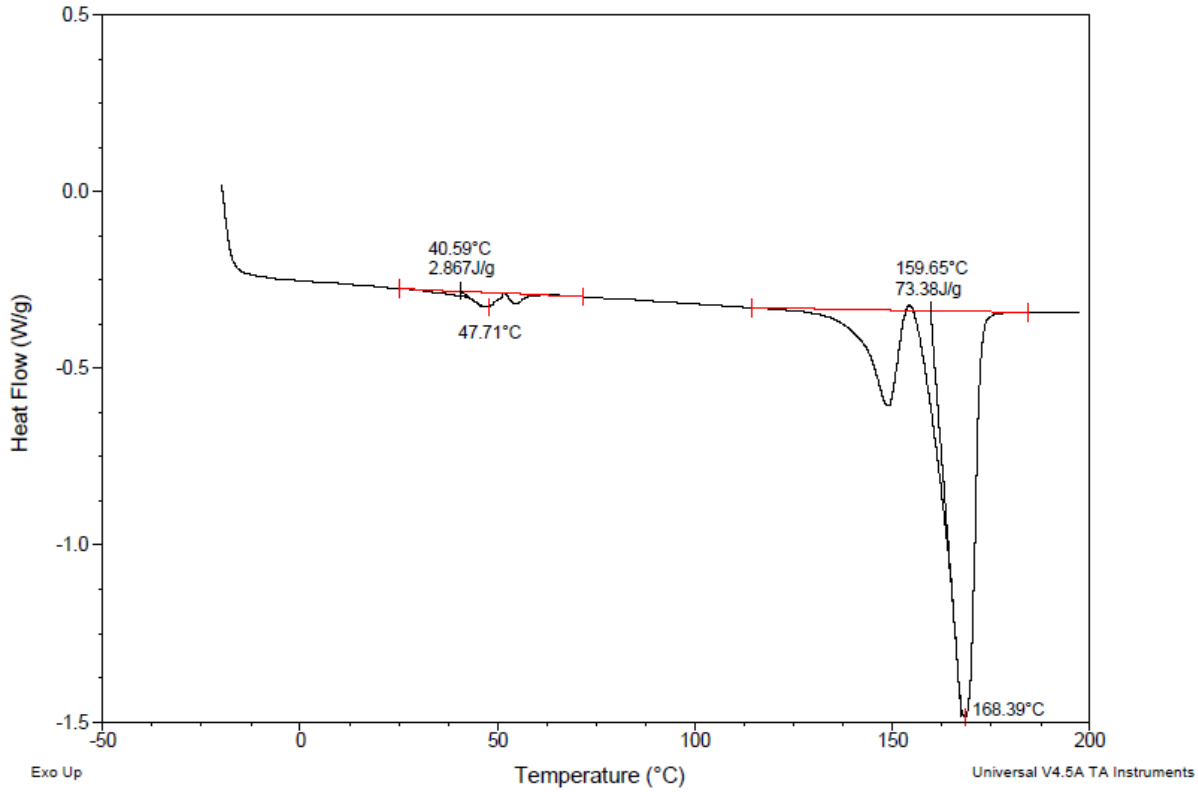


Figure 6.4.15: DSC curve of PHB+1wt% OMMT

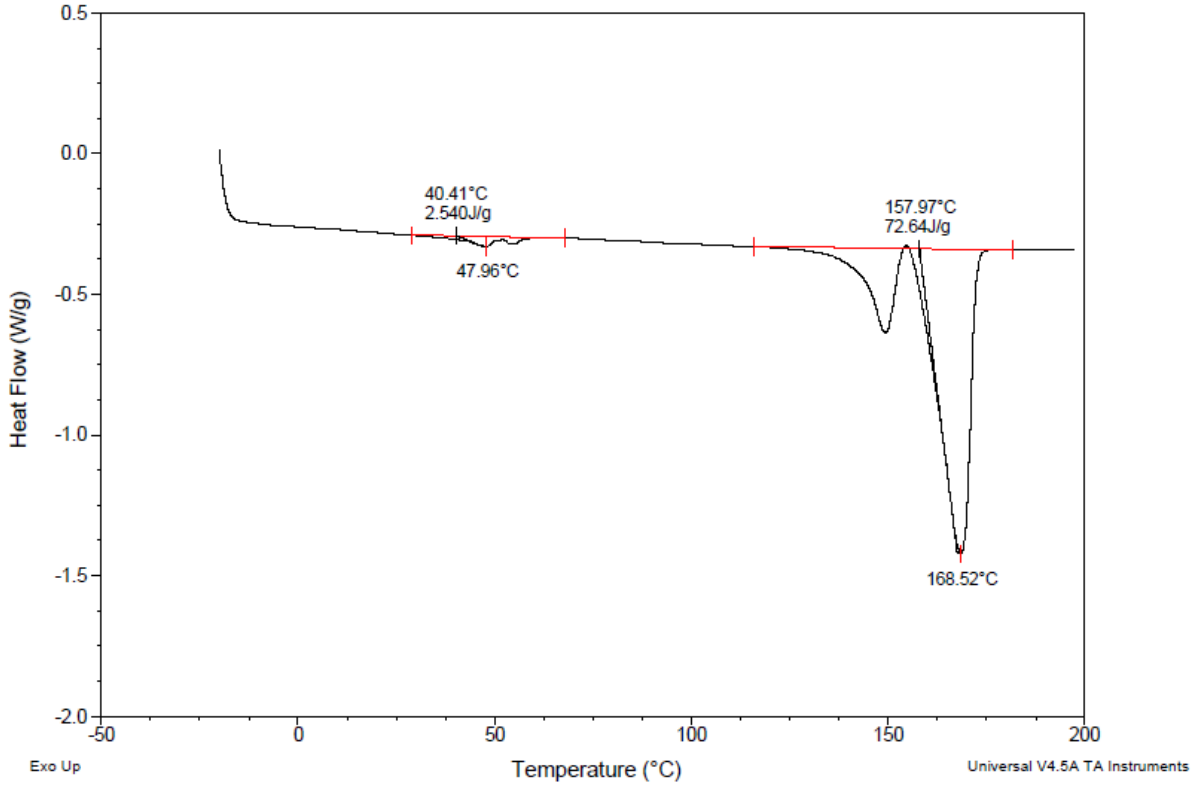


Figure 6.4.16: DSC curve of PHB+3wt% OMMT

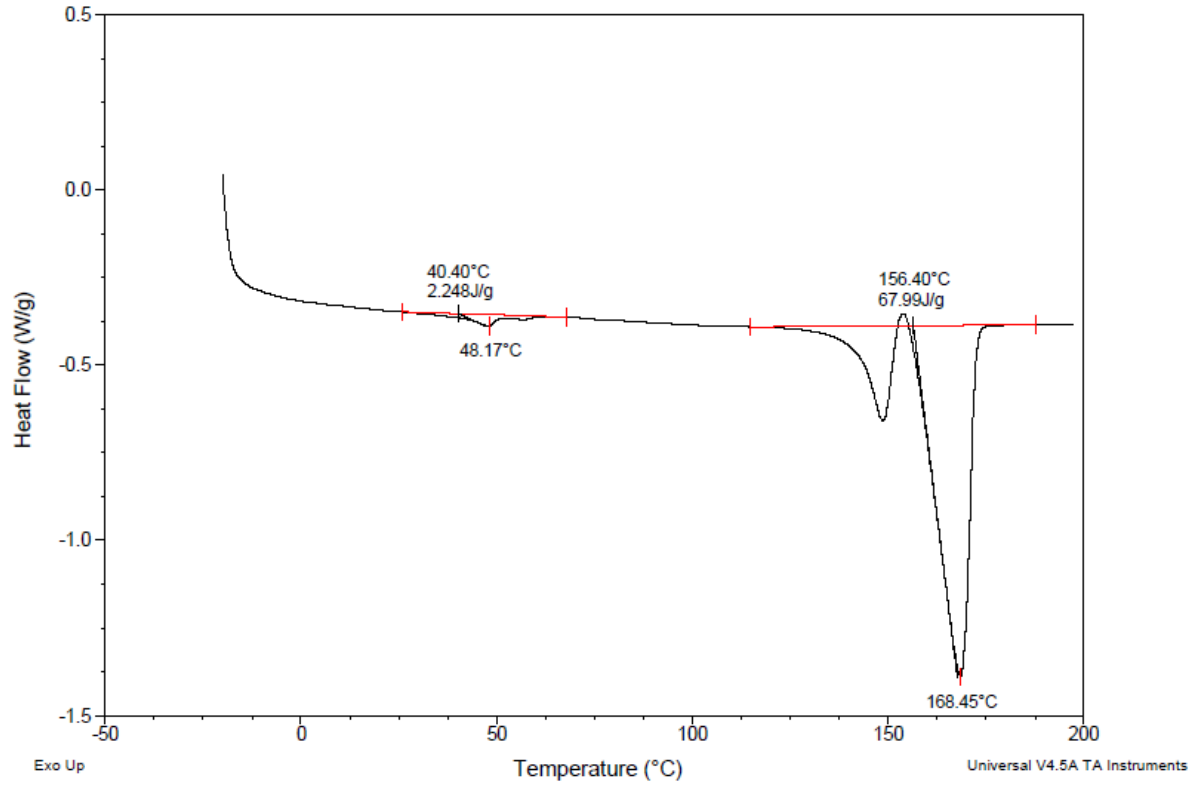


Figure 6.4.17: DSC curve of PHB+5wt% OMMT

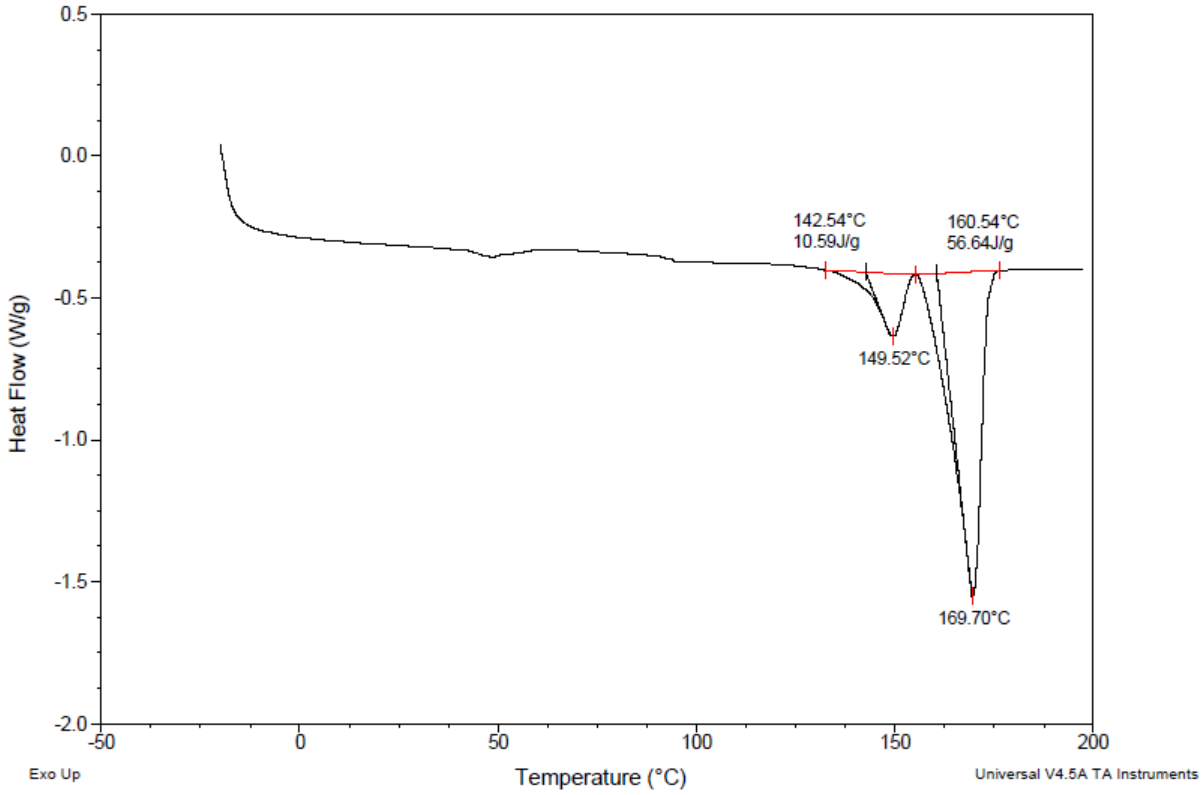


Figure 6.4.18: DSC curve of PHB+7wt% OMMT

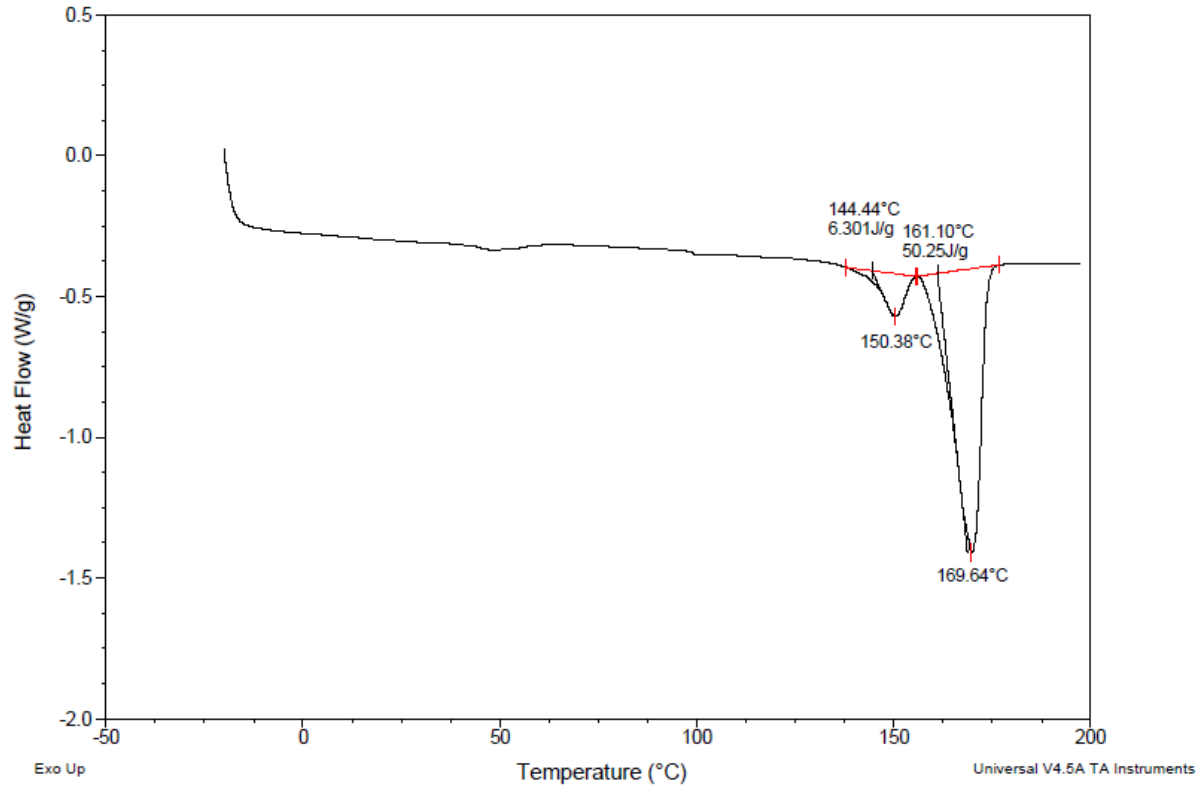


Figure 6.4.19: DSC curve of PHB+10wt% OMMT

6.4.2.3(i): Observation of DSC curve in PHB/Zeolite nanocomposites

Enthalpy of melting and melting temperature analysis of PHB/Zeolite nanocomposites was carried out by Differential Scanning Calorimetry. The analysis of result shows that 1wt% PHB/Zeolite composites demonstrate maximum increase in the thermal processing window. DSC thermal degradation curve shows that in case of pure PHB, maximum melting temperature was 169.78^{o}C and maximum thermal degradation was at 296.98^{o}C which is altered in the case of nanocomposite formed. The main aim of this study was to increase the thermal stability of the PHB by blending it with inorganic nanoclays. From all wt% blends, PHB/Zeolite 1wt% blend shows melting temperature of 171.50^{o}C and maximum degradation temperature at 300.38^{o}C. It was observed that pristine PHB window processing difference was 127.26^{o}C and PHB/Zeolite 1wt% was 128.88^{o}C. It means that there was a rise in 1.64^{o}C in the processing window. With the addition of 1wt% Zeolite in PHB matrix, the narrow thermal processing window has become wide, which increase processibility time for the molding of products at an increased range of temperature.

