PHA PRODUCTION BY *Kluyvera intermedia* UTILIZING JAMBUL SEED AS SUBSTRATE: OPTIMIZATION AND DEGRADATION STUDY

PROJECT REPORT

Submitted by

P. SASIKALA

Register No: 1020203013

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PROJECT WORK

PHASE II

APRIL 2012

This is to certify that the project entitiled

PHA PRODUCTION BY *Kluyvera intermedia* UTILIZING JAMBUL SEED AS SUBSTRATE: OPTIMIZATION AND DEGRADATION STUDY

is the bonafide record of project work done by

P. SASIKALA

Register No: 1020203013

of M.Tech. (Biotechnology) during the year 2011-2012

…… ……..

Project Guide **Head of the Department**

Submitted for the Project Viva-Voce examination held on.....

…….. ……

Internal Examiner External Examiner

DECLARATION

I affirm that the project work titled **PHA PRODUCTION BY *Kluyvera intermedia* UTILIZING JAMBUL SEED AS SUBSTRATE: OPTIMIZATION AND DEGRADATION STUDY** being submitted in partial fulfilment for the award of **M.Tech. (Biotechnology)** is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other University.

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(P. SASIKALA)

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ABSTRACT

The microbial polyesters known as polyhydroxyalkanoates (PHAs) can be produced from renewable raw materials and are degraded naturally by microorganisms that enable carbon dioxide and organic compound recycling in the ecosystem, providing a buffer to climate change. Bacteria synthesize PHA as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cells under limiting conditions of nutrients. In the present study, an attempt was made to isolate efficient PHB producing bacteria from soil. Based on dry weight, SP-Y1 produced higher PHA, compared to other isolates and it was identified as *kluyvera intermedia* by IMTECH, Chandigrah and was used for further studies. Since the use of low-cost raw materials has the potential to reduce PHA production costs, Jambul seed (*syzygium cumini*) was explored as a substrate in the Mineral Salt Media. *kluyvera intermedia* and the reference strain (*Ralstonia eutropha*) was compared for their efficiency of maximum PHA production. The % of PHA accumulation and its concentration was found to be 41.7%, 97µg/ml in *Ralstonia eutropha* and 42.2%, 100µg/ml in *kluyvera intermedia*, when the organism utilized hydrolyzed seed alone as the carbon source. Based on Design of Experiment (DOE) involving Response Surface Method (RSM) a maximal PHB production of 1.528 g/L was achieved using optimal medium composition. FTIR analysis revealed characteristic peaks at 1642.3 and 1000-1300 cm^{-1} corresponding to the stretching of the C=O bond and C–O bond of the ester group respectively. The degradability of PHA produced was explored using the best strain and fungal species and was proved to be degradable when PHA was used as sole carbon source.

***Keywords*:** Polyhydroxybutyrate, *Kluyvera intermedia*, Jambul seed powder, FTIR

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LIST OF ABBREVATIONS

µg	Microgram
DNS	Dinitro Salicylic acid
DSC	Differential scanning calorimetry
FTIR	Fourier Transform infrared spectroscopy
g/L	Gram/Litre
GPC	Gel permeation chromatography
H_2O_2	Hydrogen Peroxide
HB	Hydroxybutyrate
HCl	Hydrochloric Acid
HV	Hydroxyvalerate
IMTECH	Institute of Microbial Technology
$K_2Cr_2O_7$	Potassium Di Chromate
K_2HPO_4	Di-Potassium Hydrogen Phosphate
KCl	Potassium Chloride
KCT	Kumaraguru College of Technology
$MgSO_4$	Magnesium Sulphate
MSM	Mineral Salt Medium
MTCC	Microbial Type Culture Collection
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NMR	Nuclear magnetic resonance spectroscopy
PHA	Polyhydroxyalkanoate
SSF	Solid state fermentation
WCW	Wet Cell weight
$ZnCl_2$	Zinc Chloride

CHAPTER 1
INTRODUCTION

1.1 The solution for plastics

Plastic materials which have made entry in every sphere of human life are now causing serious environmental problems due to their non biodegradability. The intrinsic qualities of durability and resistance to degradation, over the last two decades, have been increasingly regarded as a source of environmental and waste management problem emanating from plastic materials. Replacement of non biodegradable by degradable plastics can be used in the same applications as the existing synthetic polymers (Porier *et al*., 1995).

Microorganisms are one of the most important factors influencing natural processes in environment. They participate in degradation and transformation of most of organic substances including synthetic polymeric materials. Process of material decomposition by microorganisms is called biodegradation and it could be an innovative solution for the environmental problems caused by accumulation of solid waste of synthetic polymer origin and these bacterial biopolymers could act as environmental-friendly alternative to polymers of synthetic origin (Steinbuchel *et al*., 1995).

Currently, biodegradable plastics can be categorized into three main types; Photodegradable, Starch-linked and Bacterial plastics. There has been increasing effort to study and develop the production and application of bacterial plastics (Reddy *et al*., 2003).

1.1 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are polyesters of natural origin accumulated in form of intracellular granules by a wide variety of bacterial strains. The first example of PHA to be discovered was poly (3-hydroxybutyrate) (PHB). In 1926, Lemoigne isolated and characterized PHB from bacterial strain *Bacillus megaterium*. Since that, PHA accumulation ability has been reported for many microorganisms including Gram-negative and Gram positive species (i.e. autotrophic, heterotrophic and phototrophic microrganisms, aerobes and anaerobes) as well as for some archae strains. Microorganisms usually synthesize and accumulate as a membrane enclosed inclusion in many bacteria at up to 80% of the dry cell weight. PHA can be carbon, energy and reducing power storage material under conditions of the nutrients limitation (such as nitrogen, phosphorus or iron limitation) and in presence of excess of carbon source. Under the conditions of carbon source starvation, PHA is degraded by intracellular depolymerases and subsequently metabolized as a carbon and energy sources (Anderson, and Dawes, 1990).

1.2 Properties of PHA

It has mechanical properties very similar to conventional plastics like polypropylene or polyethylene and can be extruded, moulded, spun into fibres, made into films and used to make heteropolymers with other synthetic polymers (Khanna and Srivastava, 2005). Marchessault *et al*., (1988) have also drawn attention to the potential role of PHAs as so-called biomass transducers, i.e., their use in the microbial transformation of carbohydrate feedstock via PHA into chiral depolymerization products or small molecule organic chemicals by pyrolysis. Underlying these developments has been the need to study the structure of PHB and of PHA copolymers, their physical state, the metabolic pathways involved, and the regulation of their synthesis in the microbial cell, endeavors which represent a third major area of activity (Seebach *et al*., 1985).

Biodegradability and biocompatibility are main characteristics that allow PHAs to be competitive in special market sectors. Both PHB homopolymer and PHBV copolyester have been receiving commercial interest as a promising candidate for the large-scale production of biodegradable and biocompatible thermoplastics, since this polymer exhibits a considerable range of transport and thermo-mechanical properties which depend on its 3HV content (Bonartsev *et al*., 2007).

1.3 Microorganisms involved in PHA production

Microorganisms are able to incorporate up to 60 different type monomers into their storage polymer and a series of PHAs with different monomeric composition (i.e. different physical and chemical properties) can be produced (Bonartsev *et al*., 2007). More than 100 different monomer units have been identified as constituents of the storage PHA. This creates a possibility for producing different types of biodegradable polymers with an extensive range of properties. The molecular mass of PHA is in the range of 50,000–1,000,000 Da and varies with the PHA producer. The monomer units are all in D configuration owing to the stereospecificity of biosynthetic enzymes (Senior *et al*., 1972; Dawes and Senior, 1973; Oeding and Schlegel, 1973; Wang and Bakken, 1998).

PHB was produced in an industrial scale using Gram negative bacteria such as *Cupriavidus necator* (Vandamme and Coenye, 2004), *Alcaligenes latus* and recombinant *Escherichia coli* (Choi *et al*., 1998). However, PHB of those organisms contain the outer membrane lipopolysaccharide (LPS) endotoxins that may induce a strong immunogenic reaction and is therefore undesirable for the biomedical application of the PHAs (Chen and Wu, 2005). Possible removal of endotoxin during purification of poly (3-hydroxybutyrate) from Gram negative bacteria was reported by lee *et al*., (1999). On the other hand, Gram positive bacteria lack LPS, excreting proteins at high concentration and potentially use of cheaper raw materials, therefore considered as better source of PHAs to be used for biomedical applications (Lopes *et al*., 2009).

The choice of microorganism for the industrial production of PHA varies depending on factors that include the cell's ability to utilize an inexpensive carbon source (recent attention has been paid to agricultural wastes and industrial by-products), the cost of the medium, the growth rate, the polymer synthesis rate, the quality and quantity of PHAs, and the cost of downstream processes . Although more than 300 different microorganisms synthesize PHAs, only a few, such as *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*), *Alcaligenes latus*, *Azotobacter vinelandii*, *Pseudomonas oleovorans*, *Paracoccus denitrificans*, *Protomonas extorquens* and Recombinant *E. coli*, are able to produce sufficient PHA for large-scale production (Chanprateep, 2010). Under specific conditions, *Alcaligenes eutrophus* is known to contain as much as 96% PHA and it has also been demonstrated that the PHA biosynthetic pathway from *A. eutrophus* can be cloned and expressed in *E. coli*. Polymer contents of upto 90% of the cellular dry weight obtained from *E. coli* (Lenz *et al*., 1989).

Ralstonia eutropha (formerly known as *Alcaligenes eutrophus*) is one of the PHA-producing bacteria that have the potential for commercial production of PHA due to its high polymer content depending on the carbonaceous substrates. Moreover, *R. eutropha* attracted scientific investigation because of its ability to grow on various nutrients. Many researchers verified that *R. eutropha* could use short chain fatty acid as carbon source to synthesize PHA (Boonsawang and Wongsuvan, 2010).

A number of *Bacillus* species have been reported to accumulate 9 to 67% cell dry weight (CDW) of PHA (Valappil *et al*., 2008; Adwitiya *et al*., 2009; Reddy *et al*., 2009). Furthermore, the genus *Bacillus*, in common with many other PHA-accumulating Gram-positive bacteria, accumulates co-polymers of 3-hydroxybutyrate when grown on different substrates (Satoh *et al*., 2002; Tajima *et al*., 2003).

1.4 PHA Biosynthetic pathway

Under normal growth conditions, the nutrient sources are used for the synthesis of proteins essential for the growth of bacteria. The nitrogen source depletion leads to the cessation of protein synthesis, which in turn leads to the inhibition of TCA cycle enzymes such as citrate synthase and isocitrate dehydrogenase and consequently slows down the TCA cycle. As a result, the acetyl-CoA routes to P (3HB) biosynthesis. Both the shortening of external nutrients and internal sources such as RNA or enzymes facilitate the PHA synthesis (Ramadas *et al*., 2009).

1.5 Cheaper substrates for PHA production

The main limitation in the production of PHA is the specific growth conditions required for their synthesis. Much emphasis has been placed on reducing the cost of PHA synthesis by performing biosynthesis trials in different recombinant bacterial strains and by developing more efficient fermentation and recovery processes (Rodriguez *et al*., 2011). In order to reduce the overall cost, it is important to produce PHA with high productivity and high yield. Recently, scientists have been exploring cultivation strategies involving inexpensive, renewable carbon substrates in order to reduce production cost and obtain high productivity (Ojumu, 2004). According to Lee *et al*., (1997), many carbon sources derived from wastes like whey, cane molasses and sugar beet molasses can be used in production. Recently, PHB proved to be produced from relatively cheaper substrates such as methanol (Kim *et al*., 2003; Mokhtari *et al*., 2009), rice bran and sea water (Nikel *et al*., 2005; Nath *et al*., 2008; Pandian *et al*., 2010), carbon dioxide (Ishizaki *et al*., 2001), residual fats and oils are used for the production of mcl- PHA by *Pseudomonas oleovorans* (Fuchtenbusch *et al*., 2000).

Utilization of Lignocellulosic substrate as cheap carbon sources for PHA production is only less attempted due to the difficulty involved in the hydrolysis of polysaccharides into simple sugars, for the bacteria to uptake. The enzymatic conversion can be enhanced by using non-ionic surfactants and Polyethylene Glycol (Kristensen *et al*., 2007). The use of ionic solvents in hydrolysis of cellulose to glucose was well explained by (Binder *et al*., 2010), where Ethyl Methyl Imidazolium Chloride (EMIM) was used. Cellulose subjected to hydrolysis without degradation of glucose using Zinc chloride as described in patented methods (Chen *et al*., 1984 and 1985).

1.6.1 *Syzygium cumini*

Syzygium cumini, commonly known as Jamun, a polyembryonic species (family Myrtaceae) is a widely distributed forest tree in India and other tropical and sub tropical regions of the world. The tree has a great economic importance since most of the parts like the bark, leaves, seed and fruits are used as an alternative medicine to treat various diseases. It is a seasonal fruit and is consumed fresh for its nutrient value.

