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PRODUCTION OF POLYHYDROXYALKANOATES FROM *Ralstonia eutropha* USING STRAW AS CHEAP SUBSTRATE

A PROJECT REPORT

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BONAFIDE CERTIFICATE

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ABSTRACT

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Polyhydroxyalkanoates (PHAs) are polyesters of natural origin accumulated in form of intracellular granules by a wide variety of bacterial strains. Polyhydroxyalkanoates have attracted much attention as candidates for biodegradable polymers, as they possess material properties similar to the common petrochemical based synthetic thermoplastics and elastomers currently in use. The only disadvantage found with this biopolymer is the high cost of production. Many cheap carbon sources for industrial production of PHA have been identified of which paddy straw is the least attempted one. This project was carried out with an aim to utilize Straw (Lignocellulosic substrate) as a cheap carbon source for PHA production from the predominant PHA producer *Ralstonia eutropha*. The straw was hydrolysed by non enzymatic chemical treatments and the hydrolysed straw obtained was used as the major carbon source in the Mineral Salt Media for bacterial growth and PHA production. On the 4^{th} day of bacterial growth, the PHA was extracted by solvent extraction method using chloroform. The extracted PHA was assayed for its Polyhydroxybutyrate (PHB) content in comparison with the standard Polyhydroxy-3-butyric acid spectrophotometrically at 235 nm. The effect of each component of the Mineral Salt Media upon bacterial growth and PHA accumulation were analysed. In addition to that, the effect of adding other components like Yeast extract and Sodium di-hydrogen phosphate in the Mineral Salt Media, upon bacterial growth and PHA accumulation were also analysed. The most significant components of the Mineral Salt Media were determined by the method of Plackett Burman Design. The extracted PHA was analysed for its structural properties and purity, through Fourier Transform Infra Red Spectroscopy, in comparison with the standard Polyhydroxy-3-butyric acid. The thermal properties of the extracted PHA were found from Thermogravimetric Analysis and Differential Scanning Calorimetry. From these analytical procedures it was confirmed the a multicomponent Polyhydroxyalkanoate was produced, which contained Polyhydroxybutyrate in significant amount. As a DCW of 19.2 g/L and PHA accumulation of 37.55% was achieved, it was proved that the paddy straw can be efficiently used as cheap carbon source for industrial production of the Polyhydroxyalkanoates.

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Ac-CoA- Acetyl Co-enzyme A

Ac-AcCoA- Acetoacetyl-coenzymeA

$CaCl_2.2H_2O$ – Calcium Chloride

$CoSO_4.7H_2O$- Cobalt Sulphate

$CuSO_4.5H_2O$ – Copper Sulphate

DCW- Dry Cell Weight

DO- Dissolved Oxygen

DSC- Differential Scanning Calorimetry

EDTA- Ethylene Diamine Tetra Ammonium

(EMIM)Cl- Ethyl Methyl Imidazolium Chloride

$FeCl_3.6H_2O$ – Ferric Chloride

$FeSO_4.7H_2O$ – Ferrous Sulphate

FTIR- Fourier Transform Infrared Spectroscopy

G- Glucose

HBCoA- Hydroxybutyryl-CoA

H_2SO_4- Sulphuric Acid

H_3BO_3 – Boric Acid

HCl- Hydrochloric Acid

HS- Hydrolysed Straw

KBr- Potassium Bromide

KCl- Potassium Chloride

K_2HPO_4- Di Potassium hydrogen Phosphate

$MgSO_4$ – Magnesium Sulphate Culture Collection

$MnCl_2.4H_2O$ – Manganese Chloride

MTCC- Microbial Type Culture Collection

MSM- Mineral Salt Media

Na_2CO_3 – Sodium Carbonate

NaCl- Sodium Chloride

NAD(P)H- Nicotinamide Adenine Di-Phosphate Hydrogen

Na_2MoO_4 Sodium Molybdate

$NaNO_3$- Sodium Nitrate

Na_2HPO_4- Di Sodium Hydrogen Phosphate

$NiCl_2 \cdot 6H_2O$- Nickel Chloride

$(NH_4)_2SO_4$ – Ammonium Sulphate

NMR- Nuclear Magnetic Resonance Spectroscopy

OD- Optical Density

PHA- Polyhydroxyalkanoate

PHB/ P3HB – Polyhydroxybutyrate

PHBV/[P(3HB-co-3HV)] - Polyhydroxybutyrate co valerate

T_c – Crystallisation Temperature

T_g - Glass Transition Temperature

T_m – Melting Temperature

TCA- Tri carboxylic acid cycle

TGA- Thermogravimetric Analysis

YE- Yeast Extract

$ZnCl_2$- Zinc Chloride

ZnSO4.7H20- Zinc Sulphate

INTRODUCTION

CHAPTER 1

INTRODUCTION

Polyhydroxyalkanoates (PHAs) 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), when Lemoigne isolated and characterized it from bacterial strain *Bacillus megaterium* in 1926. Microorganisms usually synthesize and accumulate PHA as the carbon, energy and reducing power storage material, under conditions of the nutrient limitation (such as nitrogen, phosphorus or iron limitation) and in presence of excess of carbon source. Under the conditions of carbon source starvation, PHA are degraded by intracellular depolymerases and subsequently metabolized (Ojumu *et. al.*, 2004).

Polyhydroxyalkanoates have attracted much attention as candidates for biodegradable polymers, because they possess material properties similar to the common petrochemical-based synthetic thermoplastics and elastomers currently in use. They can be completely degraded to carbon dioxide and water (and methane under anaerobic conditions) by micro-organisms in the environment (Du *et. al.*, 2001). The properties of pure Polyhydroxybutyrate (PHB), including thermoplastic processability, hydrophobicity, complete biodegradability and biocompatibility with optical purity suggest that PHB could be an attractive alternative to the common plastics (Sharma *et. al.*, 2005).

Ralstonia eutropha was found to achieve maximum accumulation of PHA, especially Polyhydroxybutyrate (Wu *et. al.*, 2001). PHA production from Cyanobacterium has also been extensively studied (Wu *et. al.*, 2001 and Sharma *et. al.*, 2005). It has been stated that *Synechocystis sp.* matches *Ralstonia eutropha* in PHA accumulation levels (Kumar *et. al.*, 2001). Poly-3-Hydroxybutyrate-co-3-Hydroxyvalerate, [P(3HB-co-3HV)], also called as PHBV, has proved to be an another significant biopolymer accumulated by microbes (Amirul *et.al.*, 2008 and Choi *et.al.*, 2005). Poly-3-hydroxyvalerate incorporation in the copolymer was obtained when Propionic and Valeric acid were used as precursors (Zakaria *et. al.*, 2010). *Bacillus invocatus* MTCC 9039 has emerged as an interesting organism for [P(3HB-co-3HV)] co-polymer accumulation (Sankla *et. al.*, 2010). The synthetic pathway for PHB accumulation in bacteria can be briefly stated as the condensation of two Acetyl-

Coenzyme A molecules, followed by reduction to 3-Hydroxybutyryl-Coenzyme A and finally apolymerization to yield 3-Hydroxybutyrate (Penloglou *et. al.*, 2005). The Synthetic Pathway of [P(3HB-co-3HV)] has also been shown to be similar to that of PHB (Choi *et. al.*, 2005). The concentrations of acetyl-CoA and free CoA are the key factors, regulating the activity of b-ketothiolase which catalyze the first step of polymer synthesis in addition to the high intracellular concentrations of NADPH and high ratios of NADPH/$NADP^+$ (Du *et. al.*, 2001).

The large scale industrial production of Polyhydroxyalkanoates is still under slow pace due to the need of high investment. The high cost of production of Polyhydroxyalkanoates makes it a costly endeavour to be done in large scale although it proves to be an environment friendly bioplastic. This brings out the need for an immediate identification and utilization of cheap and renewable carbon sources for industrial production of PHA. Direct carbon sources such as Glucose and Sucrose have been widely used in PHA production along with Acetate, Butyrate, Propionate and Valerate as precursors for different types of the PHA to be produced, (PHB, PHBV etc.). Use of Xylose (Tsuge *et. al.*, 2001), Pentoses (Bertrand *et. al.*, 1990) as carbon substrates for PHA production were well studied. Utilization of cheap and renewable carbon sources such as Palm oil mill effluent (Wu *et. al.*, 2009 and Zakaria *et. al.*, 2010), Waste glycerol (Cavaheiro *et. al.*, 2009), Cassava starch hydrolysate (Aremu *et. al.*, 2010), Waste Activated Sludge (Jiang *et. al.*, 2009) etc. were extensively studied.

Utilization of Lignocellulosic substrates 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)Cl was used. The method adopted by Jenkins (1930) for cellulose determination in straws yielded almost pure form of cellulose which can then be subjected to hydrolysis as described in patented methods (Chen *et. al.*, 1984; Chen *et. al.*, 1987 and Nagle, 1987), where Zinc Chloride was mostly used. Zinc chloride was proved to be efficient in cellulose degradation (Amarasekara *et. al.*, 2009).

This project was carried out with an aim to utilize Straw (Lignocellulosic substrate) as a cheap carbon substrate for PHA production from *Ralstonia eutropha*. Pure culture of *Ralstonia eutropha* MTCC 1472 was purchased from IMTECH, Chandigarh. A non-enzymatic, chemical method of straw 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 for *Ralstonia eutropha*. The growth media comprised of salts apart from the hydrolysed straw and hence can be termed as Mineral Salt Media (MSM). Maximal bacterial growth was achieved after 72-96 hours and hence intracellularly accumulated PHA was extracted at the end of 4th day using chloroform (Santhanum *et. al.*, 2010). The dry weight of the extracted powder was termed as the 'Total Multicomponent PHA' obtained from the culture. This was assayed with standard Polyhydroxybutyrate (PHB), spectrophotometrically at 235 nm, by the method of Law and Slepecky, (1960) to determine the PHB Mass (g/L) and PHB content (%). The Bacterial Cell Density, Dry Cell Weight (g/L), Residual Biomass (g/L), PHA accumulation (%) and carbon consumption were the other important parameters measured. The effects of each of the seven components of Mineral Salt Media; additional compounds in MSM such as Yeast extract, Sodium di-hydrogen phosphate; and the amount of hydrolysed straw in MSM were studied. The most significant components of the Mineral Salt Media influencing bacterial growth and PHA accumulation were identified by Plackett Burman Design (Stanbury *et. al.*, 1984).

The PHA produced was characterized by analytical procedures such as. Fourier Transform Infrared Spectroscopy (FTIR) to show the functional groups, structural properties and purity, in comparison with standard Polyhydroxybutyrate (PHB). Further Thermogravimetric Analysis (TGA) was done to determine Thermal decomposition characteristics and Differential Scanning Calorimetry (DSC) to estimate Temperatures of Glass Transition T_g, Crystallisation T_c and Melting T_m (Oliveira *et. al.*, 2007).

In comparison with earlier studies, it was concluded that a significant bacterial growth and PHA accumulation could be achieved by utilizing straw as the carbon source for bacterial growth. The different parameters of PHA production, observed on using straw were near to those observed on supplementation with pure glucose. Though lesser, significant values have been obtained for DCW and PHA accumulation (%).

1.1 Objective

- To produce Polyhydroxyalkanoates from *Ralstonia eutropha* using straw as a cheap carbon source.
- To hydrolyse straw by various chemical treatments and use it as the carbon source to grow *Ralstonia eutropha* in a Mineral Salt Media.
- To extract the accumulated PHA using chloroform from the bacterial cells.
- To assay the extracted PHA for its Polyhydroxybutyrate (PHB) content in comparison with standard Polyhydroxy-3-butyric acid, spectrophotometrically at 235 nm.
- To analyse the effect of each component of the Mineral Salt Media upon bacterial growth and PHA accumulation.
- To analyse the effect of addition of other components like Yeast extract and Sodium di-hydrogen phosphate in the Mineral Salt Media upon bacterial growth and PHA accumulation.
- To identify the most significant components of the Mineral Salt Media for bacterial growth and PHA accumulation by the method of Plackett Burman Design.
- To analyse the extracted PHA for its structural properties and purity, through Fourier Transform Infra Red Spectroscopy, in comparison with standard Polyhydroxy-3-butyric acid.
- To analyse the thermal properties of the extracted PHA by Thermogravimetric Analysis and Differential Scanning Calorimetry.