Enthalpy of melting (ΔH_M) was also increased from 57.18 J/g to 74.56 J/g. This increase in enthalpy provides more stability to the material as now more amount of heat has to be absorbed by the nanocomposite to reach its higher melting temperature. The nanocomposite prepared is better as compared to the work done by Cheng *et al.,* 2004 as enthalpy was continuous increasing

6.4.2.3(ii): Observation of DSC curve in PHB/OMMT nanocomposites

Enthalpy of melting and melting temperature analysis of PHB/OMMT nanocomposites was carried out by Differential Scanning Calorimetry. The analysis of result shows that 1wt% PHB/OMMT and 5wt% PHB/OMMT composites shows maximum increase in the thermal processing window. DSC thermal degradation curve shows that in case of pure PHB, maximum melting temperature was 169.78^{o}C and maximum thermal degradation was at 296.98^{o}C. The main aim of this study was to increase the thermal stability of the PHB by blending it with inorganic nanoclays. From all wt% blends, PHB/Zeolite 1wt% shows the melting temperature of 168.39^{o}C and maximum degradation temperature at 301.56^{o}C. It was observed that pristine PHB window processing difference was 127.26^{o}C and PHB/OMMT 1wt% was 133.17^{o}C. It means that there was a rise in 5.91 ^{o}C in the processing window. With the addition of 1wt% Zeolite in PHB matrix, the narrow thermal processing window has

become wide, which increase the processibility time for the molding of products at an increased range of temperature. Enthalpy of melting (ΔH_M) was also increased from 57.18 J/g to 73.38 J/g. This increase in enthalpy provides the material more stability as now more amount of heat has to be absorbed by the nanocomposite to reach its higher melting temperature.

The PHB/OMMT 5wt% shows the melting temperature of 168.45^oC and maximum degradation temperature at 304.72^oC. It was observed that pristine PHB window processing difference was 127.26^oC and PHB/OMMT 5wt% was 136.27^oC. It means that there was a rise in 9.01^oC in the processing window. With the addition of 5wt% OMMT in PHB matrix, the narrow thermal processing window has become wide, which increase the processibility time for the molding of products at an increased range of temperature.

Enthalpy of melting (ΔH_M) was also increased from 57.18 J/g to 67.99 J/g in case of PHB/OMMT 5wt%. The results are in coherence with work done by Maiti *et al.,* 2007 that increase in decomposition temperature occurs with increase in OMMT percentage.

This increase in enthalpy provides the material more stability as now more amount of heat has to be absorbed by the nanocomposite to reach its higher melting temperature

6.4.2.4: Mechanical Analysis

Mechanical Analysis was carried out through Instron Microtensile Testing system at Smitha Labs, Department of Textile technology, IIT Delhi as described in material and method section.

Table 6.45: Mechanical analysis of PHB +Zeolite blends

	Max Extension	Tensile stress@ MaxLoad	Tensile strain@Max Load	Modulus (Automatic)	Maximum Load	Modulus (Automatic Young's)	Energy at Break (Standard)
	(mm)	(MPa)	(%)	(MPa)	(N)	(MPa)	(J)
PHB control	0.5525	0.23	1.685	3.0525	1.35	3.4975	0.00054
1wt%	0.4225	1.045	11.0575	98.8225	0.8975	109.4	0.00201875
3wt%	0.3825	1.125	1.535	109.6625	0.88	118.875	0.000305
5wt%	0.6825	1.9675	2.735	113.86	1.5925	122.5475	0.00072
7wt%	0.495	1.9975	1.985	137.5225	1.5975	153.4775	0.00046
10wt%	0.505	1.305	2.0175	103.3275	0.975	113.7625	0.0003225

6.4.2.4(i): Observation of Mechanical Instron Testing

Mechanical properties of neat PHB and their blends with different ratios are depicted in above given Tables. Neat PHB has Maximum extension of 0.5300 mm, Tensile stress of 0.2400 MPa, Tensile strain (%) of 11.6967 MPa, Maximum load (N) of 1.4067, and Energy at break of 0.00054 J. Incorporation of Zeolite within the polymeric matrix results in intermediate properties for the blends. However, the ductility of composite PHB increased consistently with an increase in Zeolite concentration within the PHB matrix.

6.4.2.4(ii): Mechanical Properties of PHB/Zeolite Blends with variable Zeolite loading

The blend prepared at 5% ratio of PHB: Zeolite shows an optimum increase in maximum extension of 0.7567mm, tensile stress of 3.0567 MP, tensile strain of 3.0300%, modulus automation of 124.37 MPa and energy at break of 0.0008 J whereas in 7wt% showed a tensile stress of 2.3100 MPa, Maximum load of 1.6233 N, modulus automation of 142.77 MPa and energy at break of 0.0005 J with a decrease in maximum extension of 0.5067 mm as compared to pristine polymeric film. The observation suggests that some degree of interaction occurs between the macromolecules of PHB and Zeolite within the blend. Finely dispersed Zeolite nanoparticles act like a reinforcing filler within the PHB matrix. Enhanced ductility

provides better energy absorbing capability for the PHB matrix. However, the overall analysis suggested that the blend of PHB+5wt% Zeolite have a better mechanical performance as compared to the other blends as well as control polymeric matrix.

6.4.2.4a: Statistical studies of mechanical analysis of PHB+ wt% Zeolite by using ANOVA SPSS 16.0 tool

Table 6.46: Comparison of different wt% of PHB composites with PHB control in terms of maximum extension parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	0.5300± 0.04000	0.099
PHB+1wt% Zeolite	0.5033±0.13650	0.099
PHB+3wt% Zeolite	0.4233±0.06028	0.099
PHB+5wt% Zeolite	0.7567± 0.21127	0.099
PHB+7wt% Zeolite	0.5067±0.06807	0.099
PHB+10wt% Zeolite	0.5633±0.14742	0.099

Table 6.47: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of maximum extension parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% Zeolite	0.02667	1.000
	PHB+3wt% Zeolite	0.10667	.896
	PHB+5wt% Zeolite	-0.22667	.302
	PHB+7wt% Zeolite	0.02333	1.000
	PHB+10wt% Zeolite	-0.03333	.999

The analysis shows that the values were statistically non-significant (p≥0.05).

Table 6.48: Comparison of different wt% of PHB composites with PHB control in terms of tensile stress parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	.2400±.07000	.007
PHB+1wt% Zeolite	2.4333±.46918	.007
PHB+3wt% Zeolite	2.9167±.91309	.007
PHB+5wt% Zeolite	3.0567±.10786	.007
PHB+7wt% Zeolite	2.3100±1.20615	.007
PHB+10wt% Zeolite	3.2567±1.17194	.007

Table 6.49: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of tensile stress parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% Zeolite	-2.19333	.052
	PHB+3wt% Zeolite	-2.67667*	.015
	PHB+5wt% Zeolite	-2.81667*	.011
	PHB+7wt% Zeolite	-2.07000	.071
	PHB+10wt% Zeolite	-3.01667*	.006

The analysis shows that the values with superscript* were statistically significant (p≥0.05).

Table 6.50: Comparison of different wt% of PHB composites with PHB control in terms of tensile strain parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	11.4733±.83500	.000
PHB+1wt% Zeolite	2.0617±.46401	.000
PHB+3wt% Zeolite	1.6967±.23587	.000
PHB+5wt% Zeolite	3.0300±.84042	.000
PHB+7wt% Zeolite	2.0733±.20404	.000
PHB+10wt% Zeolite	1.7167±.36910	.000

Table 6.51: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of tensile strain parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% Zeolite	9.41167*	.000
	PHB+3wt% Zeolite	9.77667*	.000
	PHB+5wt% Zeolite	8.44333*	.000
	PHB+7wt% Zeolite	9.40000*	.000
	PHB+10wt% Zeolite	9.75667*	.000

The analysis shows that the values with superscript* were statistically significant (p≥0.05).

Table 6.52: Comparison of different wt% of PHB composites with PHB control in terms of modulus automation parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	3.3400±.98199	.001
PHB+1wt% Zeolite	94.9867±54.66621	.001
PHB+3wt% Zeolite	1.2512E2±32.10125	.001
PHB+5wt% Zeolite	1.2437E2±24.90441	.001
PHB+7wt% Zeolite	1.4277E2±11.79487	.001
PHB+10wt% Zeolite	1.1448E2±2.94824	.001

Table 6.53: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of modulus automation parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% Zeolite	-91.64667*	.018
	PHB+3wt% Zeolite	-121.78333*	.002
	PHB+5wt% Zeolite	-121.02667*	.002
	PHB+7wt% Zeolite	-139.42667*	.001
	PHB+10wt% Zeolite	-111.13667*	.004

The analysis shows that the values with superscript* were statistically significant (p≥0.05).

Table 6.54: Comparison of different wt% of PHB composites with PHB control in terms of maximum load parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	1.4067±.13650	.027
PHB+1wt% Zeolite	1.2958±.37819	.027
PHB+3wt% Zeolite	.9833±.31880	.027
PHB+5wt% Zeolite	1.7000±.14731	.027
PHB+7wt% Zeolite	1.6233±.06429	.027
PHB+10wt% Zeolite	1.1033±.29838	.027

Table 6.55: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of maximum load parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% Zeolite	.13650	.993
	PHB+3wt% Zeolite	.37819	.364
	PHB+5wt% Zeolite	.31880	.710
	PHB+7wt% Zeolite	.14731	.889
	PHB+10wt% Zeolite	.06429	.682

The analysis shows that the values were statistically non-significant (p≥0.05).

Table 6.56: Comparison of different wt% of PHB composites with PHB control in terms of Modulus (Automatic Young's) parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	3.8933±.98199	.000
PHB+1wt% Zeolite	128.22±54.66621	.000
PHB+3wt% Zeolite	136.08±32.10125	.000
PHB+5wt% Zeolite	132.00±24.90441	.000
PHB+7wt% Zeolite	156.28±11.79487	.000
PHB+10wt% Zeolite	119.49±2.94824	.000

Table 6.57: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of Modulus (Automatic Young's) parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% Zeolite	-124.32333*	.000
	PHB+3wt% Zeolite	-132.18333*	.000
	PHB+5wt% Zeolite	-128.10333*	.000
	PHB+7wt% Zeolite	-152.38667*	.000
	PHB+10wt% Zeolite	-115.59667*	.000

The analysis shows that the values with superscript* were statistically significant (p≥0.05).

Table 6.58: Comparison of different wt% of PHB composites with PHB control in terms of energy at break parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	.0005±.00007	.106
PHB+1wt% Zeolite	.0004±.00026	.106
PHB+3wt% Zeolite	.0003±.00004	.106
PHB+5wt% Zeolite	.0008±.00034	.106
PHB+7wt% Zeolite	.0005±.00007	.106
PHB+10wt% Zeolite	.0003±.00020	.106

Table 6.59: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of energy at break parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% Zeolite	.00019	.823
	PHB+3wt% Zeolite	.00021	.771
	PHB+5wt% Zeolite	-.00022	.749
	PHB+7wt% Zeolite	.00005	.999
	PHB+10wt% Zeolite	.00023	.691

The analysis shows that the values were statistically non-significant (p≥0.05).

Table 6.60: Mechanical analysis of PHB +OMMT blends

	Max Extention (mm)	**Tensile stress @ Max Load (MPa)**	**Tensile strain@Max Load (%)**	**Modulus (Automation) (MPa)**	**Maximum Load (N)**	**Modulus (Automatic Young's) (MPa)**	**Energy at Break (Standard) (J)**
PHB control	0.5300	0.2400	11.4733	3.3400	1.4067	3.8933	0.0005
1wt%	0.3400	3.4633	1.363	251.08	1.9467	288.56	0.0000
3wt%	0.3467	3.4967	1.3800	286	2.333	285.64	0.0003
5wt%	0.4433	3.0567	1.7667	305.02	2.4433	250.98	0.0004
7wt%	0.3167	5.9900	1.2633	265.15	1.8500	262.37	0.0003
10wt%	0.2633	3.2567	1.0533	489.94	2.6067	489.09	0.0006

6.4.2.4(iii): Mechanical Properties of PHB/OMMT Blends with variable OMMT loading

The blend prepared at 5wt % ratio of PHB: OMMT shows an optimum increase maximum extension of 0.4433mm, tensile stress of 3.0567MPa, tensile strain of 1.7667%, modulus automation of 305.02MPa, maximum load of 2.4433N, modulus automation of 250.98MPa and energy at break of 0.0004Jwhereas in 10wt% shows maximum young modulus of 489.94 MPa, maximum energy at break 0.0006J and maximum load of 2.6067MPa. The overall analysis suggested that better ductility and mechanical performance were observed in 10wt% of OMMT blend with PHB matrix.