The flower appears during the month of March to April and the fruit formation takes place about 32 days after flowering during the month of May to July. The nutritional compositions of seeds are: protein (6.3 to 8.5%), fat (1.18%), crude fiber (16.9%), ash (21.72%), calcium (0.41%), and phosphorus (0.17%), fatty acids (30%) (palmitic, stearic, oleic and linoleic), starch (41%), Carbohydrates (73.61%), dextrin (6.1%), a trace of phytosterol, and 6 to 19% tannin (Ranjan *et al*., 2011).

1.6 Optimization of PHA production

Reducing the costs of PHB production by optimizing fermentation process is the basic research objective for industrial application. Medium optimization by application of statistical optimization method, compared to the common “one-factor-at-a-time” method, proved to be powerful and useful tool (Lakshmar *et al*., 2004). RSM can be used to assess the effect and interaction of several controlled experimental factors (independent variables) that influence the selected responses (dependent variables). Furthermore, RSM has been effectively applied in industrial research and in biological studies to optimize microbial processes and interactions among various physicochemical parameters involved in biopolymer production (Yu *et al*., 2008; Sharma 2007; Mokhtari *et al*., 2009; Pandian *et al*., 2010).

1.7 Degradation of PHA

PHA can be degraded by different mechanisms and may occur inside the bacterial cell where the polyester has been synthesized and accumulated or outside the bacterial cell by other bacteria, fungi or higher organisms. They use the polyester as a carbon source after release from a dying and lysing bacterial cell or after entering the ecosystem due to a technical or medical application of PHA. Degree of crystallinity significantly affects the degradation rate of PHA and Kumagal *et al*., 1992 concluded that poly (3HB) molecules in the amorphous state are more easily hydrolyzed than in the crystalline state.

1.8.1 Extracellular degradation

In general degradation seems to follow a hydrolytic mechanism, and the enzymes catalyzing this reaction are referred to as PHA depolymerases. All PHA depolymerises consist of a single polypeptide chain and their molecular weights are in the range of 37-57 kDa and are inhibited by diisopropyl fluorophosphates, phenyl methyl sulfonyl fluoride and dithiothreitol which indicates the importance of serine residues and disulphide bonds for enzyme activity. The enzymatic degradation of linear PHA macromolecules has been reported to start from the hydroxyl- terminal groups (Sadocco *et al*., 1997). On PHA containing agar media, PHA degrading microorganisms were made visible by the formation of clearing zones around the colonies and the water insoluble PHA into water soluble cleavage products by the excreted PHA during hydrolysis (Murphy, 1993). Extracellular PHB depolymerase has been isolated from different bacteria as *Alcaligenes faecalis*, *Rhodospirillum rubrum*, *B. megaterium*, *A. beijerinckii* and *Pseudomonas lemoignei*.

PHA degradation occurs in a large variety of complex environments, which included oxic as well as anoxic environments. Degradation of PHA has been investigated in various natural environments such as soils and composts (Mergaert *et al*.,1992), natural waters (Mukai *et al*., 1964) and several microorganisms able to degrade PHA have been isolated and identified (Lee, 1996).

1.8.2 Intracellular degradation

In vivo, PHAs can be hydrolyzed by the accumulating strain itself during periods of starvation (intracellular PHA hydrolysis by intracellular PHA depolymerases). The degradation of Poly (3HB) with isolated granules from *B. megaterium*, *R. rubrum* was reported (Merrick and Doudoroff, 1964), *A. eutrophus* (Hippe and Schlegel, 1967) and *Z. ramigera* (Saito *et al*., 1992)**.**

1.8.3 Degradation by other enzymes

Whereas none of the extracellular PHA depolymerises investigated exhibited lipase activity as revealed by measuring hydrolysis of triolein, several lipases from bacteria and fungi hydrolyzed PHA as revealed by measuring the weight of PHA films (Mukai *et al*., 1993), or by a photometric assay of PHA granule suspensions (Jaeger *et al*., 1995). PHA containing hydroxyalkanoic acid with the side chains attached to the polyester backbone such as poly (3HB) were not or only marginally hydrolyzed and is relevant for the consideration of medical applications of PHA.

In the present study, PHB accumulating bacteria from soil were isolated, characterized and screened by using Nile blue staining solution. This project was carried out with an aim to utilize jambul seed (*syzygium cumini*) as a cheap carbon source for PHA production by the screened organism and pure culture of *Ralstonia eutropha* (Reference strain) MTCC 1472 was purchased from IMTECH, Chandigarh. A non-enzymatic, chemical method of seed hydrolysis was performed to remove the lignin content and release the carbon content as simple sugars. The residual white mass obtained was used as the carbon source in the growth media (Mineral Salt media) for PHA producing microorganism. The accumulated PHA was extracted using chloroform solvent (Santhanam and Sasidharan, 2010). The polymer production and yield among the native isolates was compared with the reference strain. Effect of different combination of carbon source on PHA production was studied and the purity of the extracted polymer was confirmed using FTIR spectroscopy (Oliveira *et al*., 2007 and Pandian *et al*., 2010). Optimization of the media components in the Mineral Salt Media was done based on the Placket Burman design and to determine the optimum concentration of the significant components by Response Surface Methodology. In further studies, its biodegradation capability of extracted PHA was determined by extracellular and intracellular degradation methods.

1.9 Objectives

- To study the utilization of fruit seed constituents as a nutrient medium for the production of PHA.
- Isolation and characterization of PHA producing bacteria from soil.
- To screen the PHA producing bacteria from the isolated organisms.
- To hydrolyze fruit seed by various chemical treatments and use it as the carbon source for PHA producing isolate and *Ralstonia eutropha* (Reference Strain) in a Mineral Salt Media.
- To extract the accumulated PHA by adding different combination of substrate in the production media.
- To optimize the media components in the Mineral Salt Media for maximum production of PHA.
- To analyze its structural properties using Fourier Transform Infra Red Spectroscopy (FTIR), in comparison with standard Polyhydroxy-3-butyric acid.
- To analyze its biodegradation capabilities of extracted PHA by extracellular and intracellular degradation.

CHAPTER 2
LITERATURE REVIEW

2.1 History of Polyhydroxyalkanoates (PHA)

In 1926, French microbiologist Maurice Lemoigne had discovered and firstly described a polymer, Polyhydroxyalkanoate (PHA) produced by Gram-positive bacterium *Bacillus megaterium* under nutrient stress condition such as nitrogen, phosphorous or oxygen limitation with excess carbon sources (Lenz *et al*., 1989; Sudesh *et al*., 2000). Other than 3HB was given by Wallen and Rohwedder (1974), reported heteropolymers in chloroform extracts of activated sewage sludge. They noted the presence of 3HB and 3-hydroxyvalerate (3HV; 3-hydroxypentanoate) as major constituents with C_6 and possibly C_7 3-hydroxyacids as minor components. This heteropolymer had a lower melting point than PHB and, unlike the homopolymer, was soluble in hot ethanol.

A significant development was reported by De Smet *et al*., (1983) that *Pseudomonas oleovorans*, when grown on 50% (v/v) n-octane, accumulated granules that resembled PHB inclusions by freeze-fracture electron microscopy but corresponded to an empirical formula of $C_8H_{14}0_2$ on analysis and appeared to consist principally of a polyester of 3-hydroxyoctanoic acid. The majority of the published research on PHAs other than PHB has concentrated on two bacteria: *Alcaligenes eutrophus* and *P. oleovorans*.

2.2 Properties of PHA

2.2.1 Structure and its classification

PHAs are thermoplastic or elastomeric polyesters (polyoxoesters) of R-hydroxyalkanoic acid (HA) monomers (Figure 2.2.1). Structurally, these polymers are classified on the basis of the number of carbon atoms that range from 4 to 14 and the type of monomeric units, producing homopolymers or heteropolymers. PHAs with 3–5 carbon atoms are considered as short chain length PHAs (scl-PHAs). Examples of this class include Poly (3-hydroxybutyrate), P (3HB) and Poly (4-hydroxybutyrate), P (4HB). PHAs vary in their physical and chemical characteristics owing to their varied monomer content. In contrast, polymers composed of C_6–C_{16} 3- hydroxy fatty acids or aliphatic carbon sources are referred to as medium-side-chain PHA (msc-PHA). The composition of the resulting PHA depends on the substrate used in the production media (Brandl *et al*., 1988; Huisman *et al*., 1989). The msc-PHA is also synthesized from carbohydrates, but the composition is not related to the carbon source (Huisman *et al*., 1989). Factors affecting the monomer content include: Type of microorganisms (e.g. Gram-negative or Gram-positive), Media ingredients, Fermentation conditions, Modes of fermentation (batch, fed-batch, continuous) and Recovery (Keshavarz and Roy, 2010).

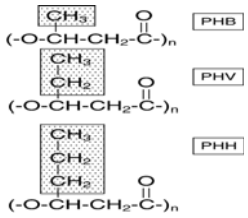


Fig 2.2.1 Chemical structure of PHAs The pendant R groups vary in chain length from one carbon (C_1) to over 14 carbons (C_{14}). Structures shown here are PHB (R=methyl), PHV (R=ethyl), and PHH (R=propyl) (Pornpa *et al*., 2007).

2.2.2 Physical, Mechanical and thermal properties

PHA is non-toxic, biocompatible and biodegradable thermoplastics that can be produced from renewable resources. They have a high degree of polymerization, highly crystalline, optically active, isotactic, piezoelectric and insoluble in water. These features make them highly competitive with polypropylene, the petro chemically derived plastics. PHB also shows good oxygen impermeability. The mechanical properties of individual PHB strongly depend on the monomer unit composition (Obruca, 2010). The mechanical properties of PHB including Young's modulus (3.5GPa) and tensile strength (MPa) are similar to those of polypropylene (Table 2.2.2), but the elongation to break of PHB (6%) is significantly lower than that of polypropylene (400%). The PHB copolymer containing 3-hydroxyvalerate unit P (3HB-*co*-3HV) has been developed and has much improved mechanical properties, the polymer becomes tougher (increase in impact strength) and more flexible (decreases in young's modulus) as the fraction of 3-hydroxyvalerate unit increases. Furthermore, the melting temperature decreases with increasing co-monomer fraction without any change in the degradation temperature. This allows the thermal processing of the copolymer without thermal degradation. Therefore the material properties can be controlled by adjusting the fraction of 3-hydroxyvalerate during the fermentation (Ojumu *et al*., 2004).

Table 2.2.2 Physical properties of various PHAs and polypropylene (Ojumu *et al*., 2004)

Property	PHB	Polypropylene
Melting point(^{o}C)	175	176
Glass-transition temp (^{o}C)	15	-10
Crystalline (%)	80	70
Young's modulus	3.5	1.7
Tensile strength (MPa)	40	34.5
Elongation to Break (%)	6	400
Impact strength (v/m)	50	45

2.2.3 Intracellular PHA granules

PHA is accumulated in cells in the form of intracellular granules. Nuclear magnetic resonance (NMR) spectroscopy of various bacteria has clearly demonstrated that the polyester in the cells occurs in a metastable amorphous state and granule morphology is under kinetic rather than thermodynamic control. The density of PHB is about 1.18- 1.24 g/cm^3. The isolated granules consist of polyester, proteins and phospholipids. The composition of PHB granules of *Bacillus megaterium*, consisted of 97.9 % polyester, 1.87 % proteins and 0.46 % lipids or phospholipids (Obruca, 2010). The fine structure of PHB granules have been well shown by Zinn *et al*., (2001) (Figure 2.2.3).

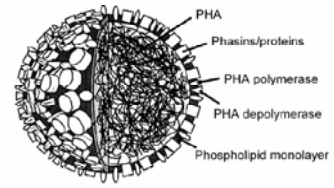


Fig 2.2.3 Structure of PHA- granules accumulated by bacteria (Zinn *et al*., 2001)

2.3 Biosynthetic pathway and regulation of PHA biosynthesis

The biosynthetic pathway of P (3HB) consists of three enzymatic reactions catalyzed by three different enzymes (Fig. 2.3.1). The first reaction consists of the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by β-ketoacyl-CoA thiolase (encoded by phbA). The second reaction is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl - CoA dehydrogenase (encoded by phbB). Lastly, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into PHB by P(3HB) polymerase, encoded by phbC (Reddy *et al*., 2003).

Lee and Choi, (1999) showed that the NAD(P)H/NAD(P) ratio is important in the metabolic regulation of PHA biosynthesis. When the NAD(P)H/NAD(P) ratio increases under nitrogen limitation, two cycle enzymes, citrate synthase and isocitrate dehydrogenase, are inhibited by NADH. It is proposed that the rate of PHA biosynthesis is controlled by β ketothiolase and acetoacetyl-CoA reductase, whereas the content of PHB is controlled by PHA synthase (Du *et al.,* 2001). The β -ketothiolase is inhibited by high concentration of CoA, and subsequently PHA biosynthesis is inhibited.