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

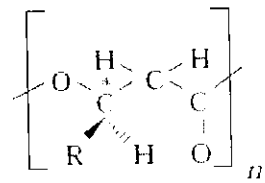
Polyhydroxyalkanoates (PHAs), the naturally accumulated biopolymers were extensively studied for their structural, chemical and mechanical properties; biosynthetic pathway and its regulation; carbon sources for large scale industrial production, analytical procedures and applications. A review of these studies follows.

2.1. Properties of Polyhydroxyalkanoates

2.1.1. Structural and Chemical Properties

PHAs are polyesters of hydroxyalkanoic acids. PHAs can be classified according to the number of carbon atoms in monomer unit as short-chain lengths (SCL) PHA consisting of 3-5 carbon atoms and medium-chain length (MCL) PHA containing 6-14 carbon atoms per monomer unit. More than 100 monomers have been found in the naturally synthesized polyester polymers. The monomer units in these microbial polyesters are all in the *R*-configuration because of the stereospecificity of the biosynthetic enzymes. Among these are 3-hydroxy- (rarely 4-6 hydroxy-) acids of 3-14 carbon atoms with saturated, unsaturated, straight or branched aliphatic side chains. Furthermore, monomers with various functional groups in the chain, such as halogen, hydroxy-, epoxy-, cyano-, carboxyl- and esterified carboxyl groups, have been discovered in MCL-PHA (Obruca, 2010).

Figure 2.1.1. General Structure of Polyhydroxyalkanoate (PHA)



$R = CH_3\text{-}C_2H_5$ Short-chain-length PHAs
$R = C_3H_7\text{-}C_{14}H_{29}$ Medium-chain-length PHAs

2.1.2. Mechanical Properties

Mechanical properties of individual PHA strongly depend on the monomer unit composition. The PHB homopolymer is a completely stereoregular polyester, with all asymmetric carbon atoms in the *R*-configuration, and it is, therefore, highly crystalline. The glass transition temperature (T_g) of PHB lies between 5 and 9° C, and the melting point (T_m) lies between 173 and 180° C. PHB polymer is decomposed at approximately 200° C, which is close to its melting temperature. Mechanical properties such as the Young's modulus (3.5 GPa) and the tensile strength (43 MPa) of PHB material are close to those of isotactic polypropylene. However, the extensions to break (5 %) for PHB is significantly lower than that of polypropylene (400 %). Therefore, PHB is stiffer and more brittle plastic material as compared to polypropylene. The incorporation of other monomer units into PHA structure can markedly improve its mechanical properties. MCL-PHA has significantly lower melting point temperature as compared to SCL-PHA. (Obruca, 2010)

2.1.3. Intracellular PHA granules

PHAs are 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 granules 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 97.7 % polyester, 1.87 % proteins and 0.46 % lipids or phospholipids (Obruca, 2010). The fine structure of PHB granules have been well shown by Jensen *et. al.*, (1971).

2.2. Biosynthesis of Polyhydroxyalkanoates

2.2.1. Synthetic Pathway

The Synthetic Pathway of PHB production by *Alcaligenes eutrophus* and *Alcaligenes latus* using fructose as carbon source has been studied extensively and a kinetic model has been developed by Penloglou *et. al.*, (2010). The carbon source is initially converted into acetyl-coenzyme (AcCoA) through the Entner–Doudoroff pathway. In the first reaction, two AcCoA molecules are condensed by the catalytic action of the enzyme 3-ketothiolase (phaA)

to form one molecule of acetoacetyl-coenzymeA (Ac-AcCoA). Subsequently, Ac-AcCoA is reduced by acetoacetyl-CoA reductase (phaB) to 3-hydroxybutyryl-CoA (3-HBCoA) at the expense of NADH. In the third enzymatic reaction, the monomer unit 3-HBCoA is polymerized into PHB following apolymerization mechanism catalyzed by synthase (phaC). Finally, under the action of depolymerase (phaZ) the accumulated PHB is hydrolyzed into 3-hydroxybutyrate (3-HB). Subsequently 3-hydroxybutyrate is converted to AcCoA which is utilized as carbon and energy source under carbon starvation conditions. Similar studies in *Bacillus sp.* are done by Tay *et. al.*, (2010).

The Synthetic Pathway of [P (3HB-co-3HV)] has also been shown to be similar to that of PHB (Choi *et. al.*, 2005). The P(3HB-co-3HV) biosynthetic pathway from glucose is likely that succinate, an intermediate of the TCA cycle, is abstracted and then converted to propionyl-CoA *via* methylmalonyl-CoA (Pathway I) by reactions catalyzed by methylmalonyl-CoA mutase and methylmalonyl-CoA decarboxylase or *via* methylmalonyl-CoA (Pathway II) by reactions catalyzed by methylmalonyl-CoA and oxaloacetate transcarboxylase. There may be another pathway for the synthesis of 3HV monomer, where threonine is metabolized to propionyl-CoA *via* branched-chain amino acid metabolism. The resulting propionyl-CoA is incorporated into P(3HB-co-3HV) by a three-step reaction catalyzed by the enzyme system of the PHB-biosynthetic pathway.

2.2.2. Regulation of PHA Biosynthesis

The concentrations of acetyl-CoA and free CoA are the key factors, regulating activity of b-ketothiolase which catalyze the first step of polymer synthesis. In addition, it has been shown that PHB biosynthesis is strongly stimulated by both high intracellular concentrations of NADPH and high ratios NADPH/$NADP^+$. Furthermore, citrate synthase and citrate dehydrogenase are significantly inhibited by coenzymes NADPH, which consequently enhances flux of acetyl-CoA into PHB biosynthetic pathway instead of TCA cycle (Du *et. al.*, 2001). It is proposed that the rate of PHA biosynthesis is controlled by b-ketothiolase and acetoacetyl-CoA reductase, whereas the content of PHB is controlled by PHA synthase. In the cyanobacterium *Synechococcus* sp. MA19, it has been found that PHA synthase is post-translationally activated by acetylphosphatase. Furthermore, the second enzyme that is involved, phosphotransacetylase, converting acetyl-CoA to acetyl phosphate is

regulated by the acetyl-CoA concentration as well as by the carbon/nitrogen ratio. Therefore, acetyl phosphate could be the signal of carbon/nitrogen balance affecting PHA metabolism (Nishioka *et. al.*, 2001).

2.3. Trends in Production of Polyhydroxyalkanoates

2.3.1. Direct Carbon sources

Glucose is considered to be the most essential source for PHA accumulation whereas Butyrate, Acetate and Valerate are considered to be essential for large scale production of PHB and [P(3HB-co-3HV)]. These are used in direct manufactured forms which are stated to be expensive, impeding commercial production. Utilization of Xylose as the carbon source for PHA production can be referred to Tsuge *et.al.* (2001). *Pseudomonas pseudoflava* is found to produce PHA from pentoses quite efficiently (Bertrand *et.al.*, 1990). There are only less reports of PHA production from pentoses or hemicelluloses, which if achieved would represent a tremendous savings in terms of substrate costs.

2.3.2. Inexpensive waste substrates

Utilization of cellulose and lignocellulosic substrates as carbon sources require an economical method to obtain reducing sugars from those complex saccharides. Zinc chloride was proved to be efficient in cellulose degradation (Amarasekara *et. al.*, 2009). Hydrolysis of lignocelluloses to obtain simple reducing sugars, especially glucose has been well explained by Sun *et. al.*, (2002) which emphasizes the importance of pretreatment of lignocellulosic substrates before enzymatic hydrolysis. The enzymatic conversion can be enhanced by using non- ionic surfactants and PolyethyleneGlycol (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)Cl was used. The method adopted by Jenkins (1930) for cellulose determination in straws yields almost pure form of cellulose which can then be subjected to hydrolysis as described in patented methods (Chen *et. al.*, 1984; Chen *et. al.*, 1987; Nagle, 1987). Renewable and cheaper raw materials, such as Palm oil mill effluent (Zakaria *et. al.*, 2010; Wu *et. al.*, 2009), Waste glycerol (Cavalheiro *et. al.*, 2009), Cassava starch hydrolysate (Aremu *et. al.*, 2010), Starch (Chen *et. al.*, 2006) have been used as nutrient supplements for bacterial PHA production as substitutes to synthetic

carbon sources. PHA production is also well investigated using Waste-Activated Sludge (Jiang *et. al.*, 2009) and other inexpensive substrates (Jiang *et. al.*, 2008 and Kulpreecha *et. al.*, 2009).

2.3.3. Microorganisms producing Polyhydroxyalkanoates

From the whole list of references stated, the following is inferred. Most bacterial and a few cyanobacterial (algae) species are proved to accumulate PHA under different growth conditions. Among Cyanobacteria, *Chloroglea fritschii, Gleocapsa PCC 6501, Microcystis aeruginosa, Trichodesmium thiebautti, Oscillatoria limosa, Gloeothece sp. PCC 6909, Synechocystis sp. PCC 6803, Synechococcus MA19, Nostoc muscorum* are known to produce PHA. In bacteria *Ralstonia eutropha* was known for highest PHA accumulation, besides which, *Comamonas sp. EB172, Agrobacterium sp., Corynebacterium glutamicum, Pseudomonas putida, Pseudomonas oleovorans, Bacillus megaterium, Azotobacter vinelandii UWD*, are also noteworthy PHA producers.

2.4. Industrial Production of Polyhydroxyalkanoates by Fermentation

2.4.1. Microbial Growth Medium

The growth of Cyanobacteria was carried out in BG-11 media, which consisted of Citric acid, Ferric citrate, EDTA, Na_2CO_3, $MgSO_4.7H_20$, $CaCl_2.2H_2O$, K_2HPO_4, $MnCl_2.4H_2O$, Na_2MoO_4, H_3BO_3, $CuSO_4.5H_2O$, $CoSO_4.7H_2O$, and ZnSO4.7H20. Nitrogen or Phosphorous starved cultures were obtained without the addition of $NaNO_3$ or K_2HPO_4. The pH of the medium was maintained to be 8.0-8.5 (Panda *et. al.*, 2006; Mallick *et. al.*, 2007 and Sharma *et. al.*, 2005). The bacterial cultures were normally grown in Nutrient agar or broth and then transferred into Mineral Salt Media (MSM) for PHA production. The composition of the MSM was $MgSO_4.7H_2O$, KH_2PO_4, $(NH_4)_2SO_4$, Na_2HPO_4, $CaCl_2.2H_20$, Yeast extract and trace elements which include $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$, H_3BO_3, $FeCl_3.6H_2O$, $CoCl_2.6H_2O$, $MnCl_2.4H_2O$, $Na_2MoO_4.2H_20$, $NiCl_2{\cdot}6H_2O$, $CuSO_4.5H_20$ and Thiourea. The pH of the medium was maintained to be 7.0 (Amirul *et. al.*, 2008; Du *et. al.*, 2001 and Yamanka *et. al.*, 2010). The Mineral Salt Media was modified and used in various studies to produce PHA from different organisms.

2.4.2. One stage and Two stage Cultivation

In a single stage cultivation method, the microbial culture was grown in the prescribed media in a shake flask or fermentor, from which the cells were harvested to obtain PHA (Choi *et. al.*, 2010). A two-stage continuous culture system was another method in which cells were grown substantially in the first-stage under balanced growth conditions and then transferred into a second-stage characterized by nitrogen limiting conditions, where PHA was synthesized. This Two stage culture system was found to be more efficient for PHA accumulation in bacteria than single stage method. (Amirul *et. al.*, 2008; Du *et. al.*, 2001; Kim *et. al.*, 1997 and Yeo *et. al.*, 2008).