6.4.2.4b: Statistical studies of mechanical analysis of PHB+ wt% OMMT by using ANOVA SPSS 16.0 tool

Table 6.61: Comparison of different wt% of PHB composites with PHB control in terms of maximum extension parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	.5300±.04000	0.028
PHB+1wt% OMMT	.3400±.08185	0.028
PHB+3wt% OMMT	.3467±.04933	0.028
PHB+5wt% OMMT	.4433±.05774	0.028
PHB+7wt% OMMT	.3167±.16623	0.028
PHB+10wt% OMMT	.2633±.05508	0.028

Table 6.62: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of maximum extension parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% OMMT	.19000	.147
	PHB+3wt% OMMT	.18333	.171
	PHB+5wt% OMMT	.08667	.815
	PHB+7wt% OMMT	.21333	.087
	PHB+10wt% OMMT	.26667*	.024

The analysis shows that the values with superscript* were statistically significant (p≥0.05).

Table 6.63: Comparison of different wt% of PHB composites with PHB control in terms of tensile stress parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	.2400±.07000	.158
PHB+1wt% OMMT	3.4633±1.34783	.158
PHB+3wt% OMMT	3.4967±.57873	.158
PHB+5wt% OMMT	3.0567±.10786	.158
PHB+7wt% OMMT	5.9900±5.20265	.158
PHB+10wt% OMMT	3.2567±1.17194	.158

Table 6.64: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of tensile stress parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% OMMT	-3.21333	.532
	PHB+3wt% OMMT	-3.24667	.522
	PHB+5wt% OMMT	-2.80667	.658
	PHB+7wt% OMMT	-5.74000	.075
	PHB+10wt% OMMT	-3.00667	.596

The analysis shows that the values were statistically non-significant (p≥0.05).

Table 6.65: Comparison of different wt% of PHB composites with PHB control in terms of tensile strain parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	11.4733±.83500	.000
PHB+1wt% OMMT	1.3633±.33606	.000
PHB+3wt% OMMT	1.3800±.21166	.000
PHB+5wt% OMMT	1.7667±.21962	.000
PHB+7wt% OMMT	1.2633±.66033	.000
PHB+10wt% OMMT	1.0533±.22189	.000

Table 6.66: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of tensile strain parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% OMMT	9.27667*	.000
	PHB+3wt% OMMT	9.26000*	.000
	PHB+5wt% OMMT	8.87333*	.000
	PHB+7wt% OMMT	9.37667*	.000
	PHB+10wt% OMMT	9.58667*	.000

The analysis shows that the values with superscript* were statistically significant (p≥0.05).

Table 6.67: Comparison of different wt% of PHB composites with PHB control in terms of modulus automation parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	334.00±.98199	.000
PHB+1wt% OMMT	305.02±97.05512	.000
PHB+3wt% OMMT	286.00±56.02524	.000
PHB+5wt% OMMT	251.08±22.15435	.000
PHB+7wt% OMMT	265.15±88.81329	.000
PHB+10wt% OMMT	489.94±38.46726	.000

Table 6.68: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of modulus automation parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% OMMT	-301.34667*	.001
	PHB+3wt% OMMT	-282.32667*	.001
	PHB+5wt% OMMT	-247.41000*	.003
	PHB+7wt% OMMT	-261.47667*	.002
	PHB+10wt% OMMT	-486.27000*	.000

The analysis shows that the values with superscript* were statistically significant (p≥0.05).

Table 6.69: Comparison of different wt% of PHB composites with PHB control in terms of maximum load parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	1.4067±.13650	.285
PHB+1wt% OMMT	1.9467±.37207	.285
PHB+3wt% OMMT	2.3333±.73276	.285
PHB+5wt% OMMT	2.4433±.08505	.285
PHB+7wt% OMMT	1.8500±.96597	.285
PHB+10wt% OMMT	2.6067±.94129	.285

Table 6.70: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of maximum load parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% OMMT	-.54000	.902
	PHB+3wt% OMMT	-.92667	.527
	PHB+5wt% OMMT	-1.03667	.416
	PHB+7wt% OMMT	-.44333	.954
	PHB+10wt% OMMT	-1.20000	.278

The analysis shows that the values were statistically non-significant (p≥0.05).

Table 6.71: Comparison of different wt% of PHB composites with PHB control in terms of Modulus (Automatic Young's) parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	3.8933±.98199	.000
PHB+1wt% OMMT	2.8856E2±92.22192	.000
PHB+3wt% OMMT	2.8564E2±62.39548	.000
PHB+5wt% OMMT	2.5098E2±25.26254	.000
PHB+7wt% OMMT	2.6237E2±85.32612	.000
PHB+10wt% OMMT	4.8909E2±42.43393	.000

Table 6.72: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of Modulus (Automatic Young's) parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% OMMT	-285.21667*	.001
	PHB+3wt% OMMT	-282.30000*	.001
	PHB+5wt% OMMT	-247.63667*	.003
	PHB+7wt% OMMT	-259.03000*	.002
	PHB+10wt% OMMT	-485.75333*	.000

The analysis shows that the values with superscript* were statistically significant (p≥0.05).

Table 6.73: Comparison of different wt% of PHB composites with PHB control in terms of energy at break parameter using one way ANOVA

	Mean± Standard deviation	**P value≥0.05**
PHB Control	.0005±.00007	.245
PHB+1wt% OMMT	.0000±.00053	.245
PHB+3wt% OMMT	.0003±.00020	.245
PHB+5wt% OMMT	.0004±.00021	.245
PHB+7wt% OMMT	.0003±.00035	.245
PHB+10wt% OMMT	.0000±.00034	.245

Table 6.74: Statistical analysis of multiple comparison of different wt% of PHB composites with PHB control in terms of energy at break parameter using One way ANOVA {POSTHOC=TUKEY ALPHA (*P<0.05)}

		Mean Difference	**P value≥0.05**
A	B	(A-B)	
PHB Control	PHB+1wt% OMMT	.00049	.446
	PHB+3wt% OMMT	.00023	.943
	PHB+5wt% OMMT	.00019	.976
	PHB+7wt% OMMT	.00026	.909
	PHB+10wt% OMMT	.00064	.209

The analysis shows that the values were statistically non-significant (p≥0.05).

6.4.3: Biodegradation Assay studies

6.4.3.1: Biodegradation study by PBS solution and its analysis (PHB+ Zeoite wt%)

One of the most attractive properties of PHB is its biodegradable nature under natural conditions.

In the current studies, biodegradation assay was carried out through Phosphate buffer saline as described in material method section. It was observed that the percentage degradation of neat PHB was 30.08%, where as this percentage was increased to 50% in case of 3wt% film of PHB+Zeolite. This increase in biodegradation percentage was also observed in case of 1wt% (44.44%), 5wt% (37.5%), 7wt% (33.28%) and 10wt% (31.5%). The results show that an increase in biodegradation was observed in all the blended films as compared to neat PHB and maximum was observed at 3wt%.

6.4.3.2: Biodegradation assay by bacteria and its analysis

Another biodegradation assay was carried out by bacterial isolate (MTCC40) as described in material and method section. The microorganisms in the presence CO_2 and H_2O hydrolyze the ester bonds of the polymeric material. It was observed that the percentage degradation of neat PHB was 31%, where as this percentage was increased to 75% in case of 10 wt% film of PHB+Zeolite. This increase in biodegradation percentage was also observed in case of 1wt% (34.28%), 3wt% (38.57), 5wt% (39. 33%), 7wt% (40%). The results show that an increase in biodegradation was observed in all the blended films as compared to neat PHB and maximum was observed at 10wt%. The results are in coherence with the studies of Maiti *et al.,* 2007 that increase in nanoclay level without any organic modification enhances the degradation level of polymer nanocomposite formed. The work is in coherence with Yu *et al.,* 2006 that increase in wt% of nanoclay enhances the mechanical properties of PHB/nanoclay composites.

Table 6.75: Biodegradation of PHB+Zeolite blended film by Phosphate Buffer Saline (PBS) and Microbial strain MTCC 40 (*E.coli*)

wt% of PHB films	**% Degradation (PBS)**	**% Degradation (MTCC 40)**
Pure PHB	30.08%	31%
PHB+1wt% Zeolite	44.44%	34.28%
PHB+3wt% Zeolite	50%	38.57%
PHB+5wt% Zeolite	37.5%	39.33%
PHB+7wt% Zeolite	33.28%	40%
PHB+10wt% Zeolite	31.5%	75%

6.4.3.3: Biodegradation study by PBS solution and its analysis (PHB+OMMT)

In the current studies, biodegradation assay was carried out by Phosphate buffer saline. It was observed that the percentage degradation of neat PHB was 30.08%, where as this percentage was increased to 62.22% in case of 3wt% film of PHB+OMMT. This increase in biodegradation percentage was also observed in case of 1wt% (33.7%), 5wt% (37.9%), 7wt% (31.79%) and 10wt% (35.26%). The results show that an increase in biodegradation was observed in all the blended films as compared to neat PHB and maximum was observed at 3wt%.

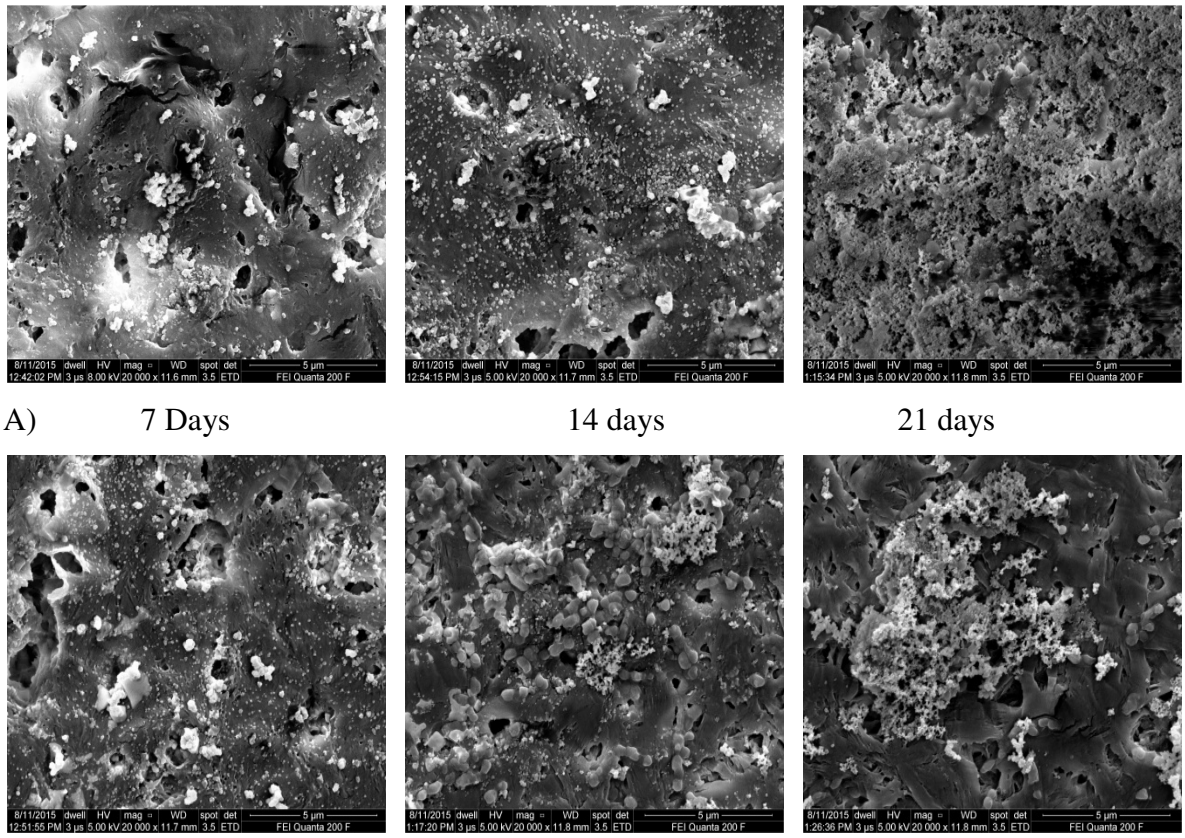
6.4.3.4: Biodegradation assay by bacteria and its analysis

Another biodegradation assay was carried out by bacterial isolate (MTCC40). It was observed that the percentage degradation of neat PHB was 31% by bacterial strain degradation, where as this percentage was increased to 59% in case of 5wt% film of PHB+OMMT. This increase in biodegradation percentage was also observed in case of 1wt% (31.4%), 3wt% (40%), 7wt% (31.9%), 10 wt% (33%). The results show that an increase in biodegradation was observed in all the blended films as compared to neat PHB and maximum was observed at 5wt%. The results of biodegradation by MTCC strain are in coherence with the results of Leet *et al.,* 2002 that increase in concentration of nanoclay after a certain level decreases the biodegradation percentage as observed that after 5wt% the degradation levels decreases.

Table 6.76: Biodegradation in PHB+OMMT blends by Phosphate Buffer Saline (PBS) and Microbial strain MTCC 40 (*E.coli*)

wt% of PHB films	**%Degradation (PBS)**	**% Degradation (MTCC 40)**
Pure PHB	30.08%	31%
PHB+1wt% OMMT	33.7%	31.4%
PHB+3wt% OMMT	62.22%	40%
PHB+5wt% OMMT	37.9%	59%
PHB+7wt% OMMT	31.79%	31.9%
PHB+10wt% OMMT	35.26%	33%

6.4.4: SIMULATED BODY FLUID ASSAY FOR APETITE LAYER FORMATION



A) 7 Days 14 days 21 days

B) 7 Days 14 days 21 days

Figure 6.4.20: Simulated Body Fluid Assay FE-SEM analysis at different time intervals of 7days, 14days and 21days A): PHB+3wt% Zeolite B): PHB+5wt% OMMT

The protocol was carried out as described in material and method to check the capability of the material for inducing osteoblast of bone cells to join and thrive over the material. Basically, SBF is the deposition of calcium particles (apatite layer) over the material in in-vitro conditions, providing the same environment which is comparable to the human blood plasma. The analysis of SBF can be done on the basis that if the deposition is taking place over the material in-vitro then this material can induce the growth of cells over it in-vivo. It can be utilized in implants and other bone tissue engineering protocols inside the live human body.

It has been already reported that zeolites can be used both in human and veterinary medicine as biological active food additives (dietic additives), drugs, drug carriers, adjuvants in anticancer therapy and antimicrobial agents as well. They are tolerated well in the body and

are highly biocompatible in living cells (Teimuraz *et al.,* 2009).

The current analysis shows positive deposition of apatite layer over the test material (A: PHB+3wt% Zeolite, B: PHB+5wt% OMMT) in course of time duration varying from 7, 14 and 21 days. This material can be utilized in bone tissue engineering protocols and in implants positively.

SUMMARY AND CONCLUSION

Continuous use of plastic in day to day life has caused their huge accumulation in the environment. Plastics are utilized in many industries including food packaging industry, paints, toys etc. A major part of plastic waste comes from its applicability in short-live food packaging industries. The plastic waste persistence in the environment causes pollution and crisis. Bio-polyesters derived from renewable and natural resources i.e. wood pulp, bio-waste, plant derived products i.e. chitin and chitosan and PHB can come in handy in the replacement of plastic waste. Continuous research on bio-plastic increased its demand specifically in various domestic and commercial industries.