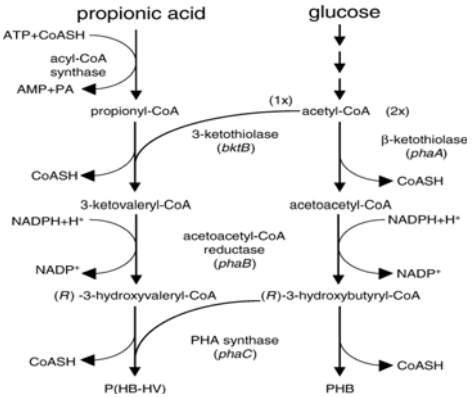


Fig 2.3.1 PHB and P (HB–HV) biosynthetic pathways in *R. Eutropha* (Pornpa *et al*., 2007)

2.4 Isolation of PHA producing microorganisms

A bacterium of *Bacillus* group was isolated from sugarcane field, which produced PHA and it was aerobic, Gram positive, rod shaped, endospore forming and catalase producing bacterium. It was able to grow up to 14% NaCl concentration, pH range from 3 to 10 and temperature range from 27°C to 70°C. Maximum yield of PHA was 38.5 mg/ml at 40°C while at 60°C yield was 34.5 mg/ml (Shukla *et al*., 2011).

A strain of poly-hydroxybutyrate (PHB) accumulating bacterium was isolated and identified as *Enterobacter aerogenes* by using biochemical and phylogenetic characterization. The accumulation of a large amount of granules in its cells cultured in the domestic wastewater medium (DWWM) were showed by transmission electron microscopy (TEM). The highest PHB yield by microorganism was up to 96.25% within 18 h (Ceyhan and Ozdemir, 2011).

Polyhydroxybutyrate producing bacteria from garden soil were isolated and characterized for their morphological, biochemical properties. Based on their 16S rRNA gene sequences, they were identified as *Bacillus mycoides* DFC1, *Bacillus cereus* DC1, *Bacillus cereus* DC2, *Bacillus cereus* DC3 and *Bacillus cereus* DC4. The highest PHB yield was observed in *Bacillus mycoides* DFC1 accumulating as high as 1.83g/L, amounting to 57.20% (w/w) of cell dry weight (Aarthi and Ramana, 2011).

Bacteria isolated from the various marine areas were screened for their ability to accumulate PHB and were compared with *Wausteria eutropha*. To increase the productivity, steps were taken to evaluate the effect of carbon sources, nitrogen sources, pH and sodium chloride concentration on PHB productivity by MK4. Significantly higher maximum biomass of 9.1 g/L with a PHB content of 4.223 g/L was obtained in a laboratory-scale bioreactor at 64 h, thus giving a productivity of 0.065 g/L/h (Arun *et al*., 2009). Soil contaminated with crude oil was screened for polyhydroxyalkanoic acid (PHA) producing bacterial strains. Biochemical tests and 16S rRNA sequencing identified bacteria of the genera *Pseudomonas*, *Acinetobacter*, *Sphingobacterium*, *Brochothrix*, *Caulobacter*, *Ralstonia*, *Burkholderia* and *Yokenella*. The phylogenetic analysis of the PHA synthase (phaC) gene of these bacteria showed a close homology with the phaC gene of different Pseudomonas species (Dalala *et al*., 2011).

Accumulation of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) [P (3HB-co-3HV)] copolymer by a local isolate, *Brevibacillus invocatus* under batch mode was investigated under glucose, acetate and propionate-supplemented conditions. Optimization of process parameters by response surface methodology (RSM) and *B.invocatus* is emerging as an interesting organism and could be exploited further for P (3HB-co-3HV) co-polymer production (Sankhla *et al*., 2010). An effective technology for production of PHB and PHBV of different molecular weight by diazotrophic bacteria of *Azotobacter* and *Rhizobium* genus has been developed by Bonartsev *et al*., (2007).

Synthesize a polyhydroxyalkanoate (PHA) containing newly reported 3-hydroxy-4-methylvalerate (3H4MV) monomer by using wild type *Burkholderia sp* and its transformed strain harboring the PHA synthase gene of *Aeromonas caviae* (phaCAc). The introduction of 3H4MV as a second monomer will improve the material properties of 3HB-based polymers. To promote the accumulation of PHA containing 3H4MV monomer, isocaproic acid was provided as co-carbon source. Approximately 1 mol% of 3H4MV was detected in wild type Burkholderia sp (Lau *et al*., 2010).

2.5 PHA production by Recombinant bacteria

Metabolic engineering is being intensely explored to introduce new metabolic pathways to broaden the utilizable substrate range, to enhance PHA synthesis and to produce novel PHA. Recombinant *E. coli* strains harbouring the *Alcaligenes eutrophus* PHA biosynthesis genes in a stable high-copy-number plasmid have been developed and used for high productivity (Zhang *et al*., 1994). Since *E. coli* can utilize various carbon sources, including glucose, sucrose, lactose and xylose, a further cost reduction in PHA is possible by using cheaper substrates such as molasses, whey and hemicellulose hydrolysate (Lee *et al*., 1994).

Natural PHA- producing bacteria have a long generation time and relatively low optimum growth temperatue. These are often hard to lyse and also contain pathways for PHA degradation. Bacteria such as *E. coli* are incapable of synthesizing or degrading PHA; however, *E. coli* grows fast, even at higher temperature and is easy to lyse. Faster growth will enable it to accumulate a large amount of polymer. The easy lyses of the cells save the cost of the purification of PHA granules (Wang and Bakken, 1998; Madison and Huisman, 1999). Hence, *E. coli* has been used to transfer PHA genes. PHB production has been studied mostly

in recombinant *E. coli* cells harbouring PHA synthesizing genes from *R. eutropha* (Lee *et al.,* 1994; Zhang *et al.,* 1994). Introduction of *Pha*CRS gene from *Rhodobacter sphaeroids* into *E. coli* does not support PHB synthesis. PHB accumulation also failed in recombinant *E. coli* cells harbouring *Pha*CV from *Chromobacter violaceum* and *Pha*BRe and PhaRe genes from *R. eutropha* (Kolibuchuk *et al.,* 1999). While the PHB synthesizing genes from *R. eutropha* have been transferred to *E. coli,* The *Pha*C genes from other organisms like *R. sphaeroides* and *C. violaceum* apparently did not function in the *E. coli* genetic background.

2.6 Screening methods for PHA accumulation in bacteria

Microbiologists have traditionally detected the presence of PHB granules in bacterial cells by staining with Sudan Black B. However, Ostle and Holt (1982) have advocated the use of Nile blue A, a water-soluble basic oxazine dye that has a greater affinity and higher specificity than Sudan Black for PHB, and that gives a bright orange fluorescence at a wavelength of 460nm. Other inclusion bodies, such as glycogen and polyphosphate, do not stain with Nile blue A, emphasizing its usefulness. Phase contrast microscopy detected brightly Refractile Cytoplasmic Inclusions (RCI) in PHB producing bacteria in axenic cultures (Cortes *et al*., 2008).

Spiekermann *et al*., (1999) recommended the use of a sensitive viable colony staining method using Nile red for direct screening of bacteria that accumulate PHA. The direct inclusion of 0.5µg/ml of dye in the medium did not affect the growth of the cells but allowed the detection of the presence of PHAs in the viable colonies at any time during growth. The PHA producers exhibited strong fluorescence when observed under UV light. Hartman (1940) was the first to suggest the use of Sudan black B, as a bacterial fat stain. Subsequently, Burdon *et al*., (1942) confirmed the greater value of this dye and modified the procedure for demonstrating intracellular fatty material in bacteria by preparing microscopic slides of bacteria stained with alcoholic Sudan black B solution and counterstained with safranin.

2.7 Cheaper substrates for PHA production

Polyhydroxyalkanoates are biodegradable polymers produced by prokaryotic organisms from renewable resources. The use of these low-cost raw materials has the potential to reduce PHA production costs; because the raw material costs contribute a significant part of production costs in traditional PHA production processes (Castilho *et al*., 2009). *Pseudomonas* sp. was capable of producing PHA from waste vegetable oil (Hwan *et al*., 2008), milk whey (Bosco and Chiampo, 2010). Whey permeate from dairy industry was hydrolyzed enzymatically to cleave its main carbon source, lactose, to glucose and galactose. The hydrolysis products were chosen as carbon sources for the production of poly-3-hydroxybutyric acid (PHB) by *Pseudomonas hydrogenovora* (Koller *et al*., 2008). A higher cell density and a greater PHB production level were obtained by using sugarcane molasses and urea as carbon and nitrogen sources (Kulpreecha *et al*., 2009). The growth of the PHB producing in the media composed of several complex carbon source comprising wheat starch, corn starch, potato starch and high PHB yield of 1.28g/L in wheat starch containing medium at the end of 48h of growth (Aarthi and Ramana, 2011).

2.8 Substrates and growth conditions for PHA production

Biopolymer obtained by SSF (Solid state fermentation) using *Ralstonia eutropha* and soy cake, sugarcane molasses as a carbon source, presented the same properties as commercial PHB, except for the higher molar mass and the lower degree of crystallinity. Solid-state fermentation is an interesting alternative for the production of PHB, allowing the production of biopolymers with adequate properties from low-cost, renewable resources (Oliveira *et al*., 2007). The culture medium used for the production of biomass and PHA was a minimal basal medium (MM) containing $CaCl_2$ (0.04g/L), KCl (0.4 g/L), $MgSO_4.7H_2O$ (2 g/L) and $FeSO_4.7H_2O$ (0.012 g/L). The basal medium contained 0.2 ml/L of the following trace elements in solution (g/L): 1.48 H_3BO_3, 1.96 $CuSO_4.5H_2O$, 1.54 $MnSO_4.H_2O$, 3.07 $ZnSO_4.7H_2O$ and 0.15 $NaMoO_4.2H_2O$. Since nitrogen, phosphorus and carbon are essential nutrients in the medium which influence the growth and accumulation of PHA and optimized the concentration of their sources ($NaNO_3$, K_2HPO_4/KH_2PO_4 and the carbon source, respectively) using RSM (Carmona *et al*., 2011, Sankhla *et al*., 2010 and Kim *et al*., 2000).

2.9 Production of Polyhydroxyalkanoates by Mixed Culture and Mixed Substrate

Salehizadeh and Van (2004) studied the recent trends in the development of PHA production using mixed microbial cultures. The PHA accumulation capacity of a mixed enriched culture, with PHA-forming bacteria kept under non aseptic conditions in comparison to the performance of pure cultures of the two isolates derived from it. The mixed culture led to better final yields of PHAs/VFAs consumed as well as faster accumulation rates (Kourmentza *et al*., 2009). The well-studied strategy for enrichment of mixed cultures with PHA production capability is based on alternating availability and unavailability of carbon substrate under aerobic conditions, so called "feast and famine" or "aerobic dynamic feeding" (ADF) conditions. The ADF process has been extensively investigated using synthetic organic acids (Dionisi *et al*., 2004) as well as fermented waste/wastewaters such as sugar cane molasses (Albuquerque *et al*., 2007), paper mill wastewater (Bengtsson *et al*., 2010) and olive oil mill effluent (Beccari *et al*., 2009).

Kim *et al*., (1997) studied the effects of different carbon sources on the growth and PHA production and reported that simultaneous supply of glucose and octanoic acid resulted in 35.9 g of PHA/L (65% of the cell dry weight). From the preceding, it appeared that mixtures of cheap growth substrates and more expensive substrates for product formation provided a valuable means of lowering PHA production costs.

2.10 Optimization of Media components

A five-level-three factor central composite rotary design was employed to find out the interactive effects of three variables, i.e. static magnetic field intensity, concentration of NH_3–N and initial pH on polyhydroxyalkanoates (PHA) production by activated sludge under the aerobic dynamic feeding (ADF) technique by Hong *et al*., (2009). Response surface methodology (RSM) was utilized for process optimization and a second-order polynomial equation was obtained by multiple regression analysis. A yield of 49.5% of dry cell weight was achieved at optimized conditions, i.e. C:N (60:1) and pH 9.0. Response surface methodology (RSM) stemmed from experimental design and was later introduced into numerical simulation in the reliability assessment of complex multivariable systems (Yu *et al*., 2006). RSM provides a systematic and efficient research strategy for studying the interaction effects of various parameters using statistical methods. It has been extensively applied in microbial fields in the recent years (Khanna and Srivastava 2005; Sharma *et al*., 2007). In this study, RSM was used to optimize the culture medium composition for biomass and PHA production by *Pseudomonas aeruginosa* 42A2 (NCIMB 40045) using an industrial oil by-product as an inexpensive carbon source triolein, several lipases from bacteria and fungi hydrolyzed PHA as revealed by measuring the weight loss of PHA films (Mukai *et al*., 1993) or by a photometric assay of PHA granule suspensions (Jaeger *et al*., 1995).

2.11 Extraction of PHA

PHA recovery processes, in addition to the costs of maintaining pure cultures and the high costs of organic substrates, is another factor that contributes to the high cost of PHA production. In the past two decades, several recovery processes have been investigated and studied in order to find the economic way to isolate and purify PHA. Hahn *et al.* (1994) recommended the method called, dispersion with sodium hypochlorite and chloroform. They claimed that this method removed most of the non-PHA cellular materials during sodium hypochlorite digestion, which facilitated the separation of PHA from the cells. In addition, digestion with hypochlorite reduced the viscosity of the chloroform phase. They also investigated on the optimum conditions for PHA recovery from *R. eutropha* using dispersions of sodium hypochlorite and chloroform. The optimum conditions from their experiments were reported to be 90 minutes digestion time with 30% sodium hypochlorite concentration and a chloroform-to-aqueous phase ratio of 1:1 (v/v). They obtained a degree of recovery of 91% and a level of purity of higher than 97% (Santhanam and Sasidharan, 2010; Sankhla *et al*., 2010).