2.4.3. Aerobic Dynamic Feeding

Aerobic dynamic feeding in a sequential batch reactor (Lemos *et. al.*, 2008) was one of the widely used methods for PHA production. It referred to alternate feast and famine cycles. Each SBR cycle consisted of a 15 min feeding, 2 h aerobiosis, 1.5 h settling and 15 min decanting of 1L of fermentation solution, which was replaced with 1L of fresh culture medium during the feeding time. The total cycle duration was 4 h. Oxygen was supplied with an air pump at an air flow rate of 1 vvm and the dissolved oxygen (DO) was around 6 mgL^{-1} and this was maintained to be 80% of the saturation value. The reactor temperature was maintained at 21-22°C and pH was maintained to be 7.0 with stirring rate of 250 rpm.

2.4.4. Solid State Fermentation

PHA production was done by Solid State Fermentation using soy cake, sugarcane molasses, using *Ralstonia eutropha*, where these solid substrates were added with the nutrient broth and moistened. The culture vessels were maintained 30 °C with humidified air for 36 h (Oliveira *et. al.*, 2007). This approach was only less attempted for PHA production.

2.4.5. Optimal growth conditions

PHA accumulation was attributed to certain limited growth conditions. The feeding of Ammonium chloride was analysed by Yeo *et. al.*, (2008) and it was found that, the

Polyhydroxybutyrate (PHB) content, the rate of PHB production and the rate of carbon substrate utilization during growth on glucose were maximum, at low dilution rate under ammonia limitation. This was also depicted as, Ammonia limitation > Potassium/Oxygen limitation > Glucose limitation. (Du *et. al.*, 2001 and Henderson *et. al.*, 1997). In another view by Savenkova *et. al.*, (1999), Nitrogen limitation did not promote PHB production whereas Oxygen limitation did. Also Phosphate limited conditions were proved to be essential for PHA accumulation (Nishioka *et. al.*, 2001). PHA production was higher in media with Ammonium sulphate as nitrogen source, whereas media with Yeast extract showed lower yield of PHA. Ammonium sulphate being simple nitrogen source was readily available than complex nitrogen source like Yeast extract (Joshi *et. al.*, 2010). To achieve a high cell density of *Ralstonia eutropha* using organic acids as carbon sources, the inhibitory effect of organic acids on cell growth must be reduced by maintaining their concentration in the culture medium at a low level (Tsuge *et. al.*, 2001). In fed-batch culture, a low ratio of propionic acid to glucose (P/G) led to high dry cell weight (DCW), P(3HB-co-3HV) content and productivity, but low HV unit fraction. On the other hand, a high P/G ratio led to, high HV unit fraction but the low P(3HB-co-3HV) content and productivity. The specific P(3HB-co-3HV) synthetic rate and the specific HV synthetic rate would decline in fed-batch cultures, which deteriorated with high P/G feeding due to the inhibitory effect of propionic acid accumulated in the culture broth (Du *et. al.*, 2001). Acidic pHs were not found suitable for PHB accumulation, so was the high alkaline pH. (Panda *et. al.*, 2006). To increase the proportion of 3HV in the resulting PHA, cells were grown with more propionate and higher initial pH (Yang *et. al.*, 2010).

2.4.6. Extraction of Polyhydroxyalkanoates

The PHA was directly extracted by the principle of solvent extraction using chloroform. The bacterial cultures were harvested by centrifugation; lipids and protein were removed from the cell pellet-using sodium hypochlorite and PHA was obtained in dry powdered form using methanol and chloroform. The PHA was precipitated from the debris with 7:3 (v/v) mixtures of methanol and water. The precipitated PHA was then washed with acetone and dried. Choloroform was added and evaporated by air drying, yielding powdered PHA (Santhanum *et. al.*, 2010). In case of cyanobacteria, the culture was suspended in methanol at 4°C (overnight) to remove pigments, centrifuged and dried. The polymer was

extracted in hot chloroform followed by precipitation with cold diethyl ether, centrifuged, washed with acetone and dissolved in hot chloroform (Sharma *et. al.*, 2005). The purity of PHA obtained by these methods was about 99% (Henderson *et. al.*, 1997).

2.5. Analytical Procedures

2.5.1. Identification of Intracellular PHA granules

Intracellular PHA granules in the bacterial cells were stained by the Oxazine dye, Nile Blue and observed under fluorescence microscopy, where fluorescent emission by the cells showed accumulation of PHA as intracellular granules (Amirul *et. al.*, 2008). The cells were also stained using Sudan Black B (Santhanum *et. al.*, 2010). In *Synechocystis sp.*, cells were fixed in glutaraldehyde, osmium tetroxide, methanolic uranyl acetate and lead citrate and then observed in an H-600 transmission electron microscope for PHA granules (Wu *et. al.*, 2001).

2.5.2. Assay of PHA- PHB Mass and PHB Content (%)

After extraction, the chloroform in the polymer was removed evaporatively, concentrated H_2SO_4 was added, and the solution was kept in a boiling water bath for 10 min. This reaction of H_2SO_4 with PHA produced crotonic acid. After cooling and thorough mixing, the absorbance of the solution was measured spectrophotometrically at 235 nm against H_2SO_4 blank (Law and Slepecky, 1960). Standard Polyhydroxybutyric acid (PHB) was assayed to estimate PHB Mass and PHB Content (%) in the extracted PHA sample.

2.5.3. Bacterial Cell Density and Dry Cell Weight

The optical density readings were usually taken at 660 nm. In some cases, the cell density was derived from optical density measurements of the culture at 450 nm. The cell density (mg/ml) was calculated using experimentally determined conversion factors, OD450=1: *Ralstonia eutropha* 0.175 g/l as per Ganzeveld *et. al.*, (1999). The dry cell weight (DCW) was determined by collecting the microbial culture, centrifuging, washing and then drying to constant weight at 80° C (Du *et. al.*, 2001).

2.5.4. Residual biomass and PHA accumulation (%)

The extent of bacterial cell accumulation with PHA and other components was determined from the parameters, PHA accumulation (%) and Residual biomass respectively. The percentage of Dry weight of extracted PHA (g/L) in the Dry cell weight (g/L) was defined as the PHA accumulation (%). The Residual biomass was defined as the mass difference between the Dry cell weight (g/L) and Dry weight of extracted PHA (g/L) as studied by Zakaria *et. al.*, (2010).

2.5.5. Gas Chromatography

PHA was also quantified by another method, where the extracted PHA was subjected to propanolysis or methanolysis in the prescence of sulphuric acid and the resulting hydroxymethyl esters were analysed using Gas chromatography (Zakaria *et. al.*, 2010). The PHB mass and content could be determined by this method in comparison with that of the standard Polyhydroxybutyric acid.

2.5.6. Gel Permeation Chromatography

The molecular weight of the sample was determined using Gel Permeation/ Size exclusion Chromatography with chloroform as eluent at 40° C. The sample was dissolved in chloroform and the solution was filtered through a membrane filter with 0.45mm pore size (Dennis *et. al.*, 1998; Oliveira *et. al.*, 2007 and Zakaria *et. al.*, 2010).

2.5.7. X-Ray Crystallography

The crystalline structures of the PHA sample were studied using a X-ray diffractometer, employing powder method which provides CuKa radiation (40 kV, 40 mA), employing the powder method. Every scan was recorded in the range of $2\theta = 5–70°$ in the step-by-step mode of 0.05° (Oliveira *et. al.*, 2007).

2.5.8. Nuclear Magnetic Resonance

The crystalline structures of the PHA sample were also studied using a NMR spectrum, using deuterated chloroform as solvent. Nuclear magnetic resonance spectra (^{13}C / ^{1}H) of samples were recorded at 75.4 MHz or 100.62 MHz (Jiang *et. al.*, 2008 and Oliveira *et. al.*, 2007).

2.5.9. Fourier Transform Infra Red Spectroscopy

To analyse the PHA structure and purity, the samples dissolved in chloroform were 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} (Oliveira *et. al.*, 2007).

2.5.10. Thermogravimetric Analysis

The thermal analysis of the extracted PHA was done in a typical TGA system with a blank aluminium pan as reference. The pan was set in TG and heated at heating rates of 10° C/min in the range of 60-400 °C under a steady flow of nitrogen (100 mL/min). The thermal decomposition of the biopolymer was well studied with a TGA curve (Zakaria *et. al.*, 2010).

2.5.11. Differential Scanning Calorimetry

Differential Scanning Calorimetry was performed to determine the thermal properties of the PHA. 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 (T_g), melting temperature (T_m), and crystallization temperature on heating (T_{hc}) were obtained. The crystallization temperature on cooling (T_{cc}) was obtained from the second cooling (Oliveira *et. al.*, 2007).

2.6. Applications of Polyhydroxyalkanoates

A Wide range of impressive PHA applications were listed (Obruca, 2010).

- PHA latex can be used to cover paper or cardboard to make water-resistant surfaces.

- Owing to their piezoelectric nature, the following can be made from PHAs; Pressure sensors for keyboards, stretch and acceleration measuring instruments, material testing, shock wave sensors, gas lighters; Acoustics: microphone, ultrasonic detectors, sound pressure measuring instruments; Oscillators: headphones, loudspeakers, for ultrasonic therapy and Atomization of liquids.

- The gas barrier properties of PHAs are useful for applications in food packaging and for making plastic beverage bottles.

- PHB or other copolymers can be used to make the non-woven cover stock and the plastic film moisture barriers in nappies and sanitary towels along with some special paramedical film applications in hospitals.

- PHAs have been used much in the medical field, to develop devices including sutures, nerve repair devices, repair patches, slings, cardiovascular patches, orthopedic pins, adhesion barriers, stents, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, bone-marrow scaffolds, tissue engineered cardiovascular devices and wound dressings.

- PHAs have been used as mulch films for agricultural purposes. Insecticides were integrated into PHA pellets and sown along with the farmer's crops. The insecticide would be released at a rate related to the level of pest activity since the bacteria breaking down the polymer would be affected by the same environmental conditions as that of the soil pests.

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1. Hydrolysis of Straw

The hydrolysis of straw was carried out by the methods derived from and Chen *et. al.*, 1984; Chen *et. al.*, 1987, Jenkins, 1930 and Nagle, 1987. Paddy straw was obtained, dried and coarsely pulverised. To about 25 g of pulverised straw, 100 mL of 10% Sodium hydroxide solution was added along with 1L of water and boiled; the residue was filtered and washed. To this 100 mL of 10% Hydrochloric acid was added along with 1L of water and boiled; again the residue was filtered and washed. This alkali- acid treatment was repeated for about 3-4 times. The washed residue was chlorinated using sodium hypochlorite - 50 mL along with the addition of 1L of water. It was kept in dark for 15 minutes and this step was repeated once more. The content was filtered and washed. To this residue 250 mL of 2% Hydrogen peroxide solution was added and kept for 5 minutes. This step was repeated after filtering and washing. The residue obtained was treated with 80% Zinc chloride solution containing 4% Hydrochloric acid. The solution was boiled, cooled, filtered, washed thoroughly and dried. The white mass obtained at last was used as the substrate for bacterial growth.

3.2. Bacterial Growth in Defined media

Pure culture of *Ralstonia eutropha* MTCC 1472 was purchased from IMTECH, Chandigarh, in freeze dried form. The culture was revived in Nutrient Broth initially and then grown in a defined mineral salt media (MSM), comprised of the following; 10 g Hydrolysed straw, 5 g Glucose, 5 g Sodium Chloride, 5 g Di-Potassium hydrogen phosphate,1 g Potassium chloride, 1 g Magnesium sulphate and 1 g Ammonium Sulphate in 1L of distilled water. The pH of the media was maintained to be 7.5 ± 0.5. The culture flasks were kept in shaker at 150 rpm at 35° C for four days. (Amirul *et. al.*, 2008, Du *et. al.*, 2001 and Yamanka *et. al.*, 2010).