The current work was carried out with the aim of finding out high PHB producing bacterial strains from various regions of Punjab, salt lakes of Rajasthan, their optimization strategies and further enhancement of properties of polyhydroxybutyrate was carried out by blending techniques with different nanoclays.

The work included the production of Polyhydroxybutyrtae with a number of approaches, which mainly included the mutagenesis studies and optimization parameters for its enhancement and further preparation of nanocomposites films of PHB with improved properties were prepared with an aim to improve its properties and applicability in biomedical fields.

The major concluding points from this present work show that a majority of bacterial isolates are producing PHB under stress conditions. The level of PHB production is enhanced after full growth of bacterial biomass has taken place. After achievement of the stationary phase major PHB accumulation takes place in these bacterial isolates.

Bacterial isolates from Majha, Malwa, Doaba regions of Punjab along with isolates from Sambhar lake were the main strains capable of producing and accumulating larger amounts of PHB inside their cells.

Under normal conditions the PHB accumulation varied from 9% to 34 % in various isolates. The highest accumulation of 34% PHB under normal condition was observed in a Sambhar isolate.

Isolates accumulated larger amounts of PHB when they were transferred to Biphasic condition with sufficient amounts of carbon and depleted conditions of nitrogen. Under such circumstances PHB accumulation enhances and observed to vary from 12 % to 51%. Again the highest accumulator was found to be the Sambhar isolate with accumulation levels shooting to 51%. These results exemplify the fact that higher carbon concentrations and depleted nitrogen content favours the PHB production.

The first step in enhancement strategies was mutating the bacteria with certain mutagenic agents which included physical UV mutagen and chemical mutagens such as Acridine Orange, Ethidium Bromide, Sodium Azide and Ethyl Methane Sulphonate.

Many of the mutagens didn't produced any major enhancement in production levels of PHB, but some of the mutagens were successful in causing mutations that can cause the enhancement of PHB accumulation levels.

Acridine orange caused enhancement of PHB production from 41 % to 51 % in S-3 bacterial strain under carbon excess presence. UV presence caused increment from 12 % to 24.99% in M-1 bacteria. UV presence caused increment from 12% to 37% in MD-1 isolates.
These results justify the fact that mutagenic agents can play a role in enhancement of PHB production in various isolates.

The next strategy for incrementing the PHB production was optimizing the carbon and nitrogen sources for observing the effect on PHB production. Presence of different carbon sources can have sufficient effect on the production levels of PHB. Major enhancement in PHB production through variation of carbon sources was seen with S-3 bacteria from 2% maltose causing enhancement from 50% to 67%. M-1 bacteria enhance its production from 25% to 46% in presence of 3% glucose. In MD-2 bacteria enhance its production from 28% to 45% in presence of 3% sucrose. These results further justifies that different carbon sources can have variation of effects on the level of PHB production. These higher production values

under different carbon sources can be employed for commercialization strategies for cheaper PHB production by industries.

Various nitrogen sources mainly caused an increase in the cell biomass along with presence of carbon. They didn't promoted enhancement in the PHB production levels. In presence of both nitrogen and carbon sources energy produced is mainly diverted towards increment in biomass and not in the accumulation of PHB. Presence of both nitrogen and carbon create a condition of positive environment and stress levels are very low or almost nil. Since the PHB accumulation is stimulated with stress condition hence presence of both nitrogen and carbon act as inhibitor in the higher accumulation of PHB.

The outcome of these studies provide that certain species have been identified which could be used under specified conditions for commercial production of PHB for industry and market use. These high PHB producer strains can further be used in recombinant technology studies for higher enhancement of PHB production. Also, cheaper substrates such as potato peel, sugarcane molasses, corn starch, soyabean oil etc. could be used, for more economical production of PHB and help in the making the polyhydroxybutyrate production at large scale with low cost approach.

Improvement in the physical and mechanical properties of polyhydroxybutyrate was achieved by blending techniques. Two types of composites were prepared and analysis was done on the basis of their thermal processing by comparing with pristine PHB. Mechanical properties were also enhanced and were analysed by Instron tensile testing. Two types of nanoclays were used in the current studies for enhancement of properties i.e. Zeolite and dimethy-dialkyl amine surface modified montomorrillonite. The thermal processing window was improved in both the nanocomposite as compared to the parental PHB.

Earlier composites that were designed with micro level particles were having 2 major components, one was the bulk polymer and other was the filler material, a third component which was minor in nature i.e. interfacial polymer. Poor interfacial bonding did not resulted into the observance of desired properties and could result in material failure under different parameters while the polymer nanocomposite utilizes the reinforcing particles at nano-meter scale and is a minor component in terms over all weight and volume. When this nanoparticles

becomes fully dispersed in the polymeric matrix, the overall bulk polymer also becomes a minor component. The entire polymer becomes an interfacial polymer and acquires changed and enhanced properties. These enhanced properties are due to interfacial polymer which becomes magnified in the final material. Hence, a polymer nanocomposite is a composite where both the filler and matrix bulk polymer are minor component and interfacial polymer dictates the material properties.

Organic treatment of the clay changes the normally hydrophilic montmorrillonite hydrophobic, and this change allows it to interface with different polymeric matrices.

Literature study reveals that Zeolite, is a natural nanoclay and is used in many filtration strategies. Zeolites have attracted much interest majorly due to their ion-exchange capabilities along with higher affinity towards divalent sorbates. Existence of zeolites in powder form in nanoscale posses the limitation. Aggregation of particles in different electrolytes causes the variations in the flow properties of the mineral, which is undesired feature for acting as sorbent. Therefore it becomes imperative for incorporation of particular additives so as to counter-act this behaviour. Particularly, incorporation of polymers can enable the mineral to act as adsorbent in an isolated confinement and enriching its applicability in aquatic systems. In this study, zeolite was incorporated into Polyhydroxybutyrate polymer matrices. It is anticipated that the resultant nanocomposites is having enhanced adsorption properties which are highly suitable for heavy-metal decontamination and usage as a filter. Zeolite has high thermal stability at 700^{o}C in air, which increases the thermal stability of polymeric matrix of PHB. PHB acts as matrix, as Zeolite is powder in form and cannot act as a good sorbent in its natural form, that is why many filters has been prepared by non-biodegradable materials.

The use of biodegradable filters is basically for the removal of heavy metal ions. In the filtration process, pre-filtration could be done by UV radiations or other sieves, where all the microbial species that are micrometer in size are removed. Metal removal during further purification by membrane filter prepared by Zeolite composites could be achieved.

It is established that zeolites can be used both in human and veterinary medicine as biological active food additives (dietic additives), drugs, drug carriers, adjuvants in anticancer therapy and antimicrobial agents as well. They are tolerated well in the body and are highly biocompatible. On the basis of this literature we can very well suggest that they could be

easily used in in-vivo applications such as nanocomposite preparation for biomedical application.

Surface morphology was analysed by FE-SEM technique. Increase in the porosity enhances the nanocomposite application. It has already been reported in many research papers that PHB is biocompatible in nature. With the increase in pore size, it could also easily be used as scaffolds material in many biomedical applications.

The supporting experiments were simulated body fluid assay for biocompatibility studies, Phosphate buffer saline and bacterial biodegradation assay studies were also carried out. SBF experiment justifies the fact that these nanocomposites strongly promote the calcium deposition through apatite layer in SBF assay. The PBS degradation assay on the other hand provides that it is biodegradable under normal body buffer conditions. So, ultimately it engineers the positive deposition of apatite layer enhancing the cell deposition and growth of cells over it and on the other hand degradation of this composite occurs with time inside the body stimulating the bone or crack joining with time when utilized for tissue engineering or bone surgical studies.

Apatite layer formation is a highly positive sign which is positively enhancing the deposition and growth of the body cells over it. On the basis of these properties these PHB nanocomposite are having strong application criteria for scaffolds formation material in artificial 3D organ printing technology and tissue engineering approaches.

BIBLIOGRAPHY

- **Amoli RI, Sharifzadeh Baei M, Pirouz F.** 2013. Polyhydroxyalkanoate Production by Cultivating *Hydrogenophaga pseudoflava* in Fed Batch Culture. Middle-East Journal of Scientific Research 14 (8): 1035-1039.
- **Abd ER, Shanshoury RE, Kenawy ER, Amara AA, Salama AF, Kishk SS.** 2013. Optimization of Polyhydroxybutyrate (PHB) production by Azotobacter vinelandii using experimental design Int J Curr Microbiol App Sci 2(11): 227-241.
- **Adwitiya P, Ashwini P, Avinash AK, Badri R, Kajal D, Vomsi P, Srividya S.** 2009 Mutagenesis of *Bacillus thuringiensis* IAM 12077 for increasing poly (-β-)hydroxybutyrate (PHB) production Turk J Biol 33 225-230
- **Adwitiya P, Ashwini P, Avinash AK, Badri R, Kajal D.** 2009. Mutagenesis of *Bacillus thuringiensis* IAM 12077 for increasing poly -βhydroxybutyrate (PHB) production. Turk J Biol 33: 225-230.
- **Alberti G, Costantino U, Casciola M, Vivani R.** 1991. Proton conductivity of zirconium carboxy n-alkyl phosphonates with an $-layered structure. Solid State Ionics 46: 61–68.
- **Aldor IS, Keasling JD.** 2003. Process design for microbial plastic factories: metabolic Engineering of Polyhydroxyalkaonates. Current Opinion in Biotechnology. 14: 475–483.
- **Alemdar A, Sain M.** 2008. Biocomposites from wheat straw nanofibers: morphology, thermal and mechanical properties. Composites Science and Technology 68(2): 557–565.
- **Alemdar A, Sain M.** 2008. Isolation and characterization of nanofibers from agricultural residues—wheat straw and soy hulls. Biores Technol 9(6): 1664–1671.
- **Alexandre M, Dubois P.** 2000. Polymer-layered silicate nanocomposites: Preparation, properties and uses of a new class of materials. Mater Sci Eng R 28: 1-63.
- **Amass W, Amass A, Tighe B.** 1998. A review of biodegradable polymers: use, current developments in the synthesis and characterization of biodegradable

polyesters, blends of biodegradable polymers and recent advances in biodegradable studies. Polymer Int 47: 89-144.

- **American Public Health Association (APHA),** Standard methods for the Examination of Water and Wastewater. 21 st ed. Published by APHA, AWWA, WPCF Washington D.C 2006.
- **Teimuraz A, Karaman P, T Kurashvili, L Eprikashvili.** 2009. Possibility of Application of Natural Zeolites for Medicinal Purposes. Bull Georg Natl Acad Sci 3 (2): 158-167.
- **Anderson AJ, Dawes EA.** 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54(4): 450-72.
- **Andrade AL.** 2011 Micro plastics in the marine environment. Marine Pollution Bulletin 62:1596-160.5
- **Aneja KR.** 2003. Experiments in Microbiology, Plant Pathology and Biotechnology ISBN : 978-81-224-1494-3, New Age International Publishers.
- **Atlas RM**. 1993. Microbial Ecology: Fundamentals and Applications, Third ed., 39-43.
- **Babu RP, O'Connor K, Ramakrishna S.** 2013**.** Current progress on bio-based polymers and their future trends. Bacterially Produced Polyhydroxyalkanoate (PHA): Converting Renewable Resources into Bioplastics. Progress in Biomaterials 2:8.
- **Bae KP, Moo-Moo K.** 2010. Applications of chitin and its derivatives in biological medicine. Int J Mol Sci 11: 5152-5164
- **Bansal V, Pramod KS, Nitin S, Omprakask P, Malviya R.** 2011. Applications of chitosan and chitosan derivatives for drug delivery. Adv Biol Res 5: 28-37.
- **Barkalow DG, Chapedelaine AH, Dzija MJ.** 2002. Improved pullulan free edible film compositions and methods of making same. PCT International Application WO 02/43657, US 01/43397.
- **Bauer R.** 1938. Physiology of Dematium pullulans de Bary. Zentralbl Bacteriol Parasitenkd Infektionskr Hyg Abt 98: 133-167.
- **Berkland C, Kim C, Pack DW.** 2001. Fabrication of PLG microspheres with precisely controlled and monodisperse size distributions. J Control Release 73(1): 59–74.

- **Bernier B.** 1958. The production of polysaccharides by fungi active in the decomposition of wood and forest litter. Can J Microbiol 4: 195-204.
- **Bhatia M, Girdhar A, Tiwari A, Nayarisseri A.** 2014. Implications of a novel Pseudomonas species on low density polyethylene biodegradation: an in vitro to in silico approach. SpringerPlus 3:497.
- **Biddlestone F, Harris A, Hay JN, Hammond T.** 1996. Physical Aging of Amorphous Poly(hydroxybutyrate). Pol Inter 39: 221-229.
- **Bonartsev AP, Myshkina VL, Nikolaeva DA, Furina EK, Makhina TA, Livshits VA, Boskhomdzhiev AP, Ivanov EA, Iordanskii AL, Bonartseva GA.** 2012. Biosynthesis, biodegradation, and application of poly(3-hydroxybutyrate) and its copolymers - natural polyesters produced by diazotrophic bacteria. Communicating Current Research and Educational Topics and Trends in Applied .Microbiology 7: 1751–1760
- **Bonartsev AP, Myshkina VL, Nikolaeva DA., Furina EK, Makhina TA, Livshits, VA, Boskhomdzhiev AP, Ivanov EA, Iordanskii AL, Bonartseva GA.** 2007. Biosynthesis, biodegradation, and application of poly(3-hydroxybutyrate) and its copolymers - natural polyesters produced by diazotrophic bacteria. Formatex 295-307.
- **Boo WJ, Sun LY, Liu J, Clearfield A, Sue HJ, Mullins MJ, Pham H.** 2007 Morphology and mechanical behaviour of exfoliated epoxy/zirconium phosphate nanocomposites. Composites Sci Technol 67: 262–269
- **Bothner B, Siuzdak G.** 2004. Electrospray ionization of a whole virus: Analyzing mass, structure, and viability. Chembiochem **5** (3). 258- 260.
- **Brandl H, Gross RA, Lenz RW, Ramona L, Clinton FR.** 1991. The accumulation of poly(3-hydroxyalkanoates) in *Rhodobacter sphaeroides*. Archives of Microbiology 155(4): 337-340
- **Brandl, H., Gross, R.A., Lenz, R.W., Fuller, R.C.** 1988. *Pseudomonas oleovorans* as a source of Poly β-Hydroxyalkaonates for Potential Applications as Biodegradable Polyesters. App Env Microbiol 54(8): 1977-1982.
- **Byrom D.** 1994.Polyhydroxyalkanoates. In: Mobley DP, editor. Plastics from microbes: microbial synthesis of polymers and polymer precursors. Munich: Hanse. 5-33.