Due to the high cost of solvent extraction, the enzymatic digestion method was developed by ICI, UK. Steps of this process include thermal treatment (100-150°C) to lyze cells and denature nucleic acids, enzymatic digestion, and washing with anionic surfactant to solubilize non-PHA cellular materials. Finally, concentrated PHA from centrifugation was bleached with hydrogen peroxide. Sodium hypochorite solubilizes non-PHA cellular materials and leaves PHA intact. Then, PHA could be separated from the solution by centrifugation. A severe degradation of polymers during sodium hypochorite digestion was frequently reported. Fifty percent reduction in the MW of the polymers was reported when the biomass was digested with sodium hypochorite (Lee, 1996).

2.12 Characterization of the polymer

2.12.1 Staining procedure

The presence of PHA as intracellular granules was confirmed by staining the cells with Sudan black-B. After the complete production of PHA under suitable growth conditions, thin smear of strains were made on a clean glass slide and was heat fixed. This slide was immersed in a filtered solution of 0.3% (w/v) Sudan black-B (in ethylene glycol) for 15 - 20 min. Then, the slide was immersed in xylem and blot dried with absorbent paper. Finally, the microscopic slide was counter-stained for 10s with (0.5% w/v) aqueous safranin. The slide was then rinsed with tap water and blot dried and examined under a microscope (Santhanam and Sasidharan, 2010).

2.12.2 Fourier Transform Infrared Spectroscopy (FTIR)

For FTIR analyses, samples were first dissolved in chloroform and then added to KBr pellets. After complete solvent evaporation, FTIR spectra were recorded. A total of 20 scans were recorded per sample at a 2 cm^{-1} resolution, between 4000 and 400 cm^{-1} by Oliveira *et al*., (2007); Santhanam and Sasidharan, (2010).

2.12.3 Nuclear magnetic resonance spectroscopy (NMR)

The crystalline structures of the PHA sample were also studied using a NMR spectrum. Nuclear magnetic resonance spectra (13C) of samples were recorded at 75.4 MHz using chloroform (CDCl3) as solvent (Oliveira *et al*., 2007; Lau *et al*., 2010).

2.12.4 Differential scanning calorimetry (DSC)

Determine the thermal properties of PHA by Differential scanning calorimetry. The samples were heated from 25 °C to 190 °C at a rate of 10 °C min^{-1}. The first and second cooling runs were carried out at rates of 190 °C $min^{-1}$and 10°C min^{-1}, respectively. From the first and second heating runs, glass transition temperature (Tg), melting temperature (Tm), and crystallization temperature on heating (Thc) were obtained. The crystallization temperature on cooling (Tcc) was obtained from the second cooling. The degree of crystallinity (XC) was determined from the ratio of the melting enthalpy of the sample (ΔHm) and the melting enthalpy of 100% crystalline PHB (ΔH°m =146 J/g) (Oliveira *et al*., 2007; Sankhla *et al*., 2010).

2.12.5 Thermogravimetric analysis (TGA)

Decomposition temperature (Td) was analyzed by thermogravimetric analysis, TGA (TA Instruments Q500), with a nitrogen atmosphere (Bengtsson *et al*., 2010). Between 1.5mg and 5mg of polymer were analyzed and the temperature was increased at 10°C/min to 350°C with a 10 min isothermal halt at 95°C to remove any residual water. The decomposition temperature was determined by observing the peak in weight loss slope (dw/dT).

2.12.6 X-ray diffraction The crystalline structure of samples was studied by using an X-ray diffract meter, which provides CuKa radiation (40 kV, 40 mA), employing the powder method. Every scan was recorded in the range of 2θ = 5–70° in the step-by-step mode of 0.05° (Oliveira *et al*., 2007).

2.12.7 Gel permeation chromatography (GPC)

Molar mass of samples was determined at 30°C using a GPC system. Samples were dissolved in chloroform at a 0.2% concentration, injection volume and flow rate was 1 ml min^{-1}. Monodisperse polystyrene standards were used in the calibration curve (Oliveira *et al*., 2007; Lee *et al*., 2008).

2.13 Quantification of PHA

2.13.1 Growth and Dry Cell Weight measurement

Cell growth was monitored by measuring the optical density of the culture broth at 600 nm. For determination of dry cell weight (DCW), the cell mass was harvested by centrifugation at 10,000 rpm for 10 min, and the supernatant was analyzed for residual sugar and nitrogen content. The harvested cells (pellet) were washed twice with distilled water, transferred to pre-weighed aluminum cups and dried to a constant weight at 80°C. The corresponding dcw for optical density of the culture broth was then plotted in the form of a graph (Sankhla *et al*., 2010; Kulpreecha *et al*., 2009; Franz *et al*., 2011).

2.13.2 Gas chromatography (GC)

PHA was quantified by gas chromatography (GC) and the extracted PHA was subjected to propanolysis or methanolysis in the presence of sulphuric acid and the resulting hydroxymethyl esters were analyzed using GC (Zakaria *et al* ., 2010). The PHB mass content be determined by this method in comparison with that of the standard PHA. GC equipped with a flow rate of 1ml/min (helium as carrier gas), sample input temperature of 230°C, column temperature of 80°C increased to 250°C at a rate of 8°C min^{-1}, interface temperature of 250°C, ion source temperature of 180°C, electron impact mode of 70 eV and scanning from 43 to 300amu (atomic mass unit) at 0.5s $scan^{-1}$ to identify the monomeric units of PHA (Dalala *et al*., 2010; Sankhla *et al*., 2010).

2.13.3 Assay of PHA

PHB content was measured as crotonic acid, formed by acid depolymerization of PHB according to Law and Slepecky. Cell pellets, harvested by centrifugation, were dissolved in methylene chloride by rapid mixing and afterwards boiled for 10 min. After the samples were cooled down, they were centrifuged at 3000 rpm for 15 min and the supernatants were carefully removed and evaporated. The remaining PHB-containing samples were digested in 2ml H_2SO_4 (96 %) at 100 °C for 30 min and subsequently diluted with concentrated H_2SO_4 and was taken for measurement at a UV spectrum between 220 and 225 nm. The presence of PHA was confirmed by the presence of a peak obtained between 230 - 240 nm (Santhanum and Sasidharan, 2010; Franz *et al*., 2011; Zakaria *et al*., 2010).

2.14 Degradation of the extracted PHA

The PHA-degrading organisms excrete extracellular PHA depolymerases that degrade PHA and utilize the decomposed products as nutrients (Jendrossek *et al*., 1996). The extracellular PHA depolymerises have been purified and characterized from many bacteria, including *Alcaligenes faecalis* and *Pseudomonas lemoignei* (Jendrossek *et al*., 1995). Most PHA-degrading bacteria apparently contain at least one depolymerase. Degradation of PHAs was determined as molecular weight loss measured by gel permeation chromatography and morphological changes in poly-3-hydroxybutyrate by scanning electron microscopy. Inhibition of the extracellular depolymerises of the three strains in the presence of p-methylphenyl sulfonylAuoride, cyanide, azide, deoxycholate or EDTA confirms the presence of an enzyme in the isolates similar to poly (3-hydroxybutyrate) degrading hydrolases reported earlier by Sadocco *et al*., (1997).

In most bacteria the biosynthesis and degradation of PHA occur via a cyclic metabolic process (Anderson and Dawes, 1990; Handrick *et al*., 2000; Jendrossek and Handrick, 2002). The accumulated polymers are readily degraded and utilized by the producer organisms as carbon and energy source by the action of intracellular PHA depolymerase (i-PHA depolymerase) when these cells undergo carbon-starved conditions.

To assess the degrading capacity of the extracted PHA obtained from *A. eutrophus*, the chloroform solvent method was used. For this, media containing PHA as the sole carbon source was used for the isolation of microbes that degrade PHA from soil and water. Nitrogen free mineral agar medium was prepared to a portion of the medium that has been melted and cooled to 45 - 50°C, a sufficient amount of sterile concentrated PHA granules suspension was added to give a final concentration of 2.5% (w/v) in the medium. Granule-agar suspension was poured over the surface of the plates of solidified medium to form a thin layer. After the overlay solidified, serially diluted samples were spread over the medium. The plates were incubated for 3 to 5 days at 37°C (Santhanum and Sasidharan, 2010).

Scanning electron microscopy of PHBV film at the end of incubation showed many changes in surface morphology, such as erosion and extensive roughening of the surface with pit formation, as compared to the untreated plastic pieces. Studying the behaviour and characteristics of the microorganisms adhering to a polymer film surface can be useful in understanding the biodegradation of PHAs in soil Shah *et al*., (2007).

2.15 Advantages of Bio-plastics

- PHB are natural polymers and thermoplastic or elastic properties with melting-points ranging from 40° to 180°C.
- Degradation in microbe active environments in 5-6 weeks.
- Degradation in process ultimately leaves behind carbon dioxide and water, which are environmentally friendly by-products. The released carbon dioxide and water are absorbed during photosynthesis in nature.
- Bio-plastics can be produced from renewable carbon resources. As long as there is fuel shortage & rise in crude oil prices traditional energy sources are safe
- Conservation of finite fossil resources like mineral oil and coal and their neutrality with regard to the emission of CO_2.
- Their biocompatibility and a low oxygen permeability which allow further applications for the production of films and coatings and special biomedicals (patch materials, stents, bone implantats, drug delivery systems, scaffolds for tissue engineering) (Ceyhan and Ozdemir, 2011).

2.16 Applications of PHA

The primary applications areas in which it features meet some market needs are:

(i) Packaging: P(HB-HV) could be used for films, blow molded bottles and as a creating on paper

(ii) Medical: P(HB-HV) biocompatibility coupled with its slow hydrolytic degradation lead to potential in reconstructive surgery. According to Lee (1996) the degradation product of PHB, D (-)-3 hydroxybutyrate has been detected in relatively large amount in human blood plasma. High biocompatibility of PHB films and medical devices implanted in animal tissues has been demonstrated (Bonartsev *et al*., 2007).

(iii) Disposable personal hygiene: P(HB-HV) could be used as the sole structural materials, it could also be used as part of degradable composites. For example PHAs could be substituted for polypropylene/polyethylene as matrix material in some composite material to achieve desirable properties comparable to these of Petro-chemical origin (Ojumu, 2004).

2.17 Economics of PHA production

It is a prerequisite to standardize all the fermentation conditions for the successful implementation of commercial PHA production systems. The price of the product ultimately depends on the substrate cost, PHA yield on substrate, and the efficiency of product formulation in the downstream processing (Lee, 1996). This implies high levels of PHA as a percentage of cell dry weight and high productivity in terms of gram of product per unit volume and time. Commercial applications and wide use of PHA is hampered due to its price. The cost of PHA using the natural producer *A. eutropphus* is US$ 16/kg which is 18 times more expensive than polypropylene. The commercially viable price should come to US $ 3-5 per kg.

In 2006, the cost of PHB was in the range of €10 per 12 $kg^{-}1$. This price was much higher than that of starch polymers and other bio-based polyesters due to high raw material costs, small production volumes, and high processing costs, particularly for purification. In 2010, the market potential of the total bioplastics market in the EU will reach 200,000-500,000 tons. The main markets are short term application for packaging and agriculture. By 2020, the bioplastics market in the EU is forecast to increase to 2-5 million tons and to expand to the textile, automotive, and agricultural sectors, including many durable applications (Chanprateep, 2010).

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials and Chemicals required

Hot air oven, Analytical weighing balance, UV –Visible spectrophotometer, Incubator shaker, Autoclave, Jaw crusher and pH meter.

Nutrient broth, Nutrient agar, DNS, Anthrone, Starch, Glucose, Hydrochloric acid, Sulphuric acid, NaOH, Chloroform, Methanol, Diethyl ether, acetone, NaCl, $(NH_4)_2SO_4$, K_2HPO_4, KCl, Mg_2SO_4, $ZnCl_2$ and H_2O_2.

3.2 Methodology

3.2.1 Isolation of bacteria from soil

Soil sample was taken from KCT campus and used for isolation of the bacteria. Around 1.0 g of sample was serially diluted in sterile distilled water and plated onto nutrient agar plates and incubated at 30˚C for 24 hours. Various colonies of different morphologies were individually picked and sub cultured on nutrient agar plates (Aarthi and Ramana, 2011). The original cultures were maintained as glycerol stock at –20°C for further use.

3.2.2 Reference strain

The reference strain for PHA production *Ralstonia eutropha* MTCC1472 was collected from the Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH) Chandigarh. All the experiments were carried out using this reference strain for comparison.