3.3. Extraction of PHA

The extraction of accumulated PHA was carried out under the principle of chloroform extraction. The bacterial culture was centrifuged at 6000 rpm to obtain the cell pellets. The cells were treated with sodium hypochlorite for 2 h whereby lipids and proteins were degraded. The cell debris was washed with 1:1 (v/v) mixture of acetone: methanol and finally treated with chloroform. It was evaporated by air drying, to yield dry powder of PHA (Santhanum *et. al.*, 2010).

3.4. Analytical procedures

3.4.1. Estimation of Glucose content in hydrolysed straw

The glucose content was estimated by the DNS assay with a standard glucose solution. To a series of standard glucose solutions, concentrations varying from 200-1000 µg, 3,5,Di NitroSalicylic 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 hydrolysed straw. Absorbance was measured at 540nm and from the standard graph, the glucose content in the hydrolysed straw sample was estimated.

3.4.2. Identification of Intracellular PHA granules

The bacterial cells were stained with Nile blue stain and visualized under UV trans illuminator or fluorecscence microscope for the emission fluorescence by the bacterial cells. The accumulation of PHA in the form of intracellular granules, would be identified from the fluorescing cells (Amirul *et. al.*, 2008).

3.4.3. Bacterial Cell Density, Dry Cell Weight and Residual Biomass

Absorbance measurement of the culture broth at 660nm was done to determine the bacterial cell density. The bacterial culture was centrifuged at 6000 rpm to obtain the cell pellet. The cell pellet was dried to estimate the dry cell weight (DCW) in units of g/l. (Du *et. al.*, 2001). Residual Biomass was estimated as the difference between dry cell weight

and dry weight of PHA extracted (Zakaria *et. al.*, 2010). This was calculated to determine the cellular weight and accumulations other than PHAs.

Residual Biomass (g/L) = DCW (g/L) - Dry weight of Extracted PHA (g/L)

3.4.4. Assay of PHA - PHB Mass, PHB Content (%) and PHA Accumulation (%)

PHA was assayed spectrophotometrically by the method of Law and Slepecky, (1960) in comparison with the Standard Polyhydroxybutyric acid, purchased from Sigma Aldrich. The standard PHB was treated with concentrated sulphuric acid, boiled for 10 min and cooled. Absorbance measurement at 235 nm yielded a standard graph from which the PHB mass in the extracted PHA sample was determined. The intracellular PHA accumulation (%) is estimated as the percentage composition of PHA present in the dry cell weight. The PHB Content (%) is estimated as the percentage composition of PHB Mass in the dry weight of extracted PHA (Zakaria *et. al.*, 2010).

$$PHA\ Accumulation\ (\%) = \frac{Dry\ weight\ of\ PHA\ extracted\ (g/L)}{DCW(g/L)} * 100\%$$

PHB Mass (g/L) = Obtained from Standard PHB Assay

$$PHB\ Content\ (\%) = \frac{PHB\ Mass\ (g/L)}{Dry\ weight\ of\ Extracted\ PHA\ (g/L)} * 100\%$$

3.4.5. Effect of Different Components of Mineral Salt Media upon Bacterial Growth and PHA accumulation

3.4.5.1. Effect of each component of Mineral Salt Media upon Bacterial Growth and PHA accumulation

The effect of each of the seven media components were analysed by growing the bacteria in a series of culture flasks, each devoid of a single component. They were labelled as B`-H`. A control flask (A`) with all the media components was grown with the test flasks

in duplicate. The culture flasks were kept in shaker at 150 RPM at 35° C for four days and PHA was extracted by the same procedure (Santhanum *et. al.*, 2010).

3.4.5.2. Effect of other components added to Mineral Salt Media upon Bacterial Growth and PHA accumulation

The effects of presence/absence of Yeast extract and a second phosphate source (Sodium di-hydrogen phosphate) added to the Mineral Salt Media were also analysed.

A set of culture flasks were incubated with and without Yeast extract (0.1%) in the Mineral Salt Media. The culture flasks were kept in shaker at 150 RPM at 35° C for four days and PHA was extracted by the same method explained (Santhanum *et. al.*, 2010).

Similarly another set of culture flasks were incubated with and without (0.15%) Sodium di-hydrogen phosphate in the Mineral Salt Media and The culture flasks were kept in shaker at 150 RPM at 35° C for four days and PHA was extracted by the same method explained (Santhanum *et. al.*, 2010).

3.4.5.3. Effect of Hydrolysed straw Content in Mineral Salt Media upon Bacterial Growth and PHA accumulation

The significance of amount of hydrolysed straw in the growth media was also studied by using varying amounts of hydrolysed straw. The straw content was varied as 0.5, 1, 1.5, 2.0, 2.5, 3.0 g in the Mineral Salt Media. The culture flasks were kept in shaker at 150 RPM at 35° C for four days and PHA was extracted by the same procedure (Santhanum *et. al.*, 2010).

3.4.5.4. Identification of Significant components of Mineral Salt Media by Plackett Burman Design

The most significant off the seven components in Mineral Salt Media were identified by Plackett Burman Design (Stanbury *et. al.*, 1984). The variables for the design were the seven media components (X-1) and the number of runs (X) was thus eight. Hence eight

experimental flasks of different high and low combinations of the media components were run in duplicate with Potassium Chloride taken as the dummy variable. The culture flasks were kept in shaker at 150 rpm at 35° C for four days and the PHA was extracted by the same procedure (Santhanum *et. al.*, 2010).

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 ($\sum A\ (H)$) and low ($\sum A\ (L)$) runs of each trail were calculated.

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

$$f\text{-}test\ value = \frac{Factor\ Mean\ Square}{Error\ Mean\ Square}$$

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

3.4.6. Fourier Transform InfraRed Spectroscopy

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

3.4.7. Thermogravimetric Analysis

The thermal decomposition properties of the extracted PHA sample were studied using a Thermogravimeter (PerkinElmer Thermal Analyser) at a ramp of 20° C/min from 50-850° C. The weight % curve was analysed for different stages of decomposition such as drying, sublimation etc. (Zakaria *et. al.*, 2010).

3.4.8. Differential Scanning Calorimetry

The thermal properties were determined using a Differential Scanning Calorimeter (Model: DSC Q200 V24.4 Build 116) by the method of Ramp - 10° C/min in a nitrogen atmosphere. The glass transition temperature (T_g), crystallisation temperature (T_c) and the melting temperatures (T_m) were determined from the DSC curve obtained (Oliveira *et. al.*, 2007).

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Estimation of Glucose content in the hydrolysed straw

The standard glucose calibration curve was obtained by performing DNS assay. Hydrolysed straw sample was also subjected to the assay to estimate the glucose content in it. From the standard calibration curve, it was inferred that the optical density reading of 0.015 g of hydrolysed straw sample corresponded to 2280 µg/mL of glucose. Thus the glucose content can be stated as 15.2% in the hydrolysed straw sample. The absorbance at 540 nm of Standard glucose and the hydrolysed straw sample are given in Tables 4.1.1. and 4.1.2. Figure 4.1.1 shows the Standard glucose calibration curve. The straw before and after hydrolysis are shown in Figures 4.1.2 and 4.1.3. The amounts of glucose liberated from the hydrolysed straw sample were in comparison with those stated by Chen *et. al.*, (1984).

Table 4.1.1: Standard Glucose Calibration – Absorbance at 540 nm

S.No	Concentration of Glucose, µg/mL	Absorbance at 540nm
1	0	0.00
2	200	0.02
3	400	0.04
4	600	0.06
5	800	0.075
6	1000	0.09
7	1200	0.12
8	1400	0.14
9	1600	0.165
10	1800	0.18
11	2000	0.2

Table: 4.1.2: Hydrolysed straw sample – Absorbance at 540 nm

S.No	Weight of the Hydrolysed straw sample, g/mL	Absorbance at 540 nm
1	0.015	0.23

Figure 4.1.1: Standard Glucose Calibration Curve

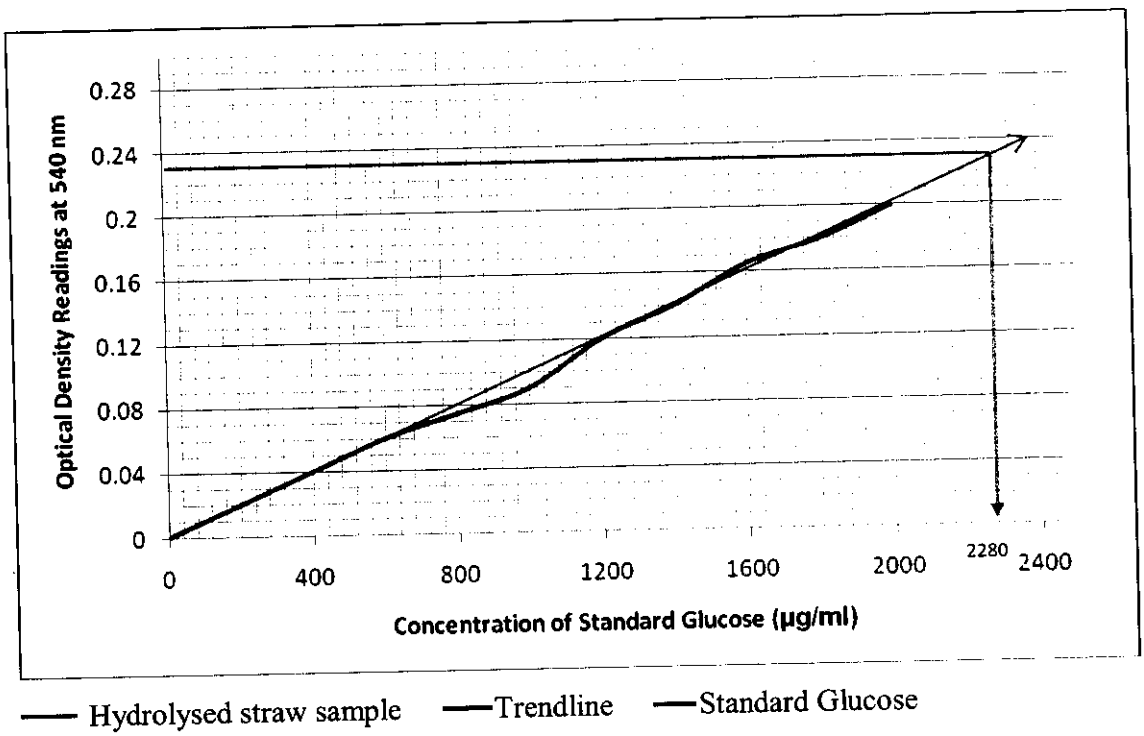


Figure 4.1.2: Straw before hydrolysis

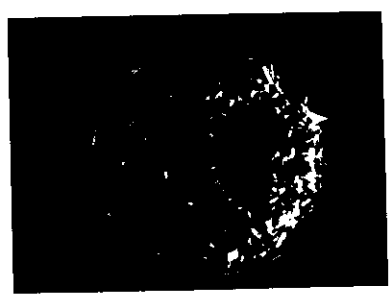


Figure 4.1.3: Straw after hydrolysis

4.2. Identification of Intracellular PHA granules

The cell culture treated with Nile blue stain was visualized under a UV transilluminator, which showed regions of fluorescence. The fluorescence confirmed the accumulation of Polyhydroxyalkanoates in the form of intracellular granules. This is depicted in Figure 4.2.1 Similar kind of studies were done earlier (Amirul *et. al.*, 2008) using fluorescence microscopy to visualize regions of intracellular PHA accumulation.

Figure 4.2.1: Nile Blue staining showing intracellular PHA granules

4.3. Bacterial Growth Characteristics

4.3.1. Growth in Simple Media

The growth of *Ralstonia eutropha* in different media was examined by measuring the absorbance at 660 nm. Bacterial growth in simple media with three different compositions was determined from the readings as tabulated (Table 4.3.1.1). It was inferred that the growth was high in the simple media containing hydrolysed straw in combination with glucose.

Growth was quite lesser in the simple media with just hydrolysed straw and much lesser in the media containing only glucose. Maximal growth was observed on the 4th day of incubation after which the growth phase started declining.