- **Cain RB.** 1992. Microbial degradation of synthetic polymers. In: Frey et al. (eds) Microbial Control of Pollution. 48th Symposium of the Society for general microbiology at University of Cardiff, pp. 293-338.
- **Cao X, Chen Y, Chang PR, Muir AD, Falk G.** 2008. Starch-based nanocomposites reinforced with flax cellulose nanocrystals. EXPRESS Polymer Letters 2(7): 502–510.
- **Catley BJ.** 1971. Utilization of carbon sources by Pullularia pullulans for the elaboration of extracellular polysaccharides. Appl Microbiol 22: 641-649.
- **Ceyhan N, Ozdemir G.** 2011. Poly-β-hydroxybutyrate (PHB) production from domestic wastewater using *Enterobacter aerogenes* 12Bi strain. Afr J Mic Res 5(6): 690-702.
- **Chanprateep S, Kulpreecha S.** 2006. Production and Characterization of Biodegradable terpolymer Poly(3-Hydroxyvalerate-co-3-hydroxyvalerate-co-4 hydroxybutyrate) by *Alcaligenes* sp-04. J Biosci Bioeng 101(1): 51-56.
- **Charron N.** 2001. Plastic Products and Industries. Statistics Canada Ref. No. 33-250-XIE.Ottawa, Canada: Manufacturing, Construction, and Energy Division.
- **Chavan PN, Bahir MM, Mene RU, Mahabole MP, Khairnar RS.** 2010. Study of nanobiomaterial hydroxyapatite in simulated body fluid: Formation and growth of apatite Mat Sci Eng B 168: 224–230.
- **Chee JY, Yoga SS, Lau NS, Ling SC, Abed RMM, Kumar S.** 2010. Current research, technology and Education topics in Applied Microbiology and Microbiology Biotechnology 1395-1404.
- **Chen GQ, Patel MK.** 2012. Plastics Derived from Biological Sources: Present and Future: A Technical and Environmental Review. Chemical Rev 112: 2082–2099.
- **Chen GQ, Wu Q.** 2005. The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials 26: 6565–6578.
- **Chen GQ.** 2009. A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem Soc Rev 38: 2434–2446.
- **Chen JY, Liu T, Zheng Z, Chen JC, Chen GQ.** 2004. Polyhydroxyalkanoate synthases PhaC1 and PhaC2 from *Pseudomonas stutzeri* 1317 had different substrate specificities. FEMS Microbiol Lett 234:231–237.
- **Chen LJ, Wang M.** 2002. Production and evaluation of biodegradable composites based on PHB-PHV copolymer. Biomaterials 23(13): 2631–2639.

- **Chen Q, Zhu C, Thouas GA.** 2012. Progress and Challenges in Biomaterials used for Bone Tissue Engineering: Bioactive Glasses and Elastomeric Composites. Progress in Biomaterials 1(2): 1-22.
- **Chen Y, Cao X, Chang PR, Huneault MA.** 2008. Comparative study on the films of poly(vinyl alcohol)/pea starch nanocrystals and poly(vinyl alcohol)/native pea starch. Carbohydrate Polymers 73: 8–17.
- **Chen GQ, Wu Q.** 2005. The Application of Polyhydroxyalkanoates as Tissue Engineering Materials. Biomaterials 26: 6565-6578.
- **Cheng KC, Demirci A, Catchmark JM.** 2011. Pullulan: biosynthesis, production, and applications. Appl Microbiol Biotechnol 92:2 9-44
- **Cheng ML, Lin YR, Lin ZZ, Liao CS, Sun YM.** 2004. Physical and transport properties of polyhydroxybutyrate/clay nanocomposite membranes. 87(96): 87.
- Chien CC, Chen CC, Choi MH, Kung SS, Wei YH. 2007. Production of poly-β-hydroxybutyrate (PHB) by *Vibrio* spp. isolated from marine environment. J Biotechnol 132: 259-263.
- **Ching-Yee L, Kumar S.** 2007. Polyhydroxyalkanoates: Bio-based microbial plastics and their properties. Malaysian Polymer Journal 2 (2): 31-57, 2007.
- **Choi JL, Lee SY.** 1999. High-Level Production of Poly(3- Hydroxybutyrate-co-3-Hydroxyvalerate) by Fed-Batch Culture of Recombinant Escherichia coli. App Envir Microbiol 65(10): 4363-4368
- **Choi HJ, Kim SG, Hyun YH, Jhon MS.** 2001. Preparation and rheological characteristics of solvent-cast poly(ethylene oxide)/montmorillonite nanocomposites. Macromol Rapid Commun 22:320–5.
- **Christopher J, Brigham A, Sinskey J.** 2012. Applications of Polyhydroxyalkanoates in the Medical Industry International Journal of Biotechnology for Wellness Industries 1: 53-60
- **Collee JG, Duguid JP, Fraser AG, Marmion BP.** 1989.Practica Medical Microbiology. 3th Ed. Mackie and McCartney 54-55.
- **Conca KR, Yang TCS**. 1993. Edible food barrier coatings. In: Ching C, Kaplan DL, Thomas EL (eds) Biodegradable polymers and packaging, Technomic Lancaster 357-369.

- **De Smet MJ, Eggink G, Witholt B, Kingma J, Wynberg H.** 1983. Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J Bacteriology 154: 870-878.
- **Di Y, Iannace S, Di ME, Nicolais L.** 2005. Poly(lactic acid)/organoclay nanocomposites: Thermal, rheological properties and foam processing. Journal of Polymer Science Part B: Polymer Physics 43: 689–698.
- **Diba M, Kharaziha M, Fathi MH, Gholipourmalekabadi M, Samadikuchaksaraei A.** 2012. Preparation and characterization of polycaprolactone/forsterite Nanocomposite porous scaffolds designed for bone tissue regeneration. Composites Sci Technol 72: 716–723
- **Doi Y, Kawaguchi Y, Koyama N, Nakamura S, Hiramitsu M, Yoshida Y, Kimura U**. 1992. Synthesis and degradation of polyhydroxyalkanoates in *Alcaligenes eutrophus*. FEMS Microbiol Rev 103: 103-108.
- **Domon B, Aebersold R.** 2006. Review - Mass spectrometry and protein analysis. Science **312** (5771): 212-217
- **Doyle C, Tanner ET, Bonfield W.** 1991. In vitro and in vivo evaluation of polyhydroxybutyrate and of polyhydroxybutyrate reinforced with hydroxyapatite. Biomaterials 12: 841–847.
- **Du G, Yu J.** 2002b. Green technology for conversion of food scraps to biodegradable thermoplastic polyhydroxyalkanoates. Environ Sci Technol 36: 5511-5516.
- **Duncan M.** 2003. U.S. Federal Initiatives to Support Biomass Research and Development. Journal of Industrial Ecology 7 (3-4) : 193–201.
- **Eggersdorfer M.J. Meyer PE.** 1992. Use of renewable resources for non-food material. FEMS Microbial Rev103: 355-364.
- **Eichhorn SJ, Dufresne A, Aranguren M.** 2010. Review: current international research into cellulose nanofibres and nanocomposites. J Mater Sci 45(1): 1–33.
- **Elhottova D, Tríska J, Petersen SO, Santrucková H.** 2000. Analysis of poly-beta-hydroxybutyrate in environmental samples by GC-MS/MS. Fresenius J Anal Chem 367(2): 157-64.

- **Elsayed NS, Aboshanab KM, Aboulwafa MM, Hassouna NA.** 2013. Optimization of bioplastic (poly-β-hydroxybutyrate) production by a promising *Azomonas macrocytogenes* bacterial isolates P173. Afr J Microbiol Res 7(43): 5025-5035.
- **El-Taweel SH, Stoll B, Hohne GWH, Mansour AA, Seliger H.** 2004. Stress–Strain Behavior of Blends of Bacterial Polyhydroxybutyrate. J Appl Polym Sci 94: 2528–2537
- **Evans J.** 2010. Bioplastics get growing. Plastics Engineering 66(2): 14-20.
- **Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM**.1989. Electrospray Ionization for Mass-Spectrometry of Large Biomolecules. Science, **246** (4926) 64-71.
- **Flechter A.** 1993. PHA as natural, biodegradable polyesters. In Plastics from bacteria and for bacteria. Springer Verlag, New York, 77-93.
- **Flora GD, Bhatt K, Tuteja U.** 2010. Optimization Of Culture Conditions For Poly Â- Hydroxybutyrate Production From Isolated *Bacillus* Species. J Cell Tissue Res 10(2): 2235-2242
- **Fomin VA**. 2001. Biodegradable polymers, their present state and future prospects. Progress In Rubber and Plastics Technology 17(3): 186-204.
- **Fortin Y1, Phoenix P, Drapeau GR.** 1990. Mutations conferring resistance to azide in Escherichia coli occur primarily in secA gene. Article J Bacteriol 172(11): 6607-10
- **Freier T, Kunze C, Nischan C.** 2002. *In vitro* and *in vivo* degradation studies for development of a biodegradable patch based on poly(3-hydroxybutyrate). Biomaterials 23: 2649-57.
- **Frost, Sullivan report.** 2009. Global bio-based plastic market, M4AI-39. Chapter 5.
- **Fuchs, JR, Nasseri BA, Vacanti JP**. 2001. Tissue Engineering: A 21st Century Solution to Surgical Reconstruction. The Annals of Thoracic Surgery 72:5 77–591.
- **Fukushima K, Fina A, Geobaldo F, Venturello A, Camino G.** 2012**.** Properties of poly(lactic acid) nanocomposites based on montmorillonite, sepiolite and zirconium phosphonate. eXPRESS Pol Letters 6 (11): 914–926.
- **Galego N, Miguens FC, Sanchez R.** 2002. Physical and functional characterization of PHASCL membranes Polymer 43: 3109.
- **Garces JM, Moll DJ, Bicerano J, Fibiger R, McLeod DG.** 2000. Polymeric nanocomposites for automotive applications. Adv Mater 12:1835-

- **Geotechnical Test Method** GTM-24 Revision#2 Geotechnical Engineering Bureau April 2007. New York State Department of Transportation
- **Giannelis EP** 1996. Polymer layered silicate nanocomposites. Adv Mater 8: 29.
- **Gomez-Guille MC, Gimenez B, Lopez CME, Montero MP.** 2011. Functional bioactive properties of collagen and gelatin from alternative sources: a review. Food Hydrocolloids 25: 1813-1827.
- **Grant WD, Kamekura M, McGenity TJ, Ventosa A.** 2001. Order I Halobacteriales Grant and Larsen 1989b, 495 vp. In: (Eds.): Boone DR, Calstenholz RW and Garrity GM. Bergey's Manual of Systematic Bacteriology. I (2nd: 294-334). Berlin. Springer-Verlag.
- **Gregory MR.** 2009. Environmental implications of plastic debris in marine settings—entanglement, ingestion, smothering, hangers-on, hitch-hiking and alien invasions. Phil. Trans R Soc B 364: 2013–2025.
- **Grigat E, Koch R, Timmermann R**. 1998. Thermoplastic and biodegradable polymers of cellulose. Polym. Degrad. Stab 59: 223.
- **Guan YL, Liu XF, Zhang YP and Yao KD**. 1998. Study of phase behavior on chitosan/viscose rayon blend film. J Appl Polym Sci 67: 1965-1972.
- **Hafdani FN, Sadeghinia N.** 2011. A Review on Application of Chitosan as a Natural Antimicrobial, World Academy of Sci, Eng Technol 50: 252-256.
- **Hahn SK, Chang YK, Kim BS, Lee SY, Chang HN.** 1993. The Recovery Of Poly(3-Hydroxybutyrate) By Using Dispersions Of Sodium Hypochlorite Solution And Chloroform . Biotechnol Tech 7(3): 209-212.
- **Hahn SK, Chang YK, Lee SY.** 1995. Recovery and Characterization of Poly(3-Hydroxybutyric Acid) Synthesized in *Alcaligenes eutrophus* and Recombinant *Escherichia coli*. App Env Microbiol 61(1): 34–39.
- **Halden R U.** 2010. Plastics and health risks. Annu Rev Public Health 31: 179-94.
- **Hasegawa M, Isogai A, Onabe F, Usuda M and Atalla RH .** 1992. Characterization of cellulose–chitosan blend films. J Appl Polym. Sci 45: 1873–1879.
- **Hasegawa N, Kawasumi M, Kato M, Usuki A, Okada A.** 1998. Preparation and mechanical properties of polypropylene-clay hybrids using a maleic anhydride-modified polypropylene oligomer J Appl Polym Sci 67: 87.

- **Hasırcıa V, Lewandrowski K, Gresser JD, Wise DL, Trantolo DJ.** 2001. Versatility of biodegradable biopolymers: degradability and an in vivo application. J Biotechnol 86: 135–150.
- **He WN, Zhang ZM, Hu P, Chen GQ.** 1999. Microbial synthesis and characterization of polyhydroxyalkanoates by strain DG17 from glucose. Acta Polymer Sin 6:709–714.
- **Heinrich D, Madkour MH, Al-Ghamdi MA, Shabbaj II, Steinbüchel A.** 2012. Large scale extraction of poly(3-hydroxybutyrate) from *Ralstonia eutropha* H16 using sodium hypochlorite. AMB Express 2:59.
- **Holland SJ, Yasin M, Tighe B.** 1990. Polymers for biodegradable medical devices VII. Hydroxy butyrate–hydroxyvalerate copolymers: degradation of copolymers and their blends with polysaccharides under in vitro physiological conditions. Biomaterials 11: 206–15.
- **Hongzhi L, Jinwen Z.** 2011. Research, progress in toughening modification of poly(lactic acid). J Polym. Sci. Part B: Polym Phys 49: 15.
- **Hrabak O.** 1992. Industrial production of Poly-β-hydroxybutyrate. FEMS Microbiol Rev 103:251-256.
- **Jandas PJ, Mohanty S, Nayak SK.** 2014. Morphology and Thermal Properties of Renewable Resource-Based Polymer Blend Nanocomposites Influenced by a Reactive Compatibilizer. ACS Sustainable Chem Eng 2: 377−386.
- **Janine M. Orban, Kacey G. Marra, and Jeffrey O.** 2002. Hollinger .Composition Options for Tissue-Engineered Bone. Tissue Engineering 4: 529-539.
- **Japanese Chitin and Chitosan Society.** 1994. Chitin and Chitosan Handbook. Tokyo, Gihodo, 460–483.
- **Jendrossek D.** 2001. Microbial degradation of polyesters. Adv Biochem Eng Biotechnology 71: 293-325.
- **Jiang Y, Song X, Gong L, Li P, Dai C, Shao W.** 2008. High poly(β-hydroxybutyrate) production by *Pseudomonas fluorescens* A2a5 from inexpensive substrates. Enzyme Microl Tech 42:167-172
    - Journal of Plastic Film and Sheeting 22: 193-211.
- **Juan ML, Gonzalez LW, Walker GC.** 1998. A Novel Screening Method for Isolating Exopolysaccharide deficient Mutants. Appl Env Microbiol 64: 4600-4602.