3.2.3 Rapid screening of native bacterial isolates for PHB production

All the bacterial isolates were qualitatively tested for PHA production by Nile blue staining method (Ostle and Holt, 1982). For rapid screening of PHA producers, nutrient agar medium was sterilized by autoclaving at 121°C for 20 minutes and cooled. The medium was poured into sterile Petri plates and allowed for solidification. Bacterial isolates were spread into agar plate and the plates were incubated at 30°C for 24 hours. Acetone solution of Nile blue (0.5µg/ml) was spread over the colonies and the plates kept undisturbed for 15 minutes. The fluorescent orange coloured colonies were taken as positive for PHA production and the isolates were assigned the code numbers as SP-1, SP-2, SP-5, SP-6, SP-Y1 and SP-G1.

3.2.4 Characterization of PHA producing bacterial isolates

The selected, most efficient PHA producing bacterial isolates were subjected to morphological test for the purpose of identification. The six potent PHA accumulating strains SP-1, SP-2, SP-5, SP-6, SP-Y1 and SP-G1 were examined for their gram reaction as per the standard procedures given by Anon (1957) and Barthalomew and Mittewer (1950).

3.2.4.1 Gram staining

Fresh culture was smeared on a clean glass slide and heat fixed. The smear was covered with crystal violet for 30 seconds and washed off with 95 percent ethyl alcohol. The slide was washed with distilled water and drained. Safranin was applied on smear for 30 seconds as counter stain, washed with distilled water and blot dried. The slide was observed under microscope for gram reaction.

3.2.5 Substrates for PHA production

Jambul fruits are seasonal cropping fruits, were easily available. Since after the usage of fruit the Jambul seed are wasted so, used as substrate for PHA production by the isolated organism and the reference strain. Indian jamun or *Syzgium cumini* fruits were procured from local market and dried in an oven to reduce moisture content and was milled into fine particles. The following parameters such as reducing sugar, starch and cellulose content in the raw seed were characterized.

3.2.5.1 Estimation of Reducing Sugar

Reducing Sugar was estimated by the DNS method with a standard glucose solution. To a series of standard glucose solutions, concentrations varying from 200-1000µg, 3, 5, Di Nitro Salicylic acid solution was added and kept in boiling water bath for 10 minutes. The reaction was stopped by adding 40% solution of Sodium Potassium Tartarate. The same procedure was followed to the solution containing raw seed. Absorbance was measured at 540nm and from the standard graph; the reducing sugar present in the raw seed sample was estimated (Miller, 1972).

3.2.5.2 Estimation of Starch

Starch estimated by anthrone method with a standard glucose solution and the concentrations varying from 20-100µg. Anthrone reagent solution was added and kept in boiling water bath for 10 minutes. The same procedure was followed to the solution containing raw seed. Absorbance was measured at 630nm and the converted glucose in the raw seed sample was estimated using the standard graph (Thayumanavan and Sadasivam, 1984).

3.2.5.3 Estimation of Cellulose

Cellulose was estimated using acetic/nitric reagent followed by anthrone method, with a standard cellulose solution (Updegroff, 1969).

3.2.6 Hydrolysis of seed

The method followed was based on Chen *et al.*, (1984). To about 25 g of pulverized seeds, 100ml of 10% Sodium hydroxide solution was added along with 1L of water and boiled. The residues was filtered and washed. To this 100 ml of 10% Hydrochloric acid was added along with 1L of water and boiled, again the residues was filtered and washed. This alkali-acid treatment was repeated for about 3-4 times. The washed residues was chlorinated using Sodium hypochlorite of 50 ml along with the addition of 1 L of water and kept in dark for 15minutes and this step was repeated once more. The content was filtered and washed. 250 ml of 2% Hydrogen peroxide was added to the residues and kept for 5 minutes. This step was repeated after filtering and washing. The finally converted cellulose was treated by 80% zinc chloride solution containing 4% Hydrochloric acid based on the procedure of Chen *et al.*, (1985). The solution was boiled, cooled, filtered, washed thoroughly and dried. The white mass obtained was used as the substrate for bacterial growth.

3.2.6.1 Estimation of Glucose content in hydrolyzed seed

The glucose content in the hydrolyzed seed was estimated by the DNS method with a standard glucose solution. The same procedure was followed as mentioned in section 3.2.5.1 (Miller, 1972).

3.2.7 Bacterial Growth in Defined Media

Pure culture of *Ralstonia eutropha* and the isolated culture was revived in Nutrient Broth initially and then grown in a defined Mineral Salt Media (MSM), comprised of the following; 10g Hydrolyzed Seed, 5g Glucose, 5g Sodium Chloride, 5g Di-Potassium Hydrogen Phosphate, 1g Potassium Chloride, 1g Magnesium Sulphate, and 1g Ammonium Sulphate in 1L of distilled water. The pH of the media was maintained to be 7.5 ± 0.5. The

culture flask was kept in shaker at 150 rpm at 35 °C for two days (Amirul *et al*., 2008, Du *et al*., 2001 and Yamanka *et al*., 2010).

3.2.8 Extraction of PHA

The PHA was directly extracted using the solvent chloroform. The bacterial cultures were harvested by centrifugation at 10,000 rpm for 10 min. The cell pellet was suspended in 1 ml of sodium hypochlorite solution and incubated at 37°C for 1 - 2 h for complete digestion of cell components except PHA, where by lipids and proteins were degraded. The mixture was centrifuged to collect PHA granules and the supernatant was discarded. The sediment was washed twice with 10 ml of distilled water and centrifuged. The PHA granules in the sediment were washed twice with acetone, methanol and diethyl ether (1:1:1) respectively. The polymer granule was dissolved with boiling chloroform and was evaporated by air drying, to yield dry powder of PHA (Santhanum and Sasidharan, 2010).

3.2.9 Identification of PHA granules

The bacterial cells were stained with Nile blue stain and visualized under UV trans-illuminator and that gives a bright orange fluorescence at a wavelength of 460nm. The accumulation of PHA in the form of granules would be identified from the fluorescing cells (Amirul *et al*., 2008).

3.2.10 Quantification of PHA

The polymer granule was dissolved in concentrated Sulphuric acid (1mg/ml) and heated at 100^{o}C for 10 min to convert PHB into Crotonic acid, which was brown coloured. The solution was cooled and the absorbance read at 260 nm against a concentrated Sulphuric acid as blank in a spectrophotometer. A standard curve was prepared with Pure PHB (Sigma, Alderich), concentrations ranging from 20-100µg/ml (Law and Slepecky, 1969). The quantity of PHA produced was determined in comparison with the standard.

3.2.11 Determination of Dry Cell Weight and % of PHA accumulation

The bacterial culture was centrifuged at 10,000 rpm to obtain the cell pellet and dried to estimate the Wet Cell Weight (WCW) in units of g/L (Du *et al*., 2001). Residual biomass was estimated as the difference between dry cell weight and dry weight of extracted PHA (Zakaria *et al*., 2010). This was calculated to determine the cellular weight and accumulation other than PHAs. The percentage of intracellular PHA accumulation is estimated as the percentage composition of PHA present in the dry cell weight.

Residual biomass (g/L) = WCW (g/L) – Dry weight of extracted PHA (g/L)

$$\text{PHA accumulation (\%)} = \frac{\text{Dry weight of extracted PHA (g/L)} \times 100\%}{\text{WCW (g/L)}}$$

3.2.12 Bacterial growth curve of SP-Y1

Screened organism (SP-Y1) from soil sample was grown in nutrient broth. From 0-12 hr the absorbance readings at 660nm were noted for every one hour interval, to determine it's doubling time.

3.2.13 Bacterial growth in Mineral Salt Media

SP-Y1 was grown in Mineral Salt Medium; their growth was monitored by taking absorbance at 660nm and determines the dry weight of accumulated PHA for a period of five days (Henderson *et al*., 1997).

3.2.14 Effect of different pH levels on PHA production by *Kluyvera intermedia*

The organism SPY-1 was grown at different pH (5.5, 6.5, 7.5, 8.5, and 9.5) in the production medium (MSM) incubating for 2 days utilizing glucose as carbon source.

3.2.15 Optimization of media components for PHAs production by statistical method

The optimization study for PHAs production was determined by statistical method including a Plackett-Burman design and Response Surface Methodology. Firstly, a Plackett-Burman design was used to screen the factors possessing a significant effect on PHAs production from *Kluyvera* sp. which were further optimized by Response Surface Methodology.

3.2.15.1 Identification of significant components by Plackett burman design

The most significant of the seven components in Mineral Salt Media were identified by Plackett Burman design (Stanbury *et al*., 1984). This technique allows for the evaluation of X-1 variables by X experiments. X must be multiples of 4. Any factors not assigned to a variable can be designed as a dummy variable. The incorporation of dummy variables into an experiment makes it possible to estimate the variance of an effect (experimental error).

The variables for the design were the seven media components (X-1) and the number of runs (X) was thus eight. Table 3.2.15.1.1 shows a Plackett-Burman design for seven variables (A-G) at high and low levels in which Factor D (Potassium Chloride), designed as dummy variable. Each horizontal row represents a trial and each vertical column represents the H (high) and L (low) values of one variable in all the trials. This design requires that the frequency of each level of a variable in a given column should be equal and that in each test the number of high and low variables should be equal.

The culture flasks of different high and low combinations of the media components were run in duplicate with Potassium Chloride taken as the dummy variable. The experimental flasks were kept in shaker for three days and the PHA was extracted by the same procedure (Santhanum and Sasidharan, 2010).

Table 3.2.15.1.1 Plackett Burman design for variables at different levels

Media components	Original (g/L)	High level (g/L) (H)	Low level (g/L) (L)
Hydrolyzed substrate	10	15	5
Glucose	5	7.5	2.5
NaCl	5	7.5	2.5
KCl	1	1.5	0.5
K_2HPO_4	5	7.5	2.5
$(NH_4)_2SO_4$	1	1.5	0.5
$MgSO_4$	1	1.5	0.5

The dry weight of PHA was taken as the yield factor. The yields of each trial were determined. The summation of yields corresponding to all the high (Σ (H)) and low (Σ (L)) runs of each trial were calculated.

- The difference between these two summations was calculated as,
  D = (ΣA (H) – ΣA (L))
- The effect of each variable was calculated as, Effect of $A = \frac{(\Sigma A\ (H) - \Sigma A\ (L))}{4}$
- The factor mean square of each variable was calculated with the following formula, Factor Mean Square = $\frac{(\Sigma\ (H) - \Sigma\ (L))^2}{8}$
- The error mean square was calculated from the factor mean square value of the dummy variable (Potassium Chloride).
- Finally, the f-test values were obtained by the following formula,
  f- test values = $\frac{\text{Factor Mean Square}}{\text{Error Mean Square}}$

From the data obtained, the most significant compounds of the Mineral Salt Media for bacterial growth and PHA accumulation were determined.

3.2.15.2 Optimization of significant components by Response surface methodology

For optimization of PHA production, the optimum values of the significant components obtained from Plackett Burmann Design was determined using a statistical experimental design by Response Surface Methodology using Box-Behnken design method. RSM is a successive, exploratory approach to establishing the relationship between more than one variable with the obtained responses. Microbial process could be optimized using this methodology (Sen 1997). The analysis yields a model developed by fitting the experimental data from the key points to a generalized smooth curve, from which a specific predicted optimized value of the response can be calculated. In addition to generating specific variable levels where the response is optimal, the response is expressed graphically as a contour plot, which delineates predicted responses over ranges in the design surface. Thus, a step by step approach for response surface analysis enables the relationship between the variables and the response to be established more efficiently than the traditional design (Launen *et a*l., 1999).

A Box–Behnken factorial design with three factors and three levels at the five centre point, was used for fitting a second-order response surface and the design obtained by using the Statistical software design expert version 8.0.7.1 computer package. A total of 17 runs were used to optimize the range and levels of chosen variables. The range and the levels of

the independent variables investigated using the Box–Behnken experimental designs in this study are shown in table 3.2.15.2.1. Regression analysis was performed on the data obtained from the design experiments. The Dry weight of PHA was taken as dependent variables or response represented as Y.

A quadratic polynomial regression model was assumed for predicted response. The model proposed for each response of Y was:

$Y_i = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$

Where, Y_i is the predicted response, x_1, x_2 and x_3 are the independent variables, β_0 is the offset term, β_1, β_2 and β_3 are the linear effects, β_{11}, β_{22} and β_{33} are the squared effects, β_{12}, β_{13} and β_{23} are the interaction term effects. The interaction of the selected medium components and their optimal concentrations for PHA production was obtained by solving the regression equations and by analyzing the response surface contour plots (Nikel *et al*., 2005).

Table 3.2.15.2.1 Response Surface Methodology for significant variables at different levels

Variables (g/L)	Level (g/L)		
	High	Middle	Low
Hydrolyzed substrate	20	12.50	5
$(NH_4)_2SO_4$	1	0.55	0.1
K_2HPO_4	2	1.25	0.50
Glucose	5		
KCl	0.5		
NaCl	0.5		
$MgSO_4$	0.5		

3.2.15.2.1 Model Validation and Confirmation

The objective of the validation study of the mathematical model was to demonstrate that the polynomial expression obtained could correctly predict and describe the response function. The experiments were carried out under the same conditions as the RSM and the percentage of deviation between the predicted and experimental values was studied (Carmona *et al*., 2011).