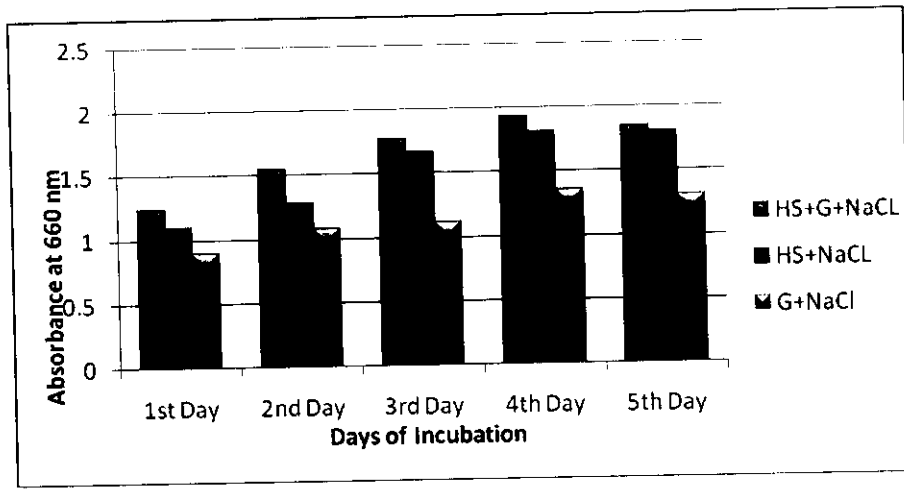
Table 4.3.1.1: Observations of Bacterial Growth in a Simple Media with three different compositions

S.No	Media Composition	No. of days of incubation	Absorbance at 660 nm
1	HS + G + NaCl	1	1.245
2	HS + G + NaCl	2	1.543
3	HS + G + NaCl	3	1.765
4	HS + G + NaCl	4	1.935
5	HS + G + NaCl	5	1.844
6	HS + NaCl	1	1.097
7	HS + NaCl	2	1.278
8	HS + NaCl	3	1.659
9	HS + NaCl	4	1.804
10	HS + NaCl	5	1.799
11	G + NaCl	1	0.897
12	G + NaCl	2	1.076
13	G + NaCl	3	1.121
14	G + NaCl	4	1.356
15	G + NaCl	5	1.309

G- Glucose HS- Hydrolysed Straw

The data presented above is depicted in a bar chart (Figure 4.3.1.1), which clearly shows that growth was higher in the media containing hydrolysed straw than the one containing only glucose. Thus it can be inferred that the carbon content of the hydrolysed straw was utilized for bacterial growth.

Figure 4.3.1.1: Bar chart showing Bacterial Growth in a Simple Media with three different compositions



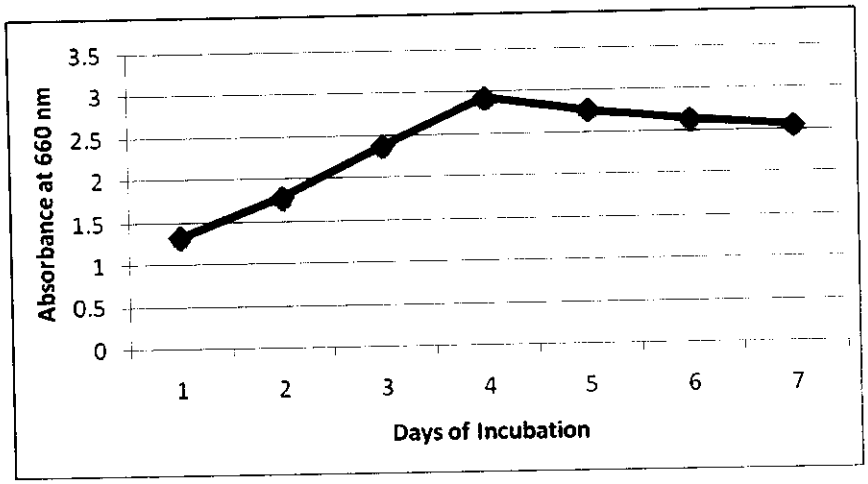
4.3.2. Growth in Mineral Salt Media

The growth of *Ralstonia eutropha* in the Mineral Salt Media was determined from the following absorbance readings at 660 nm as tabulated (Table 4.3.2.1). The growth was monitored for a period of seven days. Maximal growth was observed on 4th day of incubation, similar to the growth in simple media. Similar kind of growth characteristics were found by earlier studies, that the bacterial culture was at its log phase in 72-96 h (Amirul *et. al.*, 2008, Du *et. al.*, 2001 and Yamanka *et. al.*, 2010),

Table 4.3.2.1: Observations of Bacterial Growth in Mineral Salt Media

S.No.	No. of Days of incubation	Absorbance at 660nm
1	1	1.328
2	2	1.784
3	3	2.386
4	4	2.934
5	5	2.765
6	6	2.643
7	7	2.558

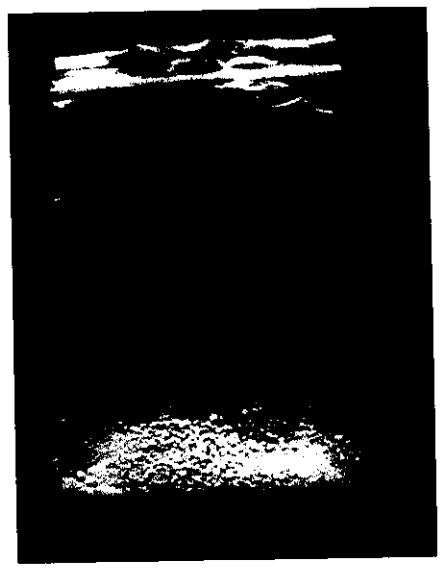
Figure 4.3.2.1: Bacterial Growth Curve in Mineral Salt Media



4.4. Extraction of PHA

The extracted PHA was an ivory white coloured powder. It was found to be sparingly soluble in water. The PHA was readily soluble in hot concentrated sulphuric acid, forming Crotonic acid, revealed by the formation of yellowish orange coloured solution. The dry weight of PHA extracted and its PHB content are quantified and tabulated as in Table 4.5.4.1. The extracted PHA is shown in Figure 4.4.1

Figure 4.4.1. Extracted PHA powder



4.5. Quantification of Polyhydroxy-3-Butyrate (PHB)

4.5.1. Assay of Standard Polyhydroxy-3-Butyric acid

The Standard Polyhydroxy-3-Butyric acid (PHB) calibration curve was obtained from the absorbance values at 235 nm of the sulphuric acid assay as tabulated in Table 4.5.1.1. The PHB content in the extracted sample was estimated (Table 4.5.1.2) from the calibration curve shown in Figure 4.5.1.1. It was inferred that the PHB Mass was 720 µg in 1000 µg of the extracted PHA sample. Thus PHB Mass was estimated to be 5.19 g/L. The standard curve obtained was similar to that observed by Law and Slepecky, (1960).

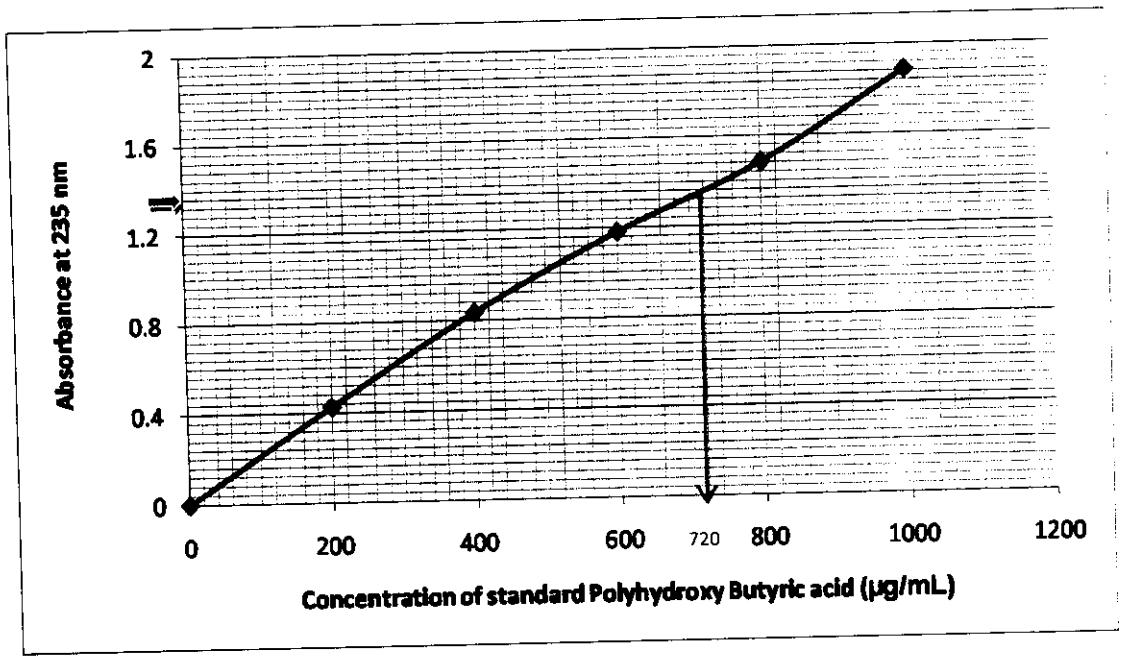
Table 4.5.1.1: Standard Polyhydroxy-3-Butyric acid Calibration-Absorbance at 235 nm

S.No.	Concentration of Standard Polyhydroxy-3-Butyric acid, µg/mL	Absorbance at 235 nm
1	0	0.00
2	200	0.426
3	400	0.834
4	600	1.18
5	800	1.48
6	1000	1.884

Table: 4.5.1.2: Estimation of PHB Mass in Extracted PHA sample

S.No.	Weight of Extracted PHA sample µg/mL	Absorbance at 235 nm
1	1000	1.36

Figure: 4.5.1.1: Standard Polyhydroxy-3-Butyric acid Calibration Curve



—— Standard PHB —— Extracted PHA sample

4.5.2. Bacterial Growth and PHA accumulation

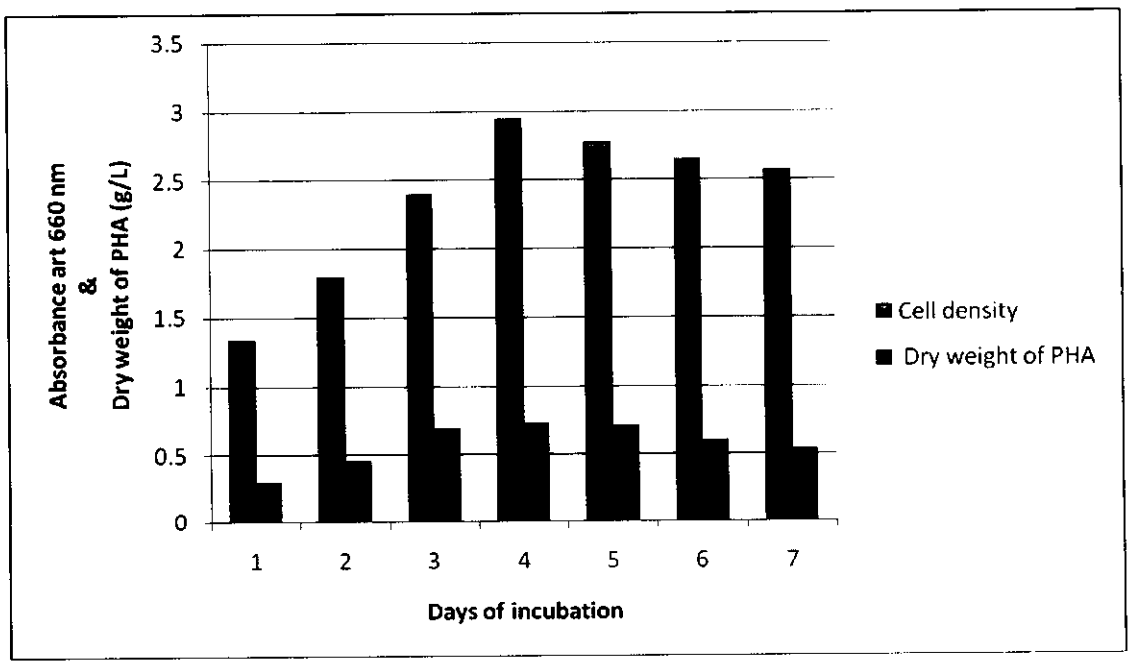
It was found that the PHA accumulation was in proportion to the bacterial cell density and Dry cell weight. Higher growth was inferred from higher optical density readings at 660nm, which corresponded to higher dry cell weight and evidently a higher PHA accumulation. The observations on bacterial growth and PHA accumulation during a seven day period of incubation are given in Table 4.5.2.1. The relationship between Bacterial growth and PHA accumulation is depicted in bar chart as shown in Figure 4.5.2.1.