- **Kalaivani R, Sukumaran V.** 2013. Isolation and identification of new strains to enhance the production of biopolymers from marine sample in Karankura, Tamil Nadu. Eur J Exp Biol 3(3): 56-64.
- **Kalia S, Vashistha S, and Kaith BS.** 2011. Cellulose nanofibers reinforced bioplastics and their applications. in Handbook of Bioplastics and Biocomposites Engineering Applications (Pilla S eds) chapter 16, Wiley-Scrivener Publishing, New York.
- **Katırcioglu H, Aslım B, Yuksekdad ZN, Mercan N, Beyatlı Y**. 2003. Production of poly- β-hydroxybutyrate (PHB) and differentiation of putative Bacillus mutant strains by SDS-PAGE of total cell protein. Afr J Biotechnol 2(6): 147–149.
- **Kawasumi M, Hasegawa N, Kato M, Usuki A, Okada A.** 1997. Macromol 30: 6333.
- **Kemavongse K, Prasertsan P, Upaichit A, Methacanon P.** 2007. Effect of Co-substrate on Production of Poly-β-hydroxybutyrate (PHB) and Copolymer PHBV from newly identified mutant *Rhodobacter sphaeroides* U7 cultivated under aerobic-dark condition. Songklanakarin J Sci Technol 29(4): 1101-1113.
- **Kemnitzer JE, McCarthy SP, Gross RA.** 1993.Preparation of predominantly syndiotactic poly (beta-hydroxybutyrate) by the tributyltin methoxide catalyzed ring-opening polymerization of racemic beta-butyrolactone. Macromolecules 26:1221–1229.
- **Khalil AK, El-Sayed M. Sherif, Abdulhakim AA.** 2011. Corrosion Passivation in Simulated Body Fluid of Magnesium/ Hydroxyapatite Nanocomposites Sintered by High Frequency Induction Heating. Int J Electrochem Sci 6: 6184 -6199.
- **Khare A, Deshmukh S.** 2006. Studies toward producing eco-friendly plastics.
- **Khatipov E, Miyake M, Miyake J, Asada Y.** 1998. Accumulation of poly-L-hydroxybutyrate by Rhodobacter sphaeroides on various carbon and nitrogen substrates. FEMS Microbiology Letters 162: 39-45.
- **Kim BS, Lee SC, Le SY, Chang HN, Chang YK, Woo SI.** 1994. Production of poly (3-hydroxybutyric acid) by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control. Biotechnol Bioeng 43: 892–898.
- **Kim BS, Lee SY, Chang H.N.** 1992. Production of poly-phydroxybutyrate by fedbatch culture of recombinant *Escherichia coli*. Biotechnol Lett 14: 811-816.

- **Kim JH, Kim JY, Lee YM and Kim KY.** 1992. Properties and swelling characteristics of cross-linked poly (vinyl alcohol)/chitosan blend membrane. J Appl Polym Sci 45: 1711.
- **Kirithika M, Rajarathinam K, Venkatesan S.** 2011. Eco Friendly Biodegradable Polymer, Poly-β-Hydroxyl Butyric Acid Production, Degradation and its Optimization Studies using *Alcaligenes* Sp. and *Pseudomonas* Sp. Dev Microbiol Molecular Biol 2 (1): 1-13
- **Knowles JC, Hastings GW, Ohta H, Niwa S, Boeree N.** 1992. Development of a degradable composite for orthopaedic use: in vivo biomechanical and histological evaluation of two bioactive degradable composites based on the polyhydroxybutyrate polymer. Biomaterials 13: 491-6.
- **Kokubo T, Kushitani H, Sakka S, Kitsugi T, Yamamuro T.** 1990. J Biomed. Mater Res 721–734.
- **Koller M, Bona R, Braunegg G, Hermann C, Horvat P, Kroutil M, Martinz J, Neto J, Varila P, Pereira L.** 2005a. Production of polyhydroxyalkanoates from agricultural waste and surplus materials. Biomacromol 6: 561–565.
- **Kshirsagar P, Suttar R, Nilegaonkar S, Kulkarni S, Kanekar P.** 2014. Scale up production of polyhydroxyalkanoate (PHA) at different aeration, agitation and controlled dissolved oxygen levels in fermenter using Halomonas campisalis MCM B-1027. J Biochem Tech (2012) 4(1): 512-517.
- **Kumaravel S, Hema R, Lakshmi R.** 2010. Production of Polyhydroxybutyrate (Bioplastic) and its Biodegradation by *Pseudomonas Lemoigne* and *Aspergillus niger*. Electronic J Chemical 7(S1): S536-S542.
- **Kunioka M, Tamaki A, Doi Y**. 1989. Crystalline and Thermal Properties of Bacterial Copolyesters: Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) and Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate). Macromolecules 22: 694-697.
- **Kuruppalil Z**. 2011. Green Plastics: An Emerging Alternative For Petroleum-Based Plastics? Int J Eng Rese & Inn 3(1).
- **Kweon H, Ha HC, Um IC, Park YH**. 2001. Physical properties of silk fibroin/chitosan blend films. J Appl Poly***m*** Sci 80: 928–934.
- **Kweon HY, Um IC, Park YH.** 2001. Structural and thermal characteristics of *Antheraea pernyi* silk fibroin/chitosan blend film. Polymer 42: 6651–6656.

- **Lajeunesse**. 2004. Plastic Bags, Chemical and Engineering News 82(38): 51.
- **Lan T.; Kaviratna, P. D.; Pinnavaia, T.** 1994. J Chem Mater**.** 1994.6, 573.
- **Lazarevic D, Austin E, Buclet N, Brandt N.** 2010. Plastic waste management in the context of a European recycling society: comparing results and uncertainties in a life cycle perspective. Resource Conserve Recycling 55: 246-259.
- **Leathers TD.** 2003. Biotechnological production and applications of pullulan. Appl. Microbiol. Biotechnol 62: 468-473.
- **Leaversuch** R**.** 1987. Industry weighs need to make polymer degradable. Modern Plastics 8: 52-55.
- **Leaversuch R**. 2002. Biodegradable polyesters; Packaging goes green. Plastics Technol 48(9): 66-73.
- **LeBaron PC, Wang Z, Pinnavaia T.** 1999. Polymer-layered silicate nanocomposites: an overview Appl. Clay Sci 15: 11.
- **Lee B, Pometto III AL, Fratzke A, Bailey TB.** 1991. Biodegradation of degradable plastic polyethylene by *Phanerochaete* and *Streptomyces* species. Applied Environment Microbial 57: 678-685.
- **Lee HC, Anuj S, Lee Y.** 2001. Biomedical applications of collagen. International J. Pharmaceutics 221:1-22.
- **Lee SY.** 1994. Suppression of filamentation in recombinant *E. coli* by amplified FtsZ activity. Biotechnol Lett 16: 1247-1252.
- **Lee SY.** 1996. Bacterial Polyhydroxyalkanoates. Biotechnol Bioeng 49: 1-14.
- **Lemoigne M.** 1926. Products of dehydration and of polymerization of P-hydroxybutyric acid. Bull Soc Chem Biol 8: 770-782
- **Li S, Juliane H, Martin KP.** 2009. Product overview and market projection of emerging biobased products. PRo-BIP 1:1-245.
- **Li SY, Dong CL, Wang SY, Ye HM, Chen GQ.** 2011. Microbial Production of Polyhydroxyalkanoate Block Copolymer by Recombinant *Pseudomonas putida*. Appl Microbiol Biotechnol 90(2): 659-669.
- **Liau CP, Ahmad MB, Shameli K, Yunus WanMd ZW, Ibrahim NA, Zainuddin N, Then Y.** 2014. Preparation and Characterization of Polyhydroxybutyrate/Polycapr-olactone Nanocomposites. Hindawi Publishing Corporation. Scientific World Journal. Article ID 572726: 9

- **Liau CP, Ahmad MB, Shameli K, Yunus WanMd ZW, Ibrahim NA, Zainuddin N, Then YY.** 2014. Preparation and Characterization of Polyhydroxybutyrate/Polycap -rolactone Nanocomposites. Hindawi Publishing Corporation. Scientific W J Article ID 572726. pp. 9.
- **Litchfield CD, Gillevet PM.** 2002. Microbial diversity and complexity in hypersaline environments: a preliminary assessment. Ind J Microbiol Biotechnol 28(1): 48-55.
- **Liu M, Zhang Y, Wu C, Xiong S, Zhou C.** 2010. Chitosan/halloysite nanotubes bionanocomposites: structure, mechanical properties and biocompatibility. Int J Biol Macromol 51: 566-575.
- **Loeb LB, Kip AF, Hudson GG, Bennett WH**. 1941. Pulses in Negative Point-to-Plane Corona. Physical Review 60 (10): 714-722.
- **Lomas AJ, Webb WR, Han JF, Chen GQ, Sun X, Zhang Z, Haj AJE, Forsyth NR.** 2013. Poly (3-Hydroxybutyrate-co-Hydroxyhexanoate)/Collagen Hybrid Scaffolds for Tissue Engineering Applications. Tissue Engineering: Part C 19(8): 577-585.
- **Loo CY and Kumar S.** 2007. Polyhydroxyalkanoates: Bio-based microbial plastics and their properties. Malaysian Pol J 2: 31-57.
- **Lu CH.** 2006. Purification and separation of polyhydroxyalkanoates from bacteria, Ms Thesis, Yuan Ze University, Taiwan.
- **Luc A, Eric P.** 2012. Biodegradable polymers. In: Environmental silicate nanobiocomposites. Green energy and technology. Springer, Hiedelberg, 13–39.
- **Luengo JM, Garcia B, Sandoval A, Naharro G, Olivera ER.** 2003. Bioplastics from microorganisms. Curr Opin Microbiol 6: 251-60.
- **Luklinska ZB, Bonfield W.** 1997.Morphology and ultrastructure of the interface between hydroxyapatite-polyhydroxybutyrate composite implant and bone. J Mater Sci Mater Med 8(6): 379-83.
- **Liu LQ, Wagner HD.** 2007. A comparison of the mechanical strength and stiffness of MWNT-PMMA and MWNT-epoxy nanocomposites. Composite Interfaces 14(4): 285 – 297.
- **Ling L, Xing W, GQ Chen.** 2009. Physical Properties and Biocompatibility of Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) Blended with Poly(3-hydroxybutyrate-co-4-hydroxybutyrate). J Biomat Sci 20: 1537–1553.

- **Ma G, Nagai M & Omi S**. 1999. Preparation of uniform (polylactide) microspheres by employing SPG emulsification technique. Colloid Surface 153 1(3): 383-394.
- **Madison LL, Huisman GW.** 1999. Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. Microbial Molecular Biology Rev 63 (1): 21–53.
- **Maeda M, Tani S, Sano A, Fujioka K**. 1999. Microstructure and release characteristics of the minipellet, a collagen based drug delivery system for controlled release of protein drugs. J Controlled Rel 62: 313-324.
- **Mahmoud MS, Ali S, Majid C**. 2013. Mechanical properties of multi-walled carbon nanotube/polyestenanocomposites. J Nanostructure Chem 3: 20.
- **Mahuya M, Patra A, Paul AK**. 2005. Production of polyhdyroxy butyrate and polyhydroxybutyrateco- valerate by *Rhodopseudomonas palustris* SP5212. World J Microbiol Biotechnol 21: 765-769.
- **Maiti P, Batt CA, Giannelis EP.** 2007. New Biodegradable Polyhydroxybutyrate/Layered Silicate Nanocomposites. Biomacromol 8: 3393-3400.
- **Maiti P, Nam PH, Okamoto M, Hasegawa N, Usuki A. 2002.** A hierarchical structure and properties of intercalated polypropylene/clay nanocomposites Macromolecules **35: 2042.**
- **Maiti P, Nam PH, Okamoto M, Kotaka T, Hasegawa N, Usuki A.** 2002. The effect of crystallization on the structure and morphology of polypropylene/clay nanocomposites Polym Eng Sci 42 (9): 1864.
- **Majid J, Elmira A T, Muhammad I, Muriel J, Stephane D.** 2010. Poly-Lactic Acid: Production, Applications,Nanocomposites, and Release Studies. Comprehensive Reviews in Food Science and Food Safety 9.
- **Marguerite R**. 2006. Chitin and chitosan: properties and applications. Progress in Polym Sci 31: 603-632
- **Marras S I, Tsimpliaraki A, Zuburtikudis I and Panayiotou C.** 2007. Surfactant-induced morphology and thermal behavior of polymer layered silicate nanocomposites. J Physics Conference Series 61: 1366–1370.

- **Marras SI, Tsimpliaraki A, Zuburtikudis I, Panayiotou C.** 2007. Thermal and colloidal behavior of amine-treated clays: The role of amphiphilic organic cation concentration. J Colloid Interface Sci 315: 520–527.
- **Martin DP, Peoples OP, Williams SF, Zhong LH.** 1999. Nutritional and therapeutic uses of 3-hydroxyalkanoate oligomers. US Patent Appl 359086.
- **Martínez-Valencia B, Carbajal-De la Torre G, Duarte Moller A, Esparza-Ponce HE, Espinosa-Medi A.** 2011. Study of bioactivity, biodegradability and mechanical
- **Maurizio A, Jan JDV, Maria EE, Sabine F, Paolo V, Maria GV.** 2005. Biodegradable starch/clay nanocomposite films for food packaging applications. Food Chem 93(3): 467-474.
- **Medvecky L, Lagashetty A; Venkataraman A.** 2005. Polymer Nanocomposites. RESONANCE 10(7): 49-57.
- **Medvecky L.** 2012. Microstructure and Properties of Polyhydroxybutyrate-Chitosan-Nanohydroxyapatite Composite Scaffolds. Scientific World J 537973.
- **Mei-Ling C, Yi-Ru L, Zhi-Zhung L, Chien-Shiun L, Yi-Ming S.** 2014. Physical and transport properties of Polyhydroxybutyrate/Clay Nanocomposite Membranes. 18: 5.
- **Merugu R, Girisham S, Reddy SM.** 2013. Production of PHB (polyhydroxybutyrate) by *rhodopseudomonas Palustris* ku003 under nitrogen limitation. Inter J App Biol Pharmaceutical Technol 1(2): 676-678.
- **Messersmith PB, Giannelis EP.** 1993. Polymer-layered silicate nanocomposites: In situ intercalative polymerization of ε-caprolactone in layered silicates. Chem Mater 5 (8): 1064-1066.
- **Mikkili I, Karlapudi AP, Venkateswarulu TC, Babu D J, Nath SB, Kodali VP.** 2014. Isolation, Screening and Extraction of Polyhydroxybutyrate (PHB) producing bacteria from Sewage sample . Inter J Pharm Tech Res 6 (2): 850-857.
- **Mikos AG, Lyman MD, Freed LE, Langer R.** 1994. Wetting of poly(L-lactic acid) and poly(DL-lactic-co-glycolic acid) foams for tissue culture. Biomat 15: 55-8
- **Miller JH.** Cold Spring Harbor Laboratory Press, New York.1992.