3.2.16 Fourier Transform Infra Red Spectroscopy

The extracted PHA samples were added with KBr and then evaporated. This sample was subjected to Fourier Transform Infra Red Spectroscopy, to analyze the PHA structure and purity. The peaks were observed from 4000-400 cm^{-1} (Oliveira *et al*., 2007 and Pandiyan *et al*., 2010).

3.2.17 Degradation of PHA

3.2.17.1 Extracellular degradation of PHA

For extracellular degradation, *Aspergillus* and *Penicillum* species were grown in the Mineral Salt Medium with PHB as the sole carbon source and incubated at 30°C with shaking (150 rpm). The composition of mineral salt medium was as follows: (K_2HPO_4 8g, KH_2PO_4 1g, $(NH_4)_2SO_4$ 0.5g, $MgSO_4$ 0.2 g, NaCl 0.1g, $FeSO_4.7H_2O$ 0.02, Na_2MoO_4 0.05 g, $MnSO_4$ 0.05g, $CuSO_4$ 0.05g, $ZnSO_4$ 0.05g, $CaCO_3$ 0.5g, Agar 15 in 1L of distilled water), pH 7 and incubated for about 10 days (Nishida and Tokiwa, 1993). After incubation, the culture broth was tested for the growth of fungi in the PHB emulsified mineral salt agar plates, which indicates that the fungi utilized PHA as a sole carbon source.

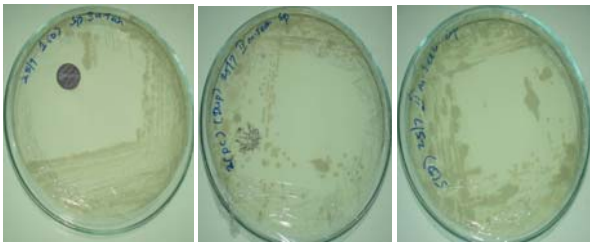
3.2.17.2 Intracellular degradation of PHA

Kluyvera intermedia were grown in the Mineral Salt Medium and the culture flask was kept in shaker at 150 rpm at 35 °C, incubated for a week for intracellular degradation of accumulated polymer. The accumulated polymers are readily degraded and utilized by the producer organisms *Kluyvera intermedia* as carbon and energy source by the action of intracellular PHA depolymerase (i-PHA depolymerase) when these cells undergo carbon-starved conditions. After three days of incubation for PHA synthesis, the culture was taken for determining its intracellular degradation of accumulated polymer. Degradation of polymers were evaluated by the decrease of polymer content of the cell mass, changes in its dry weight of extracted PHA during the course of incubation (Saha *et al*., 2007).

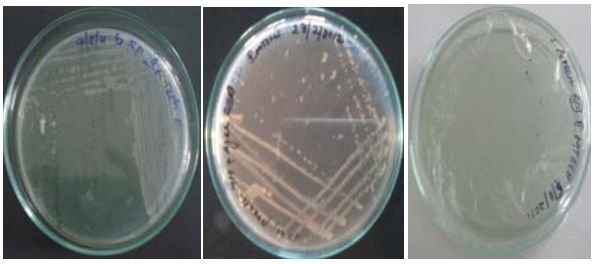
CHAPTER 4
RESULTS AND DISCUSSION

4.1 Isolation of bacteria from soil

Several bacteria were isolated from soil sample by serial dilution method. From this, nine isolates were selected for PHA production as shown in figure 4.1.1.



SP-1 SP-2 SP-5

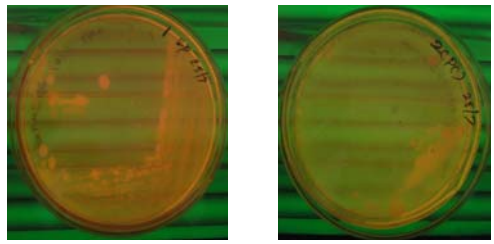


SP-6 SP-Y1 SP-G1

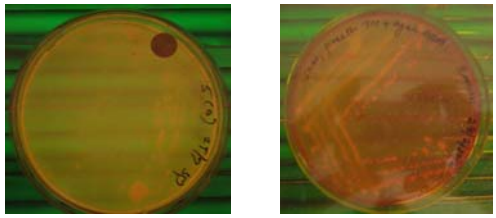
Figure 4.1.1 Isolated organisms from soil

4.2 Screening of native bacterial isolates for PHA production

Isolated bacteria were initially screened for the PHA production by Nile blue staining method (Ostle and Holt, 1982). Based on the intensity of the fluorescence were observed in the staining method, six potential PHA producers were identified out of nine isolates as shown in figure 4.2.1. The granules showed Orange fluorescence under UV trans-illuminator at a wavelength of 460nm and the results obtained was similar to Cortes *et al*., 2008.



SP-1 SP-2



SP-5 SP-Y1

Figure 4.2.1 Fluorescence of PHA granules using Nile blue staining

4.3 Characterization of PHA producing bacterial isolates

Screened bacterial strains were characterized by Gram staining as shown in table 4.3.1. All the six isolates were Gram negative and rod shaped bacteria.

Table 4.3.1 Characterization of bacterial isolates

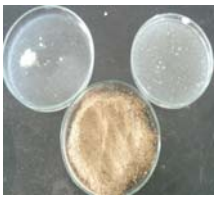
Isolated organism	Gram staining	Shape
SP1	Gram negative	Cocci
SP2	Gram negative	Small rod
SP5	Gram positive	Cocci
SP6	Gram negative	Dispersed rods
SP-Y1	Gram negative	Small rod
SP-G1	Gram positive	Small rod

4.4 Characterization of substrates

The substrate (Jambul seed) was characterized quantitatively for starch, cellulose and reducing sugars. Jambul seed contains 430µg/ml of reducing sugar, 375µg/ml of starch and 400µg/ml of cellulose was studied.

4.5 Hydrolysis of seed

The cellulose hydrolysis of seed into glucose without dergradation of glucose was done by zinc chloride method where the end product was used as substrate (Figure 4.5.1).



After hydrolysis

Before hydrolysis

Figure 4.5.1 Hydrolysis of cellulose into glucose

4.5.2 Estimation of Glucose content in hydrolyzed seed

The amount of glucose present in the hydrolysed seed was found to be 4150 µg/ml. The amount of glucose liberated from the hydrolysed seed (HS) sample was in comparison with those stated by Chen *et al*., (1984).

4.6 Selection of best isolate for PHA production

The isolates were compared with that of the reference strain *R.eutropha* for their PHA production, utilizing glucose as a sole carbon source in their growth medium. From the results obtained, it was inferred that the SP-Y1 isolate produced 0.82 g/L of PHA and was selected for further studies; PHA production was comparitively higher than other isolates as showed in figure 4.6.1.

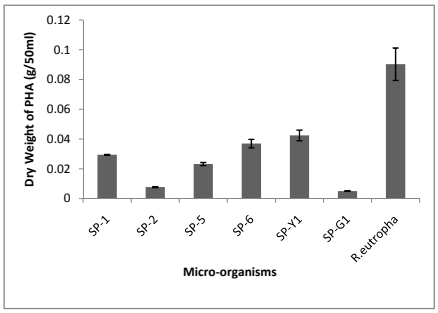


Figure 4.6.1 Comparison of PHA production among the screened isolates

4.7 Characterization and identification of best isolate SP-Y1

The selected, most efficient PHA producing bacterial isolate was subjected to a set of morphological, physiological and biochemical tests for the purpose of identification. The entire test was conducted by IMTECH, Chandigrah. From the results, it was observed that the strain SP-Y1 shares the properties of *Kluyvera* sp. (Enterobacteriaceae family). They are gram negative rods, motile, off-white pigmentation with smooth and opaque surface, catalase positive, oxidase negative and Inosital negative (Farmer *et al*., 1981).

A survival temperature ranges from 25ºC to 42ºC, pH 6-9 and salt tolerant ranges (% NaCl) from 2-8 as mentioned in table 4.7.1, 4.7.2, 4.7.3 and 4.7.4. Strain was identified as *Kluyvera* sp. and it was closely related to *Kluyvera intermedia* by IMTECH, Chandigrah (Appendix I).

4.7.1 Morphological and Biochemical Tests (IMTECH, Chandigrah)

Morphological test		**Biochemical Test**	
Tests	**Colony Morphology**	**Tests**	**Reaction**
Configuration	Circular	Methyl red	-
Margin	Entire	Voges Proskauer	+
Elevation	Raised	Casein hydrolysis	-
Surface	smooth	Citrate	+
Texture	Moist	Nitrate	+
Pigment	Off-white	Indole	-
Opacity	Opaque	Arganine dihydrolase	-
Spores	-ve	Gelatin hydrolysis	-
Motility	+ve	Starch hydrolysis	-
Gram's reaction	-	Esculin hydrolysis	+
Cell shape	Rods	Catalase test	+
		Oxidase test	-

4.7.2 Physiological and Acid production tests (IMTECH, Chandigrah)

Growth at temperature		**Growth at pH**		**Growth on NaCl (%)**		**Acid production Test**	
Temperature	**Reaction**	**pH**	**Reaction**	**NaCl (%)**	**Reaction**	**Tests**	**Reaction**
4ºC	-ve	5.0	-	2.0	+	Lactose	+
10 ºC	-ve	6.0	+	4.0	+	Maltose	+
25 ºC	+	7.0	+	6.0	+	Cellobiose	+
30 ºC	+	8.0	+	8.0	+	Raffinose	+
37 ºC	+	9.0	+	10.0	-	Inosital	-
42 ºC	+	10.0	-	11.0	-	Adonitol	-
55 ºC	-	11.0	-	12.0	-		
		12.0	-				

4.8 Bacterial growth curve of *Kluyvera intermedia*

From the figure 4.8.1, it was inferred that the doubling time changes from time to time during growth, except in the exponential phase. The doubling time of *Kluyvera intermedia* start at 7th hour intervals. For PHA production the mid log phase culture inoculum was required, for which the growth curve and doubling time were determined.

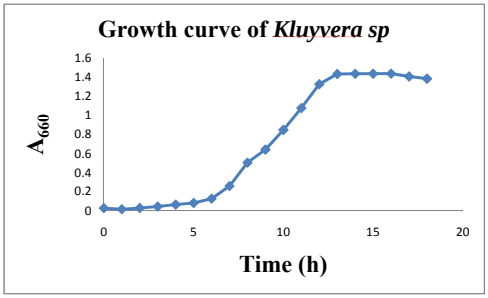


Figure 4.8.1 Growth curve of *Kluyvera intermedia*

4.9 Bacterial growth in Mineral Salt Media

It was found that the PHA accumulation was in proportion to the bacterial cell density measured at 660nm and Wet cell weight calculated. The observations on bacterial growth and PHA accumulation during a five day period of incubation are showed in table 4.9.1.

Table 4.9.1 Relationship between Bacterial growth and PHA accumulation

Number of Incubation days	**Hydrolyzed Seed as sole carbon source**		
	Absorbance at 660nm	**% PHA Accumulation**	**PHA concentration**
1	0.017 ± 0.001	10.06 ± 0.466	15.5 ± 0.71
2	0.024± 0.003	9.1 ± 2.998	22 ± 5.65
3	0.113± 0.002	11.6 ± 0.183	13 ± 0
4	0.019± 0.002	7.913 ± 1.192	70 ± 1.41
5	0.0135 ± 0.003	1.845 ± 0.452	49.5 ± 0.003

Maximal growth was observed on 3rd day of incubation and similar kind of studies was done for *Ralstonia eutropha* by Amirul *et al*., (2008), Yamanka *et al*., (2010). The studies of Henderson *et al* (1997), Du *et al* (2001) and Zakaria *et al*., (2010) have revealed similar kind of results, that the PHA accumulation is directly proportional to bacterial cell density.

4.10 pH optimization of *Kluyvera intermedia* on PHA production

For pH optimization, a level of pH was varied in the production medium by *Kluyvera intermedia*. Out of different pH levels were tested, it was observed that 8.5 pH was found to be optimum for maximum PHA production (Table 4.10.1).

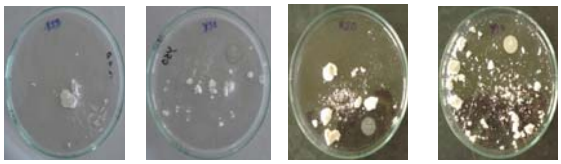
Table 4.10.1 Effect of different pH levels on PHA production by *Kluyvera intermedia*

Different pH levels	Absorbance at 660nm	% of PHA accumulation
5.5	0.434 ± 0.014	0.18 ± 0.028
6.5	1.91 ± 0.014	5.7 ± 0.14
7.5	2.08 ± 0.11	7.71 ± 0.15
8.5	2.092 ± 0.004	9.33 ± 0.16
9.5	2.038 ± 0.002	8.2 ± 0.028

4.11 Extraction and identification of PHA granules

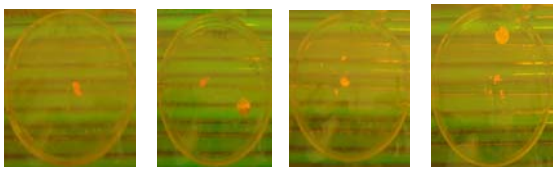
R.eutropha and the best isolate *K. intermedia* were grown in the mineral salt media containing the combination of glucose and hydrolyzed seed, and hydrolyzed seed alone for PHA production. The extracted PHA was an ivory white coloured powder was shown in figure 4.11.1. It was found to be sparingly soluble in water.