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.

Table 4.5.2.1 Observations of Bacterial Growth and PHA accumulation

S.No.	No. of Days of incubation	Absorbance at 660nm Showing cell density	Dry Weight of Extracted PHA (g/100mL)
1	1	1.328	0.287
2	2	1.784	0.452
3	3	2.386	0.68
4	4	2.934	0. 721
5	5	2.765	0.705
6	6	2.643	0.596
7	7	2.558	0.532

Figure 4.5.2.1 Bar Chart depicting the relationship between Bacterial growth and PHA accumulation



4.5.3. Bacterial Growth, PHA accumulation and Carbon consumption

From the DNS assay of the culture broths on 0^{th} and 4^{th} days, it was found that the PHA accumulation was in proportion to the carbon consumed by the bacteria to grow. The observations on bacterial growth, PHA accumulation and carbon consumption are given in Table 4.5.3.1. The relationship among these are depicted in a bar chart as in Figure 4.5.3.1.

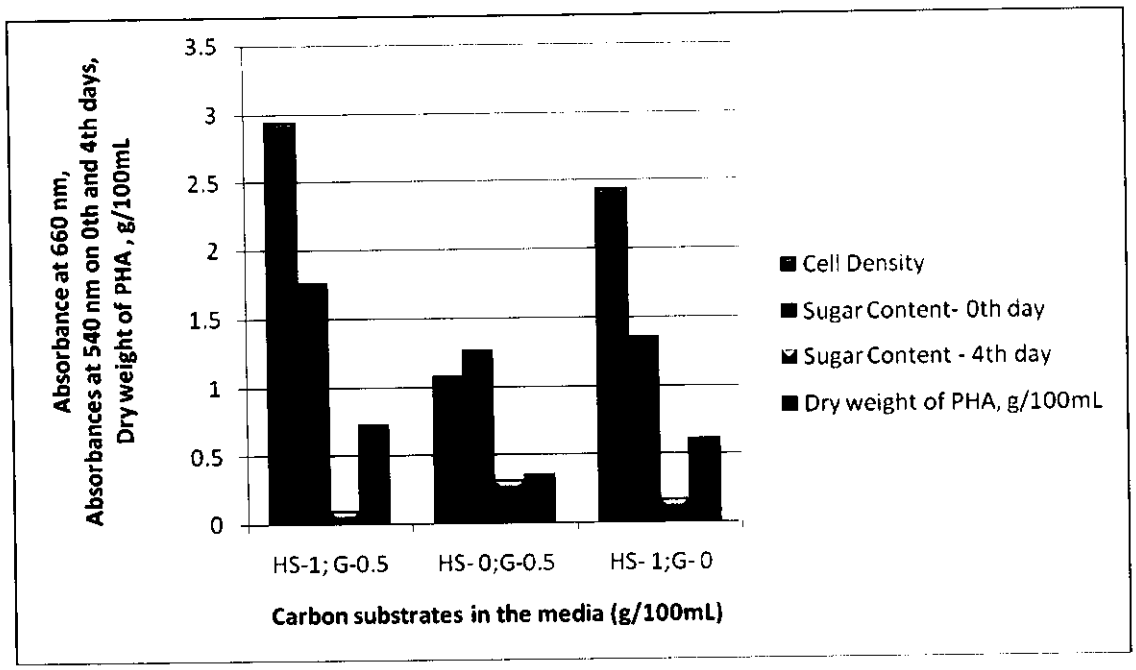
Similar results have been showed by Yang *et. al.*, 2010; Yeo *et. al.*, 2008 and Henderson *et. al.*, 1997, where PHA accumulation was higher with higher carbon supplementation in the growth media. The sugars were highly consumed during nitrogen and phosphate starvation and were accumulated in the form of PHA.

Table 4.5.3.1: Observations of Bacterial Growth, PHA accumulation and Carbon consumption

S.No.	Carbon Substrates in the growth media, g/100mL	Absorbance at 660 nm showing bacterial cell density	Absorbance at 540 nm of the 0^{th} day culture	Absorbance at 540 nm of the 4^{th} day culture	Dry weight of PHA, g/100mL
1	HS-1; G-0.5	2.934	1.75	0.09	0.72
2	HS- 0; G-0.5	1.065	1.26	0.31	0.35
3	HS- 1; G- 0	2.435	1.35	0.16	0.60

G-Glucose HS- Hydrolysed Straw

Figure 4.5.3.1 Bar Chart depicting the relationship among Bacterial growth, PHA accumulation and Carbon consumption



4.5.4 Results of Analysis of Bacterial Growth, PHA accumulation and PHB Quantification

The final results of the above methods adopted to produce Polyhydroxyalkanoates from *Ralstonia eutropha* using straw, are given in Table 4.5.4.1. The Parameters observed at the end of 4th day of incubation are, Bacterial Cell Density- OD $_{660\ nm}$, Dry Cell Weight (g/L), Dry Weight of Extracted PHA (g/L), Intracellular PHA Accumulation (%), PHB Mass in the extracted PHA sample (g/L), PHB Content in the extracted PHA sample (%) and Residual Biomass (g/L).

Table 4.5.4.1: Results of Analysis of Bacterial Growth, PHA accumulation and PHB Quantification

S.No.	Parameters observed at the end of 4^{th} day of incubation	Inference
1	Bacterial Cell Density- $OD_{660\ nm}$	2.934
2	Dry Cell Weight	19.2 g/L
3	Dry Weight of Extracted PHA	7.21 g/L
4	Intracellular PHA Accumulation	37.55%
5	PHB Mass in the extracted PHA sample	5.19 g/L
6	PHB Content in the extracted PHA sample	71.98%
7	Residual Biomass	11.99 g/L

In comparison with earlier studies, it can be concluded that a significant bacterial growth and PHA accumulation could be achieved by utilizing straw as the carbon source for bacterial growth. Du *et. al.*, (2001) have shown that a dry cell weight of 27.1 g/L, was achieved in *Ralstonia eutropha*, on supplementation with pure glucose. The dry cell weight obtained here, using straw was 19.2 g/L, near to the stated value. Further the PHA accumulation in the same bacteria by utilizing pure glucose was found to be 47-55% (Du *et. al.*, 2001 and Dennis *et. al.*, 1998). Comparatively, the utilization of straw as carbon source to grow *Ralstonia eutropha* has led to a lesser but significant PHA accumulation of 37.55%.

4.6 Effect of Different Components of Mineral Salt Media upon Bacterial Growth and PHA accumulation

4.6.1. Effect of each component of Mineral Salt Media upon Bacterial Growth and PHA accumulation

From the dry weight of PHA extracted from each of the eight test flasks. (A'-B'), each devoid of a single MSM component, the effect of each of the seven media components

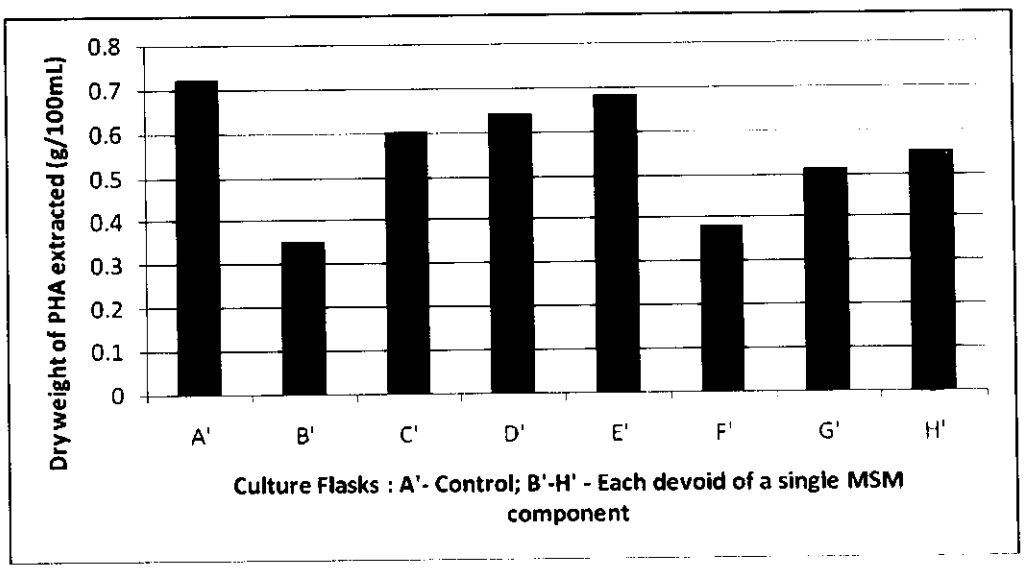
in bacterial growth and PHA accumulation was determined. The observations are tabulated (Table 4.6.1.1) and the following inferences are made.

Table 4.6.1.1: Observations of Effect of each component of Mineral Salt Media on PHA accumulation

	A'	B'	C'	D'	E'	F'	G'	H'
HS	1	*0*	1	1	1	1	1	1
G	0.5	0.5	*0*	0.5	0.5	0.5	0.5	0.5
NaCl	0.5	0.5	0.5	*0*	0.5	0.5	0.5	0.5
KCl	0.1	0.1	0.1	0.1	*0*	0.1	0.1	0.1
K_2HPO_4	0.5	0.5	0.5	0.5	0.5	*0*	0.5	0.5
$(NH_4)_2SO_4$	0.1	0.1	0.1	0.1	0.1	0.1	*0*	0.1
$MgSO_4$	0.1	0.1	0.1	0.1	0.1	0.1	0.1	*0*
PHA Extracted	0.72	0.35	0.60	0.64	0.68	0.38	0.51	0.55

A': Control; B'-H': Test Flasks each devoid of a single MSM component

Figure 4.6.1.1: Bar chart showing the Effect of each component of Mineral Salt Media on PHA accumulation



- Bacterial growth and PHA accumulation were observed to be high in culture flasks (A': Control) with all the media components being present.
- The bacterial growth and PHA accumulation was found to be significant in culture flasks with hydrolysed straw that were devoid of glucose (C'). This confirmed that even at the absence of glucose, bacterial growth and PHA accumulation were possible and significant.
- Evidently least bacterial growth and PHA accumulation were observed in the culture flasks devoid of hydrolysed straw (B'), despite the presence of glucose. This confirmed that the hydrolysed straw served the noteworthy purpose of being a cheap carbon source than direct glucose for PHA accumulation.
- The absence of sodium chloride (D') brought only negligible effects on bacterial growth and PHA accumulation. The dry weight of extracted PHA was lesser than the control but not least.
- Similarly the absence of potassium chloride (E') was not found to bring out any adverse effects. The bacterial growth and PHA accumulation was found to be equal to that of the control.
- Absence of Di-Potassium hydrogen phosphate (F') reduced the bacterial growth significantly. Hence the extracted PHA, from this culture was found to be minimum than the other cultures.
- Absence of Ammonium sulphate (G') proved to be quite significant which showed a less bacterial growth and PHA accumulation.
- Similarly absence of Magnesium sulphate (H') also proved to be quite significant showing less bacterial growth and PHA accumulation.
- The above results obtained were comparable and similar to the earlier studies (Henderson *et.al.*, 1997; Du *et.al.*, 2001 and Zakaria *et.al.*, 2010 and Yang *et.al.*, 2010).