- **Misra SK, Ansari TI, Valappil SP, Mohn D, Philip SE, Stark WJ, Roy I, Knowles JC, Salih V, Boccaccini AR.** 2010. Poly (3-hydroxybutyrate) multifunctional composite scaffolds for tissue engineering applications. Biomaterials 31(10): 2806-15.
- **Misra SK, Philip SE, Chrzanowski W, Nazhat SN, Roy I, Knowles JC, Salih V, Boccaccin AR.** 2009. Incorporation of vitamin E in poly(3hydroxybutyrate)/ Bioglass composite films: effect on surface properties and cell attachment. J Royal Society Interface 6: 401–409.
- **Misra SK, Valappil SP, Roy I, Boccaccini AR.** 2006. Polyhydroxyalkanoate (PHA)/inorganic phase composites for tissue engineering applications. Biomacromolecules 7(8): 2249-58.
- **Monte SJ.** 2002. Additives for plastics: Addcon 2001. Polymers and Polymer Composites. 10(1): 1-32.
- **Moraes MA, Nogueira GM, Weska RF, Beppu MM.** 2010. Preparation and Characterization of Insoluble Silk Fibroin/Chitosan Blend Films. Polymers 2: 719-727.
- **Morgesin R, Schinner F.** 2001. Potential of halotolerant and halophilic microorganisms for biotechnology. Extremophiles 5(2): 73- 83.
- **Mu L, Feng SS.** 2001. Fabrication, characterization and in vitro release of paclitaxel (Taxol®) loaded poly(lactic-co-glycolic acid) microspheres prepared by spray drying technique with lipid/cholesterol emulsifiers. J Control 76(3): 239- 254.
- **Mulder KF.** 1998. Sustainable production and consumption of plastics? Technological Forecasting and Social Change 58: 105-124.
- **Municipal solid waste in the United States.** 2007 Facts and figures. United States Environmental Protection Agency, Retrieved from http://www.epa.gov/osw/nonhaz/municipal/pubs/msw07-rpt.pdf
- **Murali MG, Finosh T, Jayabalan M.** 2013. Studies on Biodegradable Polymeric Nanocomposites Based on Sheet Molding Compound for Orthopedic Applications. Adv Mat Sci App 2 (2): 73-87.
- **Murray RGE, Doetsch RN, Robinow CF.** 1994. Determinative and cytological light microscopy. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR, editors. Manual of methods for general microbiology. Washington: American Society for Microbiol 21–41.

- **Muthu SS, Li Y, Hu JY, Mok PY, Ding X.** 2012. Eco-Impact of Plastic and Paper Shopping Bags Eco-Impact of Plastic and Paper Shopping Bags. J Eng Fibers and Fabrics 7 (1): 26-37.
- **Naffakh M.** 2007. Polymer Nanocomposites Based on Inorganic Fullerene-like WS2 Nanoparticles: Opportunities and Challenges. J Polym Sci Part B: Polym Phy 45: 2309.
- **Naffakh M.** 2010. Novel Melt-Processable Poly(ether ether ketone)(PEEK)/Inorganic Fullerene-like WS2 Nanoparticles for Critical Applications. J Phys Chem B 114: 11444.
- **Nielsen J, Villadsen J.** 1994. Bio reaction Engineering Principles. New York: Plenum Press.
- **Nordhoff E, Kirpekar F, Roepstorff P.** 1996. Mass spectrometry of nucleic acids. Mass Spectrometry Reviews **15** (2): 67-138.
- **Novikov LN, Novikova LN, Mosahebi A, Wiberg M, Terenghi G, Kellerth JO**. 2002. A novel biodegradable implant for neuronal rescue and regeneration after spinal cord surgery. Biomaterials 23: 3369-76.
- **Nurbas M, Kutsal T**. 2004. Production of PHB and P(HB-*co*-HV) Biopolymers by Using *Alcaligenes Eutrophus*. Iranian Pol J 13(1): 45-51.
- **Ohkoshi I, Abe H, Doi Y.** 2000**.** Miscibility and solid-state structures for blends of poly((S)-lactide) with atactic poly((R,S)-3-hydroxybu-tyrate) Polymer 41: 5985.
- **Ohtsuki C, Aoki Y, Kokubo T, Bando Y, Neo M, Nakamura T.** 1995. J Ceram Soc Japan 103 (5): 449–454.
- **Ojumu TV, Yu J, Solomon BO.** 2004. Production of Polyhydroxyalkanoates, a bacterial biodegradable polymer. Afr J Biotechnol 3(1): 18-24.
- **Okamoto M, Morita S, Taguchi H, Kim YH, Kotaka T, Tateyama H.** 2000. Synthesis and structure of smectic clay/poly(methyl methacrylate) and clay/polystyrene nanocomposites via in situ intercalative polymerization. Polymer 41(10): 3887-3890.
- **Oriakhi CO, Lerner MM**. 1995. Poly(pyrrole) and poly(thiophene)/clay nanocomposites via latex-colloid interaction. Mater Res Bull 30 (6): 723-729.
- **Orts WJ, Nobes GAR, Kawada J, Nguyen S, Yu GE, Raveneile F.** 2008. Can J Chem 86: 628.

- **Ostle A, Holt JG.** 1982. Nile Blue A as a fluorescent stain for Poly-β-hydroxybutyrate. Appl Environ Microbiol 2: 238-241.
- **Otari V, Ghosh JS.** 2009. Production and Characterization of the Polymer Polyhydroxy Butyrate-co-polyhydroxy Valerate by *Bacillus Megaterium* NCIM 2475. Current Res J Biol Sci 1(2): 23-26.
- **Pak YL, Ahmad MB, Shameli K, Yunus Wan Md ZW, Ibrahim NA, Zainuddin N.** 2013. Mechanical and Morphological Properties of Polyhydroxybutyrate/Poly(butyleneadipate-co-terephthalate)/ Layered Double Hydroxide Nanocomposites. J Nanomat Article ID 621097 pp. 8.
- **Panda B, Sharma L, Singh AK, Mallick N.** 2007. Thin layer chromatographic detection of poly-β-hydroxybutyrate (PHB) amd POLY-β-hydroxyvalerate (PHV) in cyanobacteria. Indian J Biotechnol 7: 230-234.
- **Park SJ, Lee KY, Ha WS, Park SY.** 1999. Structural changes and their effect on mechanical properties of silk fibroin/chitosan blends. J Appl Polym Sci 74: 2571–2575.
- **Park TG, Cohen S, Langer R.** 1992. Poly (L-lactic acid)/pluronic blends: characterization of phase separation behavior, degradation, and morphology and use as protein-releasing matrixes. Macromolecules 25(1): 116–122.
- **Parulekar YS, Mohanty AK.** 2005. Biobased nanocomposites from toughened bacterial bioplastic and titanate modifed layered silicate: potential replacement for reinforced tpo. J. Nanoscience Nanotech. 5(12): 2138-2143.
- **Passanha P, Kedia G, Dinsdale RM, Guwy AJ, Esteves SR.** 2014. The use of NaCl addition for the improvement of polyhydroxyalkanoate production by *Cupriavidus necator* Pearl. Bio resource Technology 163 287–294
- **Patel VR, Amiji MM**. 1996. Preparation and characterization of freeze dried chitosan poly(ethylene oxide) hydrogels for site-specific antibiotic delivery in the stomach. Pharmaceut Res 13: 588–593.
- **Patwardhan PR, Srivastava A. K.** 2004. Model-based fed-batch cultivation of *R.eutropha* for enhanced biopolymer production. Biocheml Eng J 20: 21-28.
- **Paul DR, Barlow JW and Keskkula H.** 1989. Encyclopedia of Polymer Science and Engineering Wiley, New YorK.

- **Pfeifer RJ, Hendricks CD.** 1968. Parametric Studies of Electrohydrodynamic Spraying. AIAA J **6** (3): 496-502.
- **Phillips AL.** 2008. Bioplastics boom. American Scientist 96(2): 109-110.
- **Pielichowska K, Blazewicz S.** 2010. Bioactive Polymer/Hydroxyapatite (Nano)composites for Bone Tissue Regeneration, in Biopolymers: Lignin, Proteins, Bioactive Nanocomposites. Springer-Verlag Berlin: Berlin 97-207.
- **Pluta M, Galeski A, Alexandre M, Paul MA, Dubois P.** 2002. Polylactide/montmorillonite nanocomposites and microcomposites prepared by melt blending: Structure and some physical properties. J Appl Pol Sci 86: 1497–1506.
- **Poirier Y, Nawrath C, Somerville C.** 1995**.** Production of polyhydroxyalkanoate, A family of Biodegradable plastics and elastomers in bacteria and plants, A review, Biotechnol 13: 142-150
- **Pollet E, Paul MA, Dubois P.** 2003. New aliphatic polyester layered-silicate nanocomposites. in 'Biodegradable polymers and plastics, (eds.: Chiellini E., Solaro R.) Kluwer, vol 1, New York .
- **Porter M, Yu J.** 2011. Monitoring the in situ crystallization of native biopolyester granules in *Ralstonia eutropha* via infrared spectroscopy. J Microbioll Methods, 87(1): 49-55.
- **Prashant RC, Ishwar BB, Shrikant AS, Rekha SS.** 2009. Microbial cellulose: fermentive production and applications. Food Technol Biotechnol 47:107-124.
  properties of polyurethane/nano-hydroxyapatite hybrid composites . International J Physl Sci. 6(29): 6681-6691.
- **Quesada E, Ventosa A, Rodriguez-Valera F, Ramos- Cormenzana A.** 1982.Types and properties of some bacteria isolated from hypersaline soils. J Appl Microbiol 53: 155-161.
- **Quillaguamán J, Delgado O, Mattiasson B, Hatti-Kaul R.** 2006. Poly (β-hydroxyl butyrate) production by a moderate halophile, *Halomonas boliviensis*LC1. Enzyme Microbiol Technol 38:148-154.
- **Ramos J, Millan A, Palacio F.** 2000. Production of magnetic nanoparticles in a polyvinylpyridine matrix. Polymer 41(24): 8461 -8464.

- **Ramsay BA, Lomaliza K, Chavarie C, Dubé B, Bataille P, Ramsay JA.** 1990. Production of poly-(beta-hydroxybutyric-co-beta-hydroxyvaleric) acids. Appl Environ Microbiol 56(7): 2093–2098.
- **Ramya R, Sudha PN, Dr. Mahalakshmi J.** 2012. Preparation and Characterization of Chitosan Binary Blend. Inter J Scientific Res Publications 2 (10): 2250-3153.
- **Ramya R, Venkatesan , Jayachanndran Kim S, Sudha PN.** 2012. Biomedical applications of chitosan: an overview. J Biomaterial Tissue Engg 2:100-111.
- **Ravi Kumar MNV.** 2000. A review of chitin and chitosan applications. Reactive Functional Polym 46:1-27.
- **Ray SS and Bousmina M.** 2005. Biodegradable polymers and their layered silicate nano composites: In greening the 21st century materials world. Prog Mat Sci 50(8): 962-1079.
- **Rehm Bernd HA, Mitsky Timothy A, Steinbüchel A.** 2001.Role of Fatty Acid De Novo Biosynthesis in Polyhydroxyalkanoic Acid (PHA) and Rhamnolipid Synthesis by Pseudomonads: Establishment of the Transacylase (PhaG)-Mediated Pathway for PHA Biosynthesis in *Escherichia coli*. Appl EnvMicro 3102–3109.
- **Rehm Bernd HA.** 2003. Polyester synthases: natural catalysts f or plastics. Biochemist J 376: 15–33.
- **Reis KC, Pereira J, Smith AC, Carvalho CWP, Wellner N, Yakimets I.** 2008. Characterization of polyhydroxybutyrate-hydroxyvalerate (PHB-HV)/maize starch blend films. J Food Engg 89:361–369.
- **Rekha MR, Chrndra PS.** 2007. Pullulan as a promising biomaterial for biomedical applications: a perspective. Trends in Biomaterials and Artificial Organs 20:21-45.
- **Reusch, R.N.** 1995. Low molecular weight complexed poly(3- hydroxybutyrate): a dynamic and versatile molecule in vivo. Canadian J Microbiol 1(1): 50–4.
- **Rivard C, Moens L, Roberts K, Brigham J, Kelley S.** 1995. Starch esters as biodegradable plastics: Effects of ester group chain length and degree of substitution on anaerobic biodegradation. Enzyme and Microbial Tech. 17: 848-852.
- **Roberts GAF.** 1997. Chitosan production routes and their role in determining the structure and properties of the product. In: Domard M, Roberts AF, Vårum KM (eds) Advances in Chitin Science, vol. 2, National Taiwan Ocean University, Taiwan, Jacques Andre, Lyon. pp 22-31. 1998.