The accumulation of PHA in the form of granules would be identified from the fluorescing cells as shown in figure 4.11.2. Similar kind of studies was done earlier by Amirul *et al*., (2008) using fluorescence microscopy to visualize regions of intracellular PHA accumulation.



***R. eutropha* (G+HS)** | ***K. intermedia* (G+HS)** | ***R. eutropha* (HS)** | ***K. intermedia* (HS)**

Figure 4.11.1 Extraction of PHA granules from *R. eutropha* and *K. intermedia*



***R. eutropha* (G+HS)** | ***K. intermedia* (G+HS)** | ***R. eutropha* (HS)** | ***K. intermedia* (HS)**

Figure 4.11.2 Identification of PHA from *R. eutropha* and *K. intermedia*

G – Glucose; HS - Hydrolyzed Seed

4.12 Effect of different substrate on PHA prodcution

The extracted PHA of *K. intermedia* and *R. eutropha* was quantified. Its residual biomass, % of PHA accumulation and PHA concentration was determined. From the results it was inferred that the PHA accumulation was in proportional to the wet cell weight which was earlier stated by (Zakaria *et al*., 2010 and Du *et al*., 2001). Pure cultures of *Kluyvera intermedia* and *R. eutropha* showed greater efficiency of PHA production when utilizing hydrolyzed seed as sole carbon source. For *R. eutropha*, % of PHA accumulation and PHA mass was found to be 41.77% and 97µg/ml whereas *K. intermedia were* found to be 42.26% and 100µg/ml (Table 4.12.1). Yang *et al*., (2010) stated that comparable amount of PHA was accululated with higher carbon supplementation in the growth media.

Table 4.12.1Comparison of extracted PHA by the selected isolate and *R.eutropha*

Micro-Organism	Substrate	Dry weight of Extracted PHA (g/ml)	Wet cell weight (g/ml)	Residual Biomass (g/ml)	% PHA Accumulation	PHA mass (µg/ml)
Ralstonia eutropha	G+HS	0.003±0.01	0.104±0.34	0.1±0.01	2.38	11
	HS	0.044±0.12	0.105±0.44	0.061±0.01	41.77	97
SPY-1	G+HS	0.002±0.01	0.025±0.03	0.023±0.02	9.52	38
	HS	0.06±0.24	0.142±0.11	0.082±0.34	42.26	100

G – Glucose; HS - Hydrolyzed Seed

4.13 Optimization of media components

4.13.1 Identification of significant components by Plackett burman design

From the table 4.13.1.1, bacterial growth, dry cell weight and PHA accumulation were taken for analysis in Plackett Burman design. The dry weight of extracted PHA was taken as the Yield factor, with which the effect and the mean square were calculated for each variable. Since potassium chloride was taken as the dummy variable, the error mean square was calculated from Factor D. The f-test values were finally calculated to determine the most significant factors of the Mineral Salt Media in bacterial growth and PHA accumulation (Stanbury *et al*., 1984).

Based on f-test values, only three factors among eight investigated parameters including Hydrolyzed Substrate, K_2HPO_4 and $(NH_4)_2SO_4$ concentration showed significant effects on PHB production (Table 4.13.1.2). These parameters were further optimized through a Response Surface Methodology (Box-Behnken design method).

Similar kind of optimization studies, (Cavalheiro *et al*., 2009;Panda *et al*., 2006;Sankhla *et al*., 2005;Yang *et al*., 2010 and Zakaria *et al*., 2010) carried out earlier were suggested nearly the same factors, such as carbon, nitrogen and phosphate sources, as the most influencing components in the growth media for bacterial growth and PHA accumulation. The studies of Panda *et al*., (2006), can be of more relevance to this study, where carbon supplementation was proved to increase PHA accumulation. The impact of nitrogen and phosphate sources, as studied by Sankhla *et al*., (2005), has also brought a similar idea about bacterial growth and PHA accumulation.

Table 4.13.1.1 Observations from Plackett Burman design

	A' HS	B' G	C' NaCl	D' KCl	E' K_2HPO_4	F' $(NH_4)_2SO_4$	G' $MgSO_4$	Absorbance at 660nm	%PHA accumulation
1	H	H	H	H	H	H	H	1.106	0.164
2	H	L	L	L	H	L	H	0.801	0.169
3	L	L	H	L	L	H	H	1.372	0.049
4	L	H	L	H	L	H	H	1.069	0.137
5	H	H	H	L	H	H	L	1.103	0.182
6	H	L	L	H	L	L	L	0.089	0.042
7	L	H	H	H	H	L	L	1.699	0.214
8	L	L	L	L	L	L	L	1.413	0.121

Table 4.13.1.2 Analysis of yields from different trials of Media Design

	A' HS	B' G	C' NaCl	D' KCl	E' K_2HPO_4	F' $(NH_4)_2SO_4$	G' $MgSO_4$
Σ(H)	0.641	0.591	0.558	0.436	0.707	0.499	0.467
Σ(L)	0.253	0.303	0.336	0.458	0.187	0.395	0.427
Difference	0.388	0.288	0.222	-0.022	0.52	0.104	0.04
Factor Mean Square	0.019	0.01	$6.161X10^{-3}$	$6.05X10^{-5}$	0.034	$1.352X10^{-3}$	$2X10^{-4}$
f-Test Values	314.04	165.289	101.834	1	561.983	22.347	3.305

4.13.2 Optimization of process parameter for maximum PHA production

To validate the exact optimum values of Hydrolyzed Substrate, Ammonium Sulphate and Di-Potassium Hydrogen Phosphate as well as their interactions using statistical

design (Box–Behnken factorial design), in which the range between the optimum points from Plackett-Burman were selected. Using the Box–Behnken method, a set of 17 experiments with three factors at the five center point was conducted. The design matrix of the variables in coded units along with its corresponding PHA response was given in table 4.13.2.1 and 4.13.2.2.

Table 4.13.2.1 Box-Behnken matrix representing the effect of significant variables affecting PHA production by *Kluyvera intermedia*

Std	Run	Factor 1: Hydrolyzed Seed (g/L)	Factor 2: $(NH_4)_2SO_4$ (g/L)	Factor 3: K_2HPO_4 (g/L)	Response PHA (g/L)
4	1	20	1	1.25	1.08
16	2	12.5	1	2	0.7
17	3	12.5	1	0.5	0.9
15	4	12.5	0.55	1.25	1.07
11	5	20	0.55	0.5	1.12
10	6	20	0.1	1.25	0.88
8	7	12.5	0.55	1.25	1.07
13	8	12.5	0.55	1.25	1.07
12	9	12.5	0.55	1.25	1.07
1	10	20	0.55	2	1.38
3	11	12.5	0.55	1.25	1.07
14	12	5	0.1	1.25	0.02
2	13	5	0.55	0.5	0.04
5	14	5	0.55	2	0.042
9	15	12.5	0.1	2	0.4
7	16	5	1	1.25	0.014
6	17	12.5	0.1	0.5	0.344
7	16	5	1	1.25	0.014
6	17	12.5	0.1	0.5	0.344

Table 4.13.2.2 Design Summary

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio
Y1	PHA	g/L	17	Polynomial	0.014	1.38	0.722	0.471	98.6

4.13.2.1 Model Fitting: ANOVA, Regression and Prediction Equations

For predicting the optimal point, a second-order polynomial function was fitted to correlate the relationship between independent variables and response represented by the amount of PHA produced. The second-order polynomial equation was given below, where Y is Dry weight of PHA (g/l), A is Hydrolyzed Substrate (w/v), B is Ammonium Sulphate (w/v) and C is Di-Potassium Hydrogen Phosphate (w/v).

$$PHA(g/l) = -1.51677+0.16345\ A+2.05179\ B+0.72952\ C+0.015259\ AB+0.011467\ AC-0.18963\ BC-4.55111E\text{-}003\ A^2-1.55802\ B^2-0.29956\ C^2 \longrightarrow \text{Equation-1}$$

The media component in the form of Carbon source (Hydrolyzed substrate), nitrogen source (Ammonium Sulphate) and phosphorus source (Di-Potassium Hydrogen Phosphate) when given at a higher level had a significant effect on PHA production, as evident from equation 1.

The goodness of fit of the model was checked by the determination coefficient (R^2). The R^2 value was always between 0 and 1 and the closer the R^2 was to 1.0, the stronger the model and the better it predicted the response (Haaland, 1989). The coefficient of determination (R^2) was calculated to be 0.9702 from the quadratic model, indicating that the model could explain 97% of the variability in the production of PHA (table 4.13.2.1.1). The values presented above are higher than those of experimental second-order equations reported by Nikel *et al*., (2005), who assessed the influence of industrial byproducts as a carbon source for the production of PHB by *E. coli*. In those experiments, the reported R^2 value was 0.957 for the PHB production model.

Table 4.13.2.1.1 Model Summary Statistics

Source	Std. Dev.	R-Squared
Linear	0.284469555	0.703691153
2FI	0.317547486	0.715981233
Quadratic	0.12284892	**0.970244284**
Cubic	0	1

The statistical significance of the model equation was evaluated by the F-test for analysis of variance (ANOVA), which showed that the regression is statistically significant at Prob > F" less than 0.05 confidence level. Values of "Prob > F" less than 0.05 indicate the model terms are significant (table 4.13.2.1.2). The model is highly significant, as is evident from the model F-value and a very low probability value (P-value 0.0002).

The lack of fit measures the failure of the model to represent data in the experimental domain at points which are not included in the regression. The lack of fit of the regression model was not significant, which is good and indicates that the model equation was adequate for predicting the PHA production under any combination of values of the variables.

Table 4.13.2.1.2 Analysis of variance (ANOVA) for the regression model of PHB production obtained from the experimental results.

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	3.444700059	9	0.382744451	25.36099085	**0.0002**
A-HS	2.358792	1	2.358792	156.2956751	**< 0.0001**
B- $(NH_4)_2SO_4$	0.1378125	1	0.1378125	9.131579944	**0.0193**
C-K2HPO4	0.0017405	1	0.0017405	0.115327092	0.7441
AB	0.010609	1	0.010609	0.702961862	0.4295
AC	0.016641	1	0.016641	1.102647596	0.3286
BC	0.016384	1	0.016384	1.085618545	0.3321
A^2	0.275941053	1	0.275941053	18.28410182	**0.0037**
B^2	0.419116842	1	0.419116842	27.77105814	**0.0012**
C^2	0.119546316	1	0.119546316	7.921246183	**0.0260**
Residual	0.105643	7	0.015091857		
Lack of Fit	0.105643	3	0.035214333		
Pure Error	0	4	0		
Cor Total	3.550343059	16			

The P-values are used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The low values of P of less than 0.05 indicate the more significant correlation of coefficients. The smaller the magnitude of the P, the more significant is the corresponding coefficient. It was observed that the coefficients of the quadratic terms of HS and $(NH_4)_2SO_4$ concentration (P = <0.0001, 0.0193, respectively) for PHA production was highly significant.

4.13.2.2 Model Diagnostics

Before accepting any model, the adequacy of the model should be checked using an appropriate statistical method (Lee and Guilmore, 2005). Experimental and calculated analyses of PHA production were the diagnosis method chosen in this study to test the model's assumptions. In Figure 4.13.2.2.1, the data for the real responses are plotted against the predicted responses. Points above or below the diagonal lines of correlation coefficient R2 represent areas where the prediction has been overestimated or underestimated. Since the plot is very close to $y = x$ ($R^2 = 0.9702$) as shown in figure 1 and all the points are very close to the diagonal lines. Therefore, it could be summarized that the models adequately explain the experimental range studied and the significance of the regression coefficients was determined by Student's t test (table 4.13.2.2.1).