4.6.2. Effect of other components added to Mineral Salt Media upon Bacterial Growth and PHA accumulation

The culture flasks devoid of Yeast extract (0.1%) showed an equal and significant bacterial growth and PHA accumulation to those flasks with yeast extract. Thus the presence of Yeast extract in the proposed Mineral Salt Media was found to be insignificant for

bacterial growth PHA accumulation. The observations are given in Table 4.6.2.1. A similar result was obtained by Joshi *et. al.*, 2010, stating that ammonium sulphate was enough to serve the purpose of nitrogen source in the growth media for PHA production than yeast extract in the Mineral Slat Media.

Table 4.6.2.1: Observations of Effect of adding Yeast extract in Mineral Salt Media upon Bacterial growth and PHA accumulation

S.No.	Media Components	Optical Density at 660 nm	Dry weight of PHA extracted (g/100mL)
1	MSM + 0.1% YE	2.95	0.723
2	MSM	2.934	0.72

YE- Yeast extract

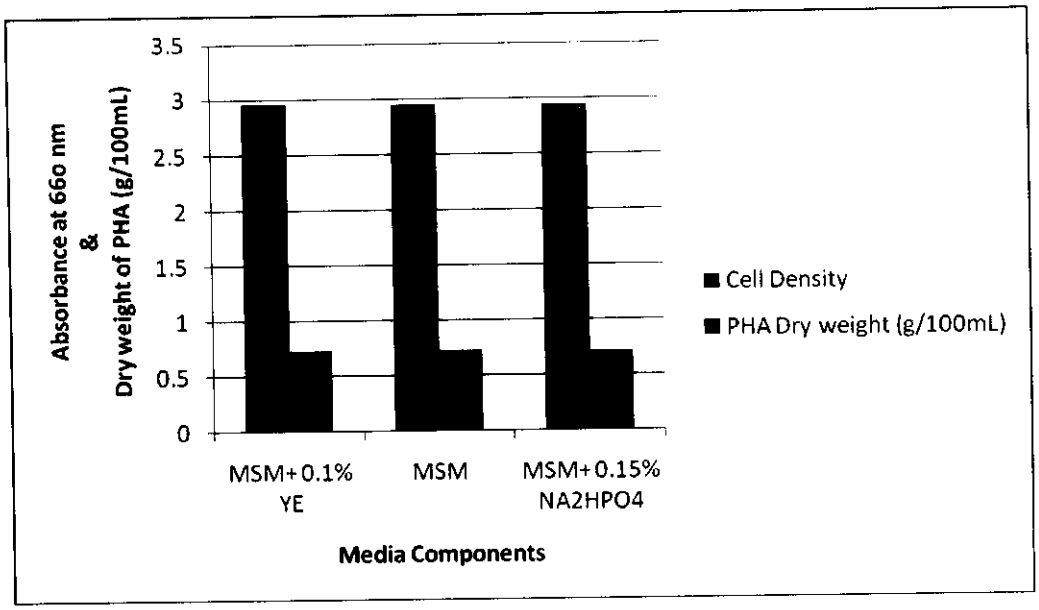
Similarly the culture flasks with a second phosphate source, Sodium di-hydrogen phosphate (0.1%) showed only negligible variations in bacterial growth and PHA accumulation. Thus the presence of another phosphate source in the proposed Mineral Salt Media was also found to be insignificant for bacterial growth and PHA accumulation. The observations are tabulated as follows (Table 4.6.2.2). Nishioka *et. al.*, 2001 have provided a comparable result in this case, stating the importance of phosphate sources in the growth media for PHA accumulation.

Table 4.6.2.2: Observations of Effect of adding Sodium di-hydrogen phosphate in Mineral Salt Media upon bacterial growth and PHA accumulation

S.No.	Media Components	Optical Density at 660 nm	Dry weight of PHA extracted (g/100mL)
1	MSM + 0.15% NaH_2PO_4	2.92	0.71
2	MSM	2.934	0.72

These negligible effects on adding Yeast extract and Sodium di-hydrogen phosphate in MSM upon bacterial growth and PHA accumulation are depicted in bar chart. Figure 4.6.2.1.

Figure 4.6.2.1 Bar chart depicting negligible Effects of adding Yeast extract and Sodium di-hydrogen phosphate in Mineral Salt Media upon bacterial growth and PHA accumulation



4.6.3. Effect Hydrolysed Straw Content in Mineral Salt Media upon bacterial growth and PHA accumulation

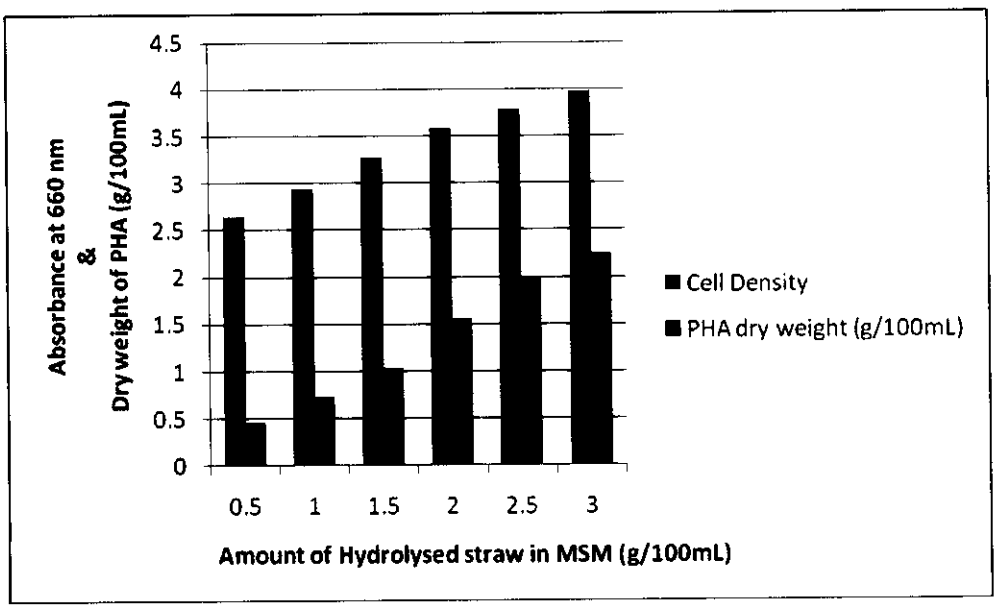
From the culture flasks grown with varied amounts of hydrolysed straw, it was evidently inferred that, the bacterial growth and PHA accumulation were found to be in direct proportion to the amount of hydrolysed straw in the MSM. Higher hydrolysed straw content contributed to higher PHA accumulation and vice versa, as shown below (Table 4.6.3.1). These significant effects of varied amounts of hydrolysed straw in the Mineral Salt Media upon the bacterial growth and PHA accumulation were shown in a bar chart (Figure 4.6.3.1). The carbon consumption studies done earlier (Henderson *et. al.*, 1997; Panda *et. al.*, 2006 Yang *et. al.*, 2010) revealing similar facts were taken into reference here.

Table 4.6.3.1: Observations of Effect Hydrolysed Straw Content in Mineral Salt Media upon bacterial growth and PHA accumulation

S.No.	Amount of HS in MSM (g/100mL)	Absorbance at 660 nm	Dry weight of PHA (g/100mL)
1	0.5	2.636	0.45
2	1.0	2.934	0.72
3	1.5	3.252	1.02
4	2.0	3.578	1.54
5	2.5	3.769	1.97
6	3.0	3.964	2.24

HS- Hydrolysed Straw

Figure 4.6.3.1 Bar chart depicting Significant Effects of the varied Amounts of Hydrolysed straw in Mineral Salt Media upon bacterial growth and PHA accumulation



4.6.4. Identification of Significant components of the Mineral Salt Media by Plackett Burman Design

From the observations as tabulated below (Tables 4.6.4.1 and 4.6.4.2), the bacterial growth, dry cell weight and PHA accumulation were taken for analysis in Plackett burman design. The Bar chart of Figure 4.6.4.1 shows PHA accumulation in different trials of Plackett Burman Design The dry weight of extracted PHA was taken as the Yield factor, with which the effect and mean square were calculated for each variable. Since potassium chloride was taken as the dummy variable, the error mean square was calculated from D'. The f- test values were finally calculated to determine the most significant factors of the MSM in bacterial growth and PHA accumulation. The f- test values were depicted in a pie chart, as in Figure 4.6.4.2, to identify the most significant components.

Table 4.6.4.1: Observations from Plackett Burman Design

	A' HS	B' G	C' NaCl	D' KCl	E' K_2HPO_4	F' $(NH_4)_2SO_4$	G' MgSO4	Optical Density at 660 nm	Dry Cell Weight (g/L)	Dry Weight of PHA extracted(g/L)
1	H	H	H	H	H	H	H	2.674	1.067	7.62
2	H	L	L	L	H	L	H	2.416	0.996	7.12
3	L	L	H	L	L	H	H	1.692	0.733	5.24
4	L	H	L	H	L	H	H	1.757	0.767	5.5
5	H	H	H	L	H	H	L	2.564	0.974	6.96
6	H	L	L	H	L	L	L	2.096	0.890	6.36
7	L	H	H	H	H	L	L	1.652	0.686	4.9
8	L	L	L	L	L	L	L	1.428	0.442	3.16

HS- Hydrolysed Straw G- Glucose

Figure 4.6.4.1 Bar chart showing PHA accumulation in different trials of Plackett Burman Design

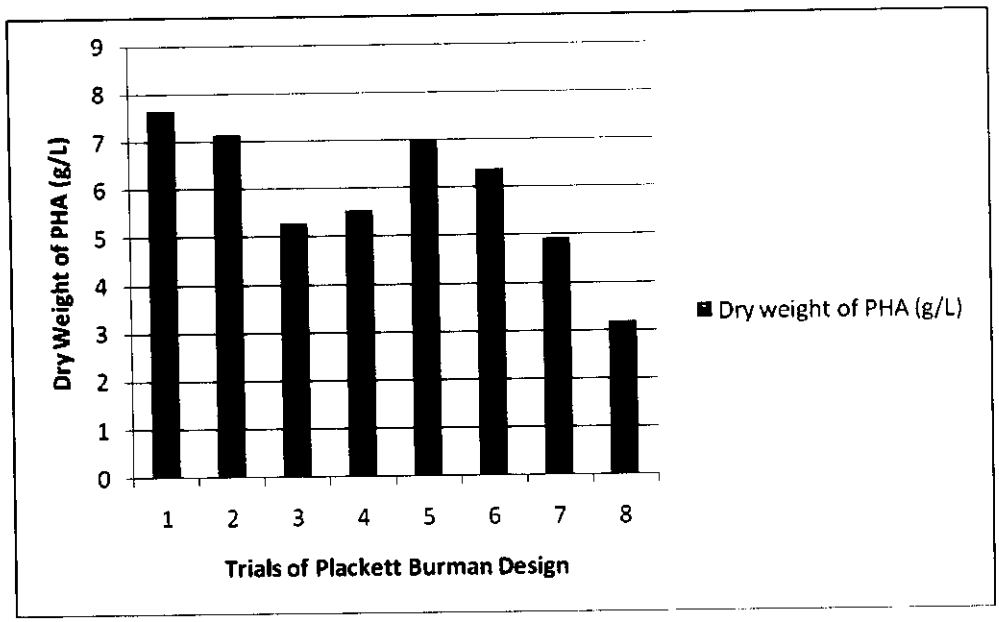
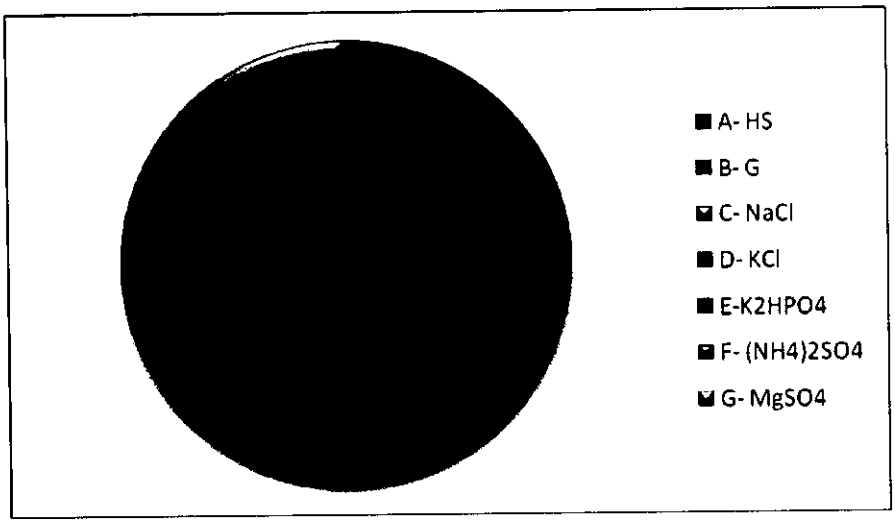