- **Rodriguez-Contreras Alejandra, Martin Koller, Miguel Miranda de Sousa Dias, Margarita Calafell, Gerhart Braunegg, María Soleda Marqués-Calvo.** 2013. Novel Poly[(*R*)-3-Hydroxybutyrate]-Producing Bacterium Isolated from a Bolivian Hypersaline Lake Food. Technol Biotechnol 51(1): 123–130.
- **Rodriguez-Valera F.** 1988. Characteristics and microbial ecology of hypersaline environments. In: (Ed.): Rodriguez-Valera F. Halophilic Bacteria I: 3-30. Boca Raton: CRC Press.
- **Rohini RD, Rawal SK** .2006.Synthesis and characterization of poly hydroxybutyrate from *Bacillus thurengenesis*. Ind J Biotechnol 5: 276-283
- **Roohi A, Ahmed I, Iqbal M, Jamil M.** 2012. Preliminary isolation and characterization of halotolerant and halophilic bacteria from salt mines of Karak, PAakistan. Pak J Bot 44: 365-370.
- **Rosca ID, Watari F, Uo M.** 2004. Microparticle formation and its mechanism in single and double emulsion solvent evaporation. J Control Release 99 (2): 271-280.
- **Rubin AL, Stenzel KH, Miyata T, White MJ, Dune M.** 1973.Collagen as a vehicle for drug delivery: preliminary report. J of Clinical Pharmacology 13: 309-312
- **Saharan BS, Ankita, Sharma D.** 2012. Bioplastics-For Sustainable Development: A Review. Int J Microbiol Res Tech 1(1): 11-23.
- **Saharan BS, Sharma AD.** 2012. Bioplastics-For Sustainable Development: A Review. Int J Microbiol Res Tech 1(1): 11-23.
- **Sangkharak K, Prasertsan P** .2007. Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. Electron J Biotechnol 11(3) doi: 10.2225/vol11-issue3-fulltext-2
- **Savenkova L, Gercberga Z, Nikolaeva V, Dzene A, Bibers I, Kalina M.** 2000. Mechanical properties and biodegradation characteristics of PHB-based films. Process Biochem 35: 537–579
- **Sena RF, Costelli MC, Gibson LH, Coughlin RW.** 2006. Enhanced production of pullulan by two strains of *A. pullulans* with different concentrations of soybean oil in sucrose solution in batch fermentations. Brazilian J Chem Eng 2:507-515.
- **Shah KR.** 2012. FTIR analysis of polyhydroxyalkanoates by novel *Bacillus sp.* AS 3-2 from soil of Kadi region, North Gujarat. J Biochem Tech 3(4): 380-383

- **Sherwood PMA.** 1990. Practical Surface Analysis by Auger and X-ray Photoelectron Spectroscopy 2nd Ed., edited by D. Briggs, and M.P. Seah, Wiley.
- **Shi H, Lan T, Pinnavaia TJ.** 1996. On the nature of polyimide-clay hybrid composites. Chem Mater 8: 1584.
- **Shishatskaya EI, Khlusov IA, Volova TG.** 2006. A hybrid PHB-hydroxyapatite composite for biomedical application: production, in vitro and in vivo investigation. J Biomater Sci-Pol Edition 17(5): 481-498.
- **Shokri A, Larsson G.** 2004. Characterisation of the *Escherichia coli* membrane structure and function during fedbatch cultivation. Microb Cell Fact 3: 9.
- **Shum-Tim D, Stock U, Hrkach J.** 1999. Tissue engineering of autologous aorta using a new biodegradable polymer. Ann Thorac Surg 68(6): 2298-304.
- **Singh KM, Verma G.** 2010. Green nanocomposites based on thermoplastic starch and steam exploded cellulose nanofibrils from wheat straw. Carbohydrate Polymers 82(2): 337–345.
- **Singh P, Parmar N.** 2011. Isolation and characterization of two novel polyhydroxybutyrate (PHB)-producing bacteria. Afr J Biotechnol 10(24): 4907-4919.
- **Singh RS, Saini GK, Kennedy JF.** 2008. Pullulan: microbial sources, production and applications. Carbohydrate Polym 73: 515-531
- **Sinha RS, Okamoto M.** 2003. Polymer/layered silicate nanocomposites: A review from preparation to processing. Prog Pol Sci 28: 1539–1641.
- **Sodian R, Sperling JS, Martin DP, Stock U, Mayer JE, Jr., Vacanti JP.** 1999. Tissue engineering of a trileaflet heart valveearly in vitro experiences with a combined polymer. Tissue Eng 5(5): 489-494.
- **Sreeju SN, Babu MM, Mariappan C, Selvamohan T.** 2011. Effect of physical and chemical mutagens on biopolmer producing strains and RAPD analysis of mutated strains. Arch Appl Sci Res 3(6): 233-246
- **Steinbüchel A, Fuchtenbusch B.** 1998. Bacteria and other biological systems for polyester production. TIBTECH 16: 419-427.
- **Steinbuchel A, Lutke-Eversloh T.** 2003. Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. Biochem Eng J 16: 81–96.

- **Steinbüchel A.** 1991. Polyhydroxyalkanoic acids. In: Byrom D (ed) Biomaterials: novel materials from biological sources. Stockton 124-213.
- **Stevens ES.** 2002. Green Plastics: An introduction to the new science of biodegradable plastics. Princeton university Press. ISBN: 9780691049670
- **Stevens ES.** 2003. What makes green plastics green? BioCycle 44(3): 24.
- **Stock UA, Vacanti JP.** 2001. Tissue engineering: current state and prospects. Annual review of Medicine. 52:443-451.
- **Stock UA, Vacanti JP.** 2001. Tissue Engineering: Current State and prospects. Annu Rev Med 52: 443-51.
- **Suzuki T, Yamane T, Shimizu S.** 1986. Mass production of poly-beta-hydroxybutyric acid by fed-batch culture with controlled carbodnitrogen feeding. Applied Microbial Biotechnol 24: 370-374.
- **Taylor MS, Daniels AU, Andriano KP, Heller J. J.** 1994. Appl Biomater 5: 151.
- **Tekin E, Ateş M, Kahraman O.** 2012. Poly-3-hydroxybutyrate-producing extreme halophilic archaeon: *Haloferax* sp. MA10 isolated from Çamaltı Saltern, İzmir. Turk J Biol 36: 303-312.
- **Trigui H, Masmoudi S, Brochier-Armanet C, Maalej S, Dukan S.** 2011. Characterization of *Halorubrum sfaxense* sp. nov., a New Halophilic Archaeon Isolated from the Solar Saltern of Sfax in Tunisia. Int J Microbiol 8.
- **Tripathi AD, Yadav A, Jha A, Srivastava S.** 2012. Utilizing of Sugar Refinery Waste for Production of Bio-Plastic Under Submerged Fermentation Process. J Pol Env 1-8.
- **Tripathi L, Wu LP, Chen J, Chen GQ.** 2012. Synthesis of Diblock copolymer poly-3-hydroxybutyrate -block-poly-3-hydroxyhexanoate [PHB-b- PHHx] by a β-oxidation weakened Pseudomonas putida KT2442. Microbial Cell Factories 11(44): 1-11.
- **Tsuge, T.** 2002. Metabolic Improvements and Use of Inexpensive Carbon Sources in Microbial Production of Polyhydroxyalkanoates. J Biosci Bioeng 94(6): 579-584.
- **Upasani VN.** 2007. Microbiological studies on Lake (Salt of Earth) Rajasthan, India. Proceedings of Taal: The 12th World Lake Conferences 448-450.
- **Usuki A, Kojima Y, Kawasumi M, Okada AY, Kurauchi T, Kamigaiti OJ.** 1993. Mater Res 8: 1179.

- **Utracki L.** 2004. A.: Basic elements of polymeric nanocomposites technology. in 'Clay- containing polymeric nanocomposites' (ed.: Utracki L. A.) vol 1, Rapra, Shropshire.
- **Vaia RA, Ishii H, Giannelis EP.** 1993. Chem Mater 5: 1694.
- **Vaia RA, Teukolsky RK, Giannelis EP.** 1994. Chem Mater 6: 1017.
- **Vaia RA, Vasudevan S, Krawice W, Scanlon LG, Giannelis EP.** 1995. Adv Mater 7: 154.
- **Valerie D, Vinod DV.** 1998. Pharmaceutical applications of chitosan. Pharmaceutical Sci Technol Today 1:246-253
- **Viciosa MT, Dionísio M.** 2004. Molecular mobility and fragility in N-ethylene glycol dimethacrylate monomers. J. Non-Crys Solids 341: 60-67.
- **Virginia E, Marie G, Eric P, Luc A.** 2011. Structure and properties of glycerol plasticized chitosan obtained by mechanical kneading. Carbohydrate Polym 83: 947-952
- **Vroman I, Tighzert L.** 2009**.** Biodegradable Polymers. Materials 2: 307-344.
- **VSPK Sankara AJ, P Babu A.** 2007. Production of PolyHydroxyButrates (PHBs) by marine bacteria. Curr Res Microbiol Biotechnol 1(1): 23-25.
- **Walter JA, Caroline JC, Caroline JJ, Anjanapriya S.** 2013.Toxic and mutagenic impact of ethidium bromide traces on the growth of Escherichia coli KL226 Int J Curr Sci 8: e 28-32.
- **Wang F, Lee SY.** 1997. Poly(3-Hydroxybutyrate) Production with High Productivity and High Polymer Content by a Fed-Batch Cultureof Alcaligenes latus under Nitrogen Limitation. App Env Microbiol 3703–3706.
- **Wang M, Chen LJ, Ni J, Weng J, Yue CY.** 2001. Manufacture and evolution of Bioactive and biodegradable materials and scaffolds for tissue engineering. J Mat Sci: Materials in Medicine 12: 855- 860.
- **Wang Y, Chen R, Cai JY, Liu Z, Zheng Y, Wang H, Li Q, He N**. 2013. Biosynthesis and Thermal Properties of PHBV Produced from Levulinic Acid by *Ralstonia eutropha*. PLoS One. 4; 8(4): e60318.

- **Wang YW, Wu QO, Chen GQ.** 2004. Attachment, proliferation and differentiation of osteoblasts on random biopolyester poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) scaffolds. Biomaterials 25: 669–675.
- **Warth H, Mulhaupt R, Schatzle J.** 1997. Thermoplastic cellulose acetate and cellulose acetate compounds prepared by reactive processing. J Appl Polym Sci 64: 231–242.
- **Weber CLJ.** 2011 Biobased packaging materials for the food industry. Report by the EU Directorate 12.
- **Wei YH, Chen WC, Huang CK, Wu HS, Sun YM, Lo CW, Janarthanan OM.** 2011. Screening and Evaluation of Polyhydroxybutyrate-Producing Strains from Indigenous Isolate *Cupriavidus taiwanensis* Strains. Int J Mol Sci 12: 252-265
- **Willett JL, Shogren RL.** 1995. "Woodfiber-Plastic Composites" Madison, Winsconsin, Proceedings No.7293, 7644.
- **Williams SF, Martin DP, Horowitz DM, Peoples OP.** 1999. PHA applications: addressing the price performance issue I. Tissue engineering. Int J Biol Macromol 25 (1-3):111-21.
- **Win NN, Stevens WF.** 2001. Shrimp chitin as substrate for fungal chitin deacetylase. Appl Microbiol Biotechnol 57: 334-341.
- **Winfred FD, Robards AW.** 1973.Ultra structural study of Poly hydroxy butyrate granules from *Bacillus cereus*. J Bacteriol 114:1271-1280.
- **Witholt B, Kessler B.** 2002. Perspectives of medium-chain-length poly (hydroxyalkanotes), a versatile set of bacterial bioplastics. Curr Opin Biotechnol 10: 279-85.
- **Wu H, Liu C, Chen J, Chang PR, Chen Y, Anderson DP.** 2009. Structure and properties of starch/$-zirconium phosphate nanocomposite films. Carbohydrate Polymers 77: 358–364.
- **Wu W, He T, & Chen JF.** 2006. Study on in situ preparation of nano calcium carbonate/PMMA composite particles. Mater Lett 60(19): 2410 -2415.
- **Wu Q, Sun SQ, Yu PHF. , Chen AXZ, Chen GQ.** 2000. Environmental dependence of microbial synthesis of polyhydroxyalkanoate Acta Polymer Sin 6: 751-756.

- **Xianmo D, Jianyuan H, Minglong Y, Chengdong X, Shuji Z.** 2001. Miscibility, thermal behaviour, morphology and mechanical properties of binary blends of poly[(R)-3-hydroxybutyrate] with poly(g-benzyl-L-glutamate). Polym Int 50: 37-44.
- **Xiao C, Gao S, Wang H and Zhang L.** 2000.Blend films from chitosan and konjac glucomannan solutions. J Appl Polym Sci 76: 509.
- **Xu C, Qiu Z.** 2011**.** Crystallization behavior and thermal property of biodegradable poly(3-hydroxybutyrate)/multi-walled carbon nanotubes nanocomposite. Polym Adv Technol 22: 538–544.
- **Xu RJ, Manian E, Snyder AJ.** 2007. J Macromolecules 34:337.
- **Yamashita M, Fenn JB.** 1984. Negative ion production with the electrospray ion source. J Phy Chem 88 (20): 4671-4675.
- **Yamashita M, Fenn JB.** 1984a. Electrospray ion source. Another variation on the free-jet theme. J Phy Chem 88 (20): 4451-4459.
- **Yan S, Yin J, Cui L, Yang Y, Chen X.** 2011. Apatite-forming ability of bioactive poly(l-lactic acid)/grafted silica nanocomposites in simulated body fluid. Colloids and Surfaces B: Biointerfaces 86 (1): 218–224.
- **Yan YF, Krishnaiah D, Rajin M, Bono A.** 2009. Cellulose extraction from palm kernel cake using liquid phase oxidation. J Engg Sci Tech 4: 57-68.
- **Yang M, Zhu S, Chen Y, Chang Z, Chen GQ, Gong Y, Zhao N, Zhang X.** 2004. Studies on bone marrow stromal cells affinity of poly (3- hydroxybutyrate-co-3-hydroxyhexanoate). Biomaterials 25: 1365–1373.
- **Yano K, Usuki A, Okada A.** 1997. Synthesis and properties of polyimide-clay hybrid films. J. Polym. Sci., Part A: Polym Chem 35: 2289.
- **Yao KD, Liu J, Cheng GX, Lu XD, Tu HL, Lopes Da Silva JA.** 1996. Swelling behavior of pectin/chitosan complex films. J Appl Polym Sci 60: 279–283.
- **Yilgor P, Tuzlakoglu K, Reis RL, Hasirci N, Hasirci V.** 2009. Incorporation of a Sequential BMP-2/BMP-7 Delivery System into Chitosan- Based Scaffolds for Bone Tissue Engineering. Biomaterials 30: 3551–3559.
- **You M, Peng G, Li J, Ma P, Wang Z, Shu W, Peng S, Chen GQ.** 2011. Chondrogenic differentiation of human bone marrow mesenchymal stem cells on polyhydroxyalkanoate (PHA) scaffolds coated with PHA granule binding protein PhaP fused with RGD peptide. Biomaterials 32: 2305-2313.

- **Yu J.** 2001. Production of PHA from starchy waste water via organic acids. J Biotechnol 86: 105-112.
- **Zajic JE, LeDuy A.** 1973. Flocculant and chemical properties of a polysaccharide from Pullularia pullulans. Appl Microbiol 25: 628-635.
- **Zakaria MR, Ariffin H, Johar NAM, Aziz S A, Nishida H, Shirai Y, Hassan MA**. 2010. Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxybutyrate) copolymer from wild type *Comamonas sp*. EB172. Polym Degrad Stab 95: 1382-1386.
- **Zanetti M, Camino G, Reichert P, Mulhaupt R.** 2001. Thermal behaviour of poly(propylene) layered silicate nanocomposites, Macromolecular Rapid Communications 22(3): 176-180.
- **Zhang L, Deng X, Zhao S, Huang Z.** 2007. Biodegradable polymer blends of poly(3-hydroxybutyrate) and starch acetate. Polym Int, 44 (1): 104–110.
- **Zhang Y, Han JH.** 2006. Plasticization of pea starch gilms with monosaccharides and polyols. J Food Sci 71: E253–E261.
- **Zhu ZK, Yin J, Cao F, Shang XY, Lu QH.** 2000. Photosensitive polyimide/ silica hybrids. Adv. Mater 12(14): 1055-1057.
- **Zuburtikudis I, Marras SI, Tornikidou K, Tsimpliaraki A, Panayotidou E, Cristofidou G.** 2008. Enhanced thermal stability of biodegradable poly(3-hydroxybutyrate)/layered silicate nanocomposites. AIChE Annual Meeting Philadelphia PA: American Institute of Chemical Engineers: 908/901-900/907.