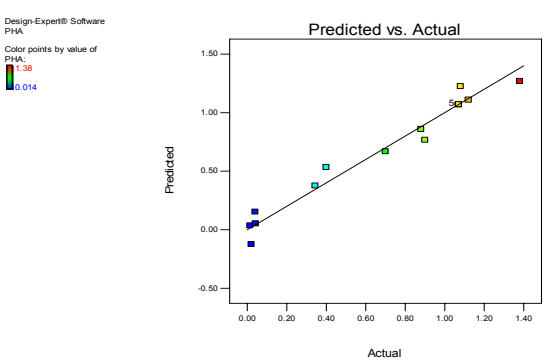


Table 4.13.2.2.1 Diagnostics Case Statistics on PHA production

Standard Order	Actual Value	Predicted Value	t Test	Run order
1	1.38	1.26775	1.827448	10
2	0.04	0.15225	-1.82745	13
3	1.07	1.07	0	11
4	1.08	1.22425	-2.34841	1
5	0.042	0.05275	-0.17501	14
6	0.344	0.376	-0.52097	17
7	0.014	0.03525	-0.34595	16
8	1.07	1.07	0	7
9	0.4	0.5335	-2.1734	15
10	0.88	0.85875	0.345953	6
11	1.12	1.10925	0.175012	5
12	1.07	1.07	0	9
13	1.07	1.07	0	8
14	0.02	-0.1243	2.348413	12
15	1.07	1.07	0	4
16	0.7	0.668	0.520965	2
17	0.9	0.7665	2.173401	3

Figure 4.13.2.2.1 Predicted Vs Actual values on PHA production

4.13.2.3 Optimization and Response Surface Graphs; the Effect of Variables

The contour plot described by the regression model was drawn to illustrate the combined effects of each independent variable upon the response variable. PHA production for different concentrations of the components such as HS, $(NH_4)_2SO_4$ and K_2HPO_4 can be predicted from these plots. The maximum predicted production of PHA was indicated by the surface confined in the response surface diagram.

Figure 4.13.2.3.1 showed a plot at varying substrate (HS) and $(NH_4)_2SO_4$ concentrations at fixed value for K_2HPO_4. It was observed that PHA production increased as the concentration of HS increases, since HS as a carbon source for PHA production. PHA production increases when $(NH_4)_2SO_4$ concentration increased up to a specific point, but then it began to decrease as the concentration of $(NH_4)_2SO_4$ increases. Moreover, the highest C/N ratio (30:1) was also observed in this treatment which in turn affected the cell growth and PHB production (Luengo *et al*., 2003 and Reddy *et al*., 2003).

Figure 4.13.2.3.2 showed a plot at varying substrate (HS) and K_2HPO_4 concentrations at fixed value for $(NH_4)_2SO_4$. Higher PHA production was found at high level of substrate (HS) concentration and increase in K_2HPO_4 concentration up to certain point and further increases in K_2HPO_4 concentration decreases PHA production. Similar kind of studies on PHA production was reported by Carmona *et al*., (2011). At the optimum level of nitrate, the highest concentration of the carbon source favoured PHA production. Under these conditions, the maximum predicted PHA was obtained at minimum phosphate concentration level.

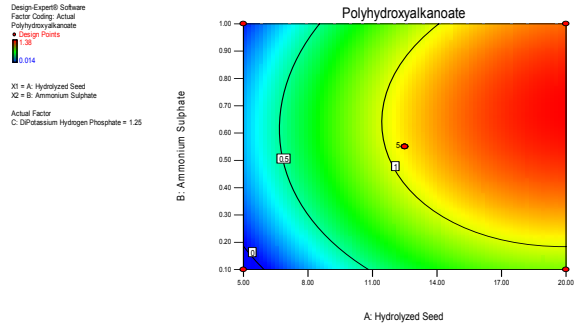


Figure 4.13.2.3.1 Influence of HS and $(NH_4)_2SO_4$ on PHA production keeping K_2HPO_4at its optimum level

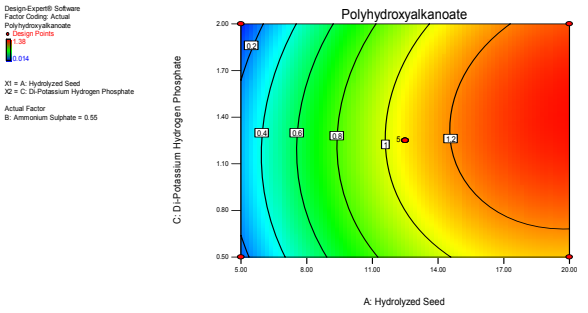


Figure 4.13.2.3.2 Influence of HS and K_2HPO_4 on PHA production keeping $(NH_4)_2SO_4$ at its optimum level

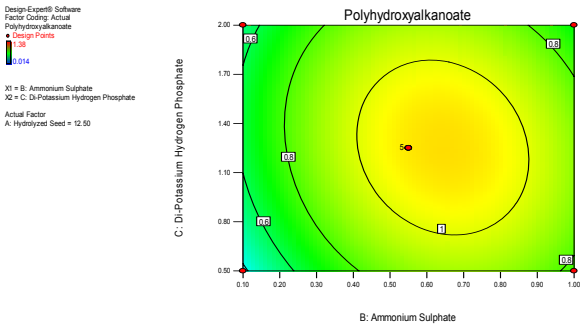


Figure 4.13.2.3.3 Influence of $(NH_4)_2SO_4$ and K_2HPO_4 on PHA production keeping HS at its optimum level

Figure 4.13.2.3.3 showed a plot at varying $(NH_4)_2SO_4$ and K_2HPO_4 concentrations at fixed value for substrate (HS). PHA production increases with increase in $(NH_4)_2SO_4$ and K_2HPO_4 concentrations up to a certain level PHA production rate decreases. The PHA concentration reached a peak value at the mid-value of $(NH_4)_2SO_4$ and K_2HPO_4 concentrations. Boonsawang and Wongsuvan, (2008), also reported that PHA production was favoured at low phosphate and nitrogen concentrations.

With the optimized medium the production of PHA obtained was 1.38 g/L in the following ranges of the tested variables: HS concentration = 20g/L; $(NH_4)_2SO_4$ = 0.55g/L; K_2HPO_4 = 2g/L. Glucose, Sodium Chloride, Potassium Chloride and Magnesium Sulphate are less significant parameters for PHA production than the other medium components, however the presence of minimum amounts are necessary for its production. The results collectively showed that PHB production by *K. intermedia* was about 0.15-folds increased when cultivated in the optimal medium developed by BBD, as compared to MSM. Therefore, the statistical experimental design proved to be a powerful and useful tool for enhancing PHB production and confirm the necessity of the optimization process.

It can be concluded that the higher amount of PHA produced with excess of carbon source (HS) under nutrient limiting conditions of $(NH_4)_2SO_4$ and K_2HPO_4 concentrations. Otherwise excess nutrient was diverted towards biomass build up but decreased PHB accumulation (Lakshmar *et al*., 2004; Sangkharak and Prasertsan, 2007). Therefore, in order to increase PHA production, a cheap alternative carbon source should be used.

4.13.2.4 Model Validation and Confirmation

PHA production yielded an average maximum concentration of 1.528 g/L PHA compared to a predicted response, using the concentrations specified by RSM for the PHA model, of 1.38 g PHA/L. Based on the results of RSM studies, the concentration of PHA obtained 1.528 g/L was found to be higher than the results shown by Fernandez *et al*., (2005), in which the maximum PHA obtained was found to be from 0.03 to 0.10 PHA g/L by presence of phosphate (3.00 g/L) and with the excess of carbon source (80.00 g/L). From the results showed that the predicted and experimental values are not significantly different and with 9.6 % deviation which indicating that this model is effective.

4.14 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectrum of the extracted PHA sample was compared with that of the Standard Polyhydroxy-3-Butyric acid. From the FTIR spectra obtained, the band observed in sample found between the spectral range (3570-3200cm^{-1}),(1055-100cm^{-1}/1005-925 cm^{-1}) corresponds to H-X stretch region, Methylene (>CH_2) respectively (Coates, 2000).

According to the findings of Coates, (2000) and Sharma *et al*., (2011), the peak found at 1642.43 in the sample corresponds to C=O stretch and according to Sharma *et al*., (2011), the peak found in the sample at 632.55 corresponds to presence of alkene C-H bend. Based on the results of Olivera *et al*., (2007), a series of intense bands located at 1000–1300 cm^{-1} correspond to the stretching of the C–O bond of the ester group. The peak found at 1110.4 in sample was found exactly at the peak range of standard PHA (Figure 4.14.1 and 4.14.2).

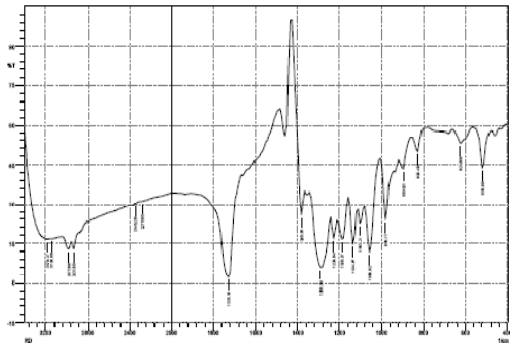


Figure 4.14.1 FTIR spectra of Standard Polyhydroxy-3-Butyric acid

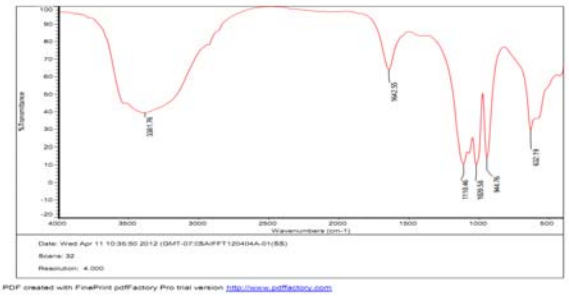


Figure 4.14.2 FTIR spectra of PHA sample

4.15 Degradation of PHA

4.15.1 Extracellular degradation

After incubation, the water insoluble PHA was converted into water soluble PHA, which indicates that the growth of *Aspergillus* and *Penicillum* species utilizing PHA as the sole carbon source in the Mineral Salt Media (Figure 4.15.1.1a and 4.15.1.2). On PHA containing agar media, the growth of PHA degrading microorganisms (*Aspergillus* and *Penicillum*) was observed on the petri plate (Figure 4.16.1.1b). Similar kind of degradation activity was detected by the formation of a clear zone below and around the fungal colony of *Cephalosporium* sp. and *Aspergillus fumigates*. These two genera are known to grow on the surface of the assay medium and show clear zones within a week (Brandl *et al*., 1995; Lee *et al*., 2005). Lodhi *et al*., (2011) identified poly (3- hydroxybutyrate) (PHB) degrading *Aspergillus fumigatus* and optimization of culture conditions for the maximum production of PHB depolymerase.

Before incubation **After incubation** **Plate assay**

Figure 4.15.1.1 a) Liquid media for extracellular degradation by *Penicillum* species
b) Plate assay for extracellular degradation by fungi

Before incubation **After incubation**

Figure 4.15.1.2 Extracellular degradation by *Aspergillus* species

4.15.2 Intracellular degradation

During starvation period, the accumulated polymers were readily degraded and utilized by the producer organism as carbon and energy source by the action of intracellular PHA depolymerises. The dry weight of the extracted PHA was reduced for a period of days by depolymerisation reaction (Table 4.15.2.1). Yoon and Choi (1999) described the magnitudes of degradation of two different copolymers, Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) and Poly(3-hydroxybutyrate-co4 hydroxybutyrate) accumulated in *Hydrogenophaga pseudoflava* and determined the relative substrate specificity of its intracellular PHA depolymerase as in the order 3HB > 3HV > 4HB. Likewise, different substrate specific and structurally dissimilar intracellular PHA depolymerases have been reported from *Pseudomonas oleovorans* for its short chain length and medium chain length PHAs (Stuart *et al*., 1996; Foster *et al*., 1996).

Table 4.15.2.1 Changes in the Dry weight of PHA during the course of their intracellular degradation

Number of days	Hydrolyzed seed as sole carbon source	
	Wet cell weight (g/50ml)	Dry weight of extracted PHA(g/50ml)
1	0.815 ± 0.004	0.082 ± 0.004
2	1.5075 ± 0.034	0.172 ± 0.002
3	1.978 ± 0.07	0.2245 ± 0.01
4	1.12 ± 0.144	0.112 ± 0.007
5	0.9935 ± 0.106	0.036 ± 0.001
6	0.805 ± 0.009	0.021 ± 0.009
7	0.431 ± 0.021	0.0125 ± 0.003

CHAPTER 5
CONCLUSION

The success in the biodegradable plastic strategy largely depends on the isolation of potent PHA producing bacteria and optimizing culture parameters for maximum PHA biosynthesis. Dry weight of PHA was higher in *Kluyvera intermedia*, compared to other isolates and thus used for the entire experimental studies. It was inferred that the jambul seed can be utilized as a carbon source in the Mineral Salt Media, for the screened bacteria to grow and accumulate PHA in the production medium when it was compared with the Reference strain (*Ralstonia eutropha*). The results showed that the % of PHA accumulation was found to be 41.7% in *Ralstonia eutropha* and 42.2% in SPY-1. RSM proved to be a powerful and useful tool for enhancing PHB production by *K. intermedia*, and after the optimization studies a final yield of 1.528g/l was obtained. Biodegradation of extracted PHA by fungal species (*Aspergillus* and *Penicillum*) was studied by observing its turbidity reduction before and after incubation. The degradation capability of accumulated bacteria (*K. intermedia*) was observed by reducing in their dry weight of PHA.

From the results, it was concluded that *Kluyvera intermedia* identified as the best PHA producing organism, when jambul seed (*syzygium cumini*) used as cheap carbon source in the production medium.

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