Table 4.6.4.2: Analysis of yields of different trials and determination of significant factors

	A'	B'	C'	D'	E'	F'	G'
$\sum$ (H)	28.06	24.98	24.72	24.38	26.6	25.32	25.48
$\sum$ (L)	18.8	21.88	22.14	22.48	20.26	21.54	21.38
Difference	9.26	3.1	2.58	1.9	6.34	3.78	4.1
Effect	2.315	0.775	0.645	0.475	1.585	0.945	1.025
Factor Mean Square	10.71	1.201	0.832	0.45	5.02	1.78	2.10
f- Test Values	23.8	2.66	1.848	1	11.15	3.95	4.66

Figure 4.6.4.2 Pie Chart depiction of f-test values to identify the significant components of the Mineral Salt Media



From the above tabulations and figures, it can be inferred that the hydrolysed straw forms the most significant factor in the Mineral Salt Media upon bacterial growth and PHA accumulation. Next is the Di-Potassium hydrogen phosphate making the second most significance in bacterial growth and PHA accumulation. The third level of significance was attributed to two factors, Magnesium sulphate and Ammonim sulphate. This result was in found to be in fairness with the results obtained in the previous test, 'Effect of each component of MSM upon bacterial growth and PHA accumulation', where the presence of hydrolysed straw, Di-Potassium hydrogen phosphate, Magnesium sulphate and Ammonim sulphate were confirmed to be significant. It was also proved that presence of glucose and sodium chloride was not much significant.

Similar kind of optimization studies, (Cavalheiro *et. al.*, 2009; Panda *et. al.*, 2006; Lee *et. al.*, 1995; Sankhla *et. al.*, 2005; Yang *et. al.*, 2010 and Zakaria *et. al.*, 2010) carried out earlier have 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.

4.7. FTIR Spectroscopy

The FTIR spectrum of the extracted PHA sample was compared with that of the Standard Polyhydroxy-3-Butyric acid. From the spectra, it can be inferred that, the extracted sample is a multicomponent Polyhydroxyalkanoate (PHA), containing significant amount of Polyhydroxybutyrate. The peaks were observed to be similar in the regions of 3800- 3300; 2900- 2100; 1458.23; 1057.03; 600-450, cm^{-1}. These regions correspond to ester carbonyl groups present in hydroxy acids. Two of these peaks were exactly the same, confirming the presence of PHB in the extracted PHA sample. The FTIR spectra of the Standard PHB and the extracted PHA sample are given in Figures 4.7.1 and 4.7.2.

Figure 4.7.1: FTIR Spectra of Standard Polyhydroxy-3-Butyric acid

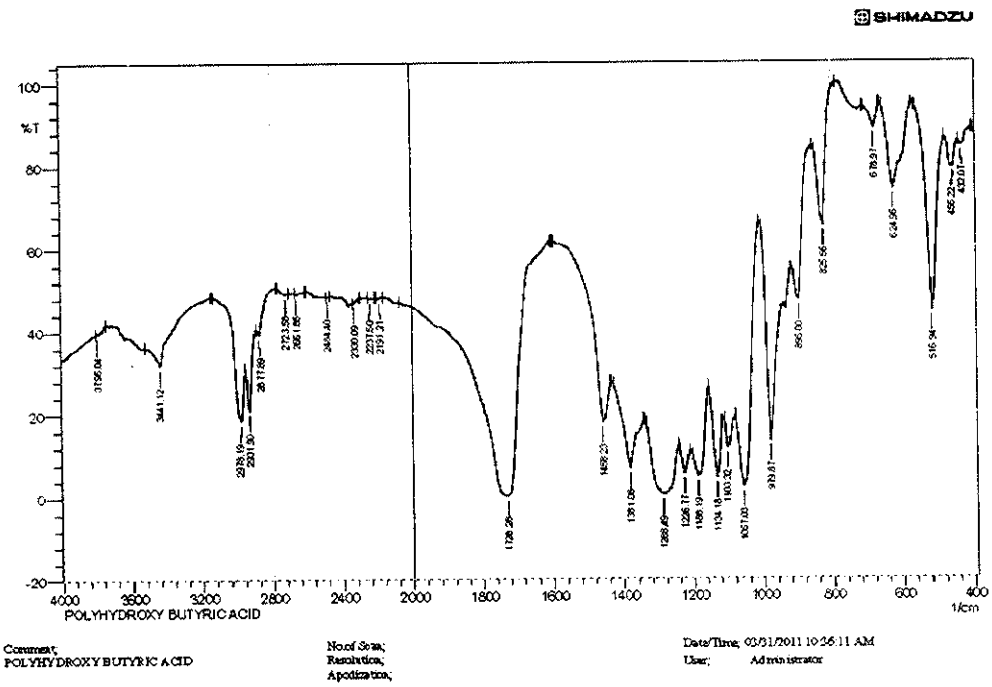
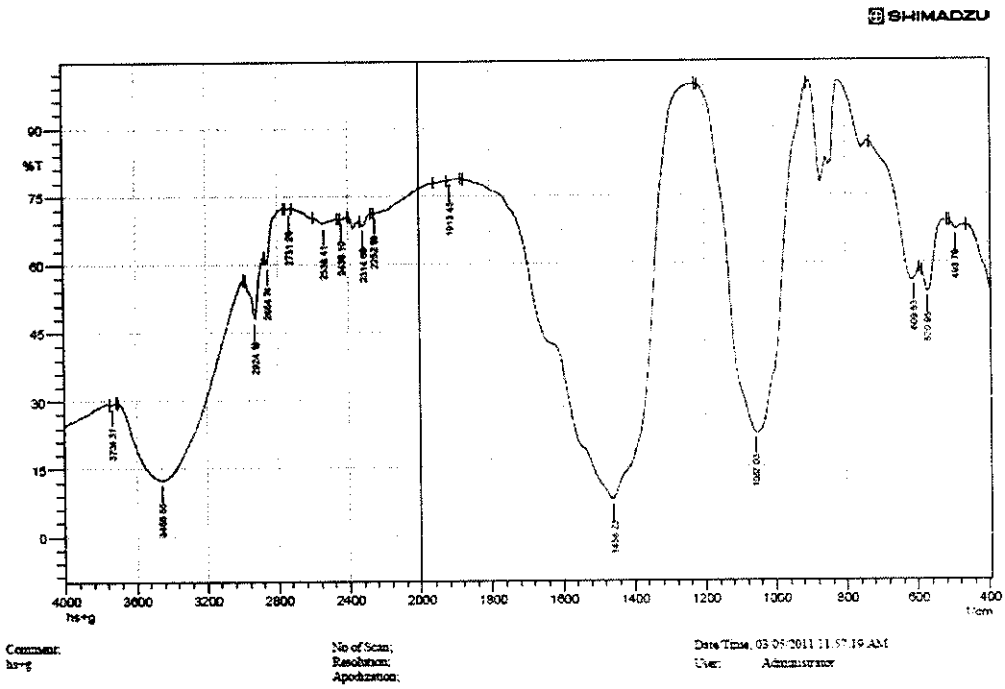


Figure 4.7.2: FTIR of Extracted PHA sample

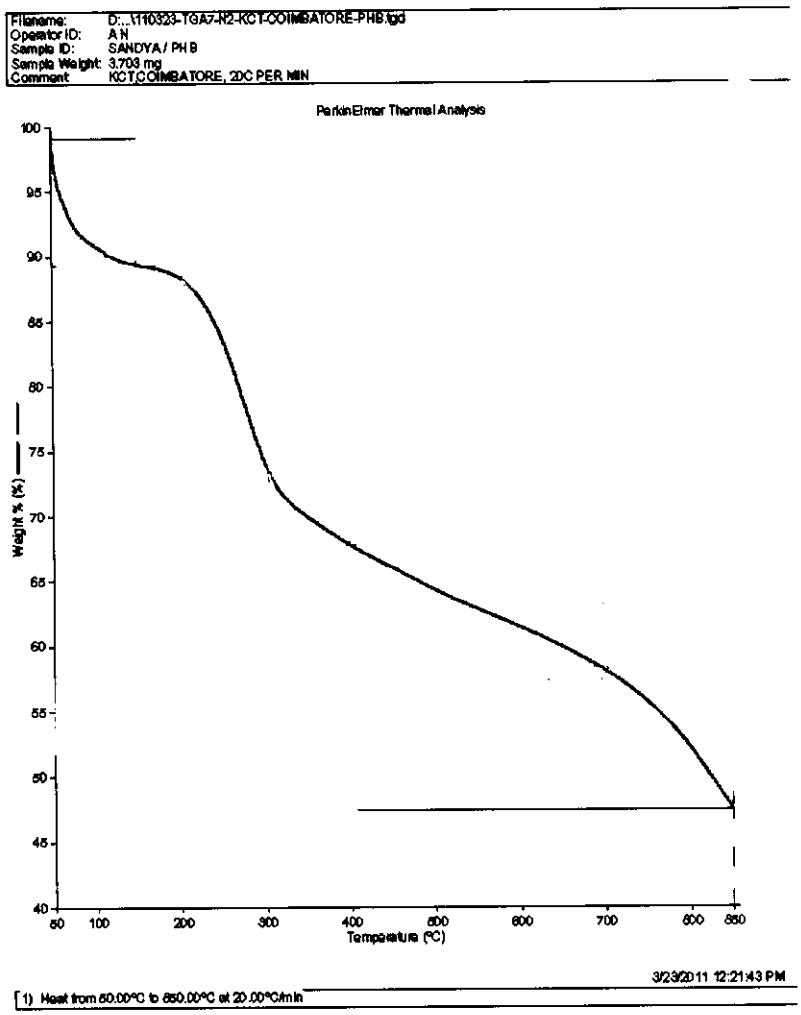


The FTIR analysis by Oliveira *et. al.*, 2007 was taken into consideration since it showed similar kind of spectra. The functional groups identified in that were similar to that of the extracted PHA sample.

4.8. Thermogravimetric Analysis

The TGA Curve along weight % shows the thermal degradation properties such as, Drying around 150°C, Desorption above 400°C followed by sublimation and decomposition. These are in correspondence with the thermal degradation properties of Polyhydroxyalkanoates studied by Zakaria *et. al.*, (2010). Also the data corresponded to standard polymer degradation characteristics. Further it was also inferred that the sample contained many hydroxyalkanoate compositions since the degradation temperatures were lower than that of the standard values. The degradation of the PHA sample was complete at 850°C as inferred from the TGA curve given in Figure 4.8.1

Figure 4.8.1: Thermogravimetric Analysis Curve of extracted PHA



4.9. Differential Scanning Calorimetry

From the Differential Scanning Calorimetric curve given in Figure 4.9.1, the following properties of the extracted PHA sample were inferred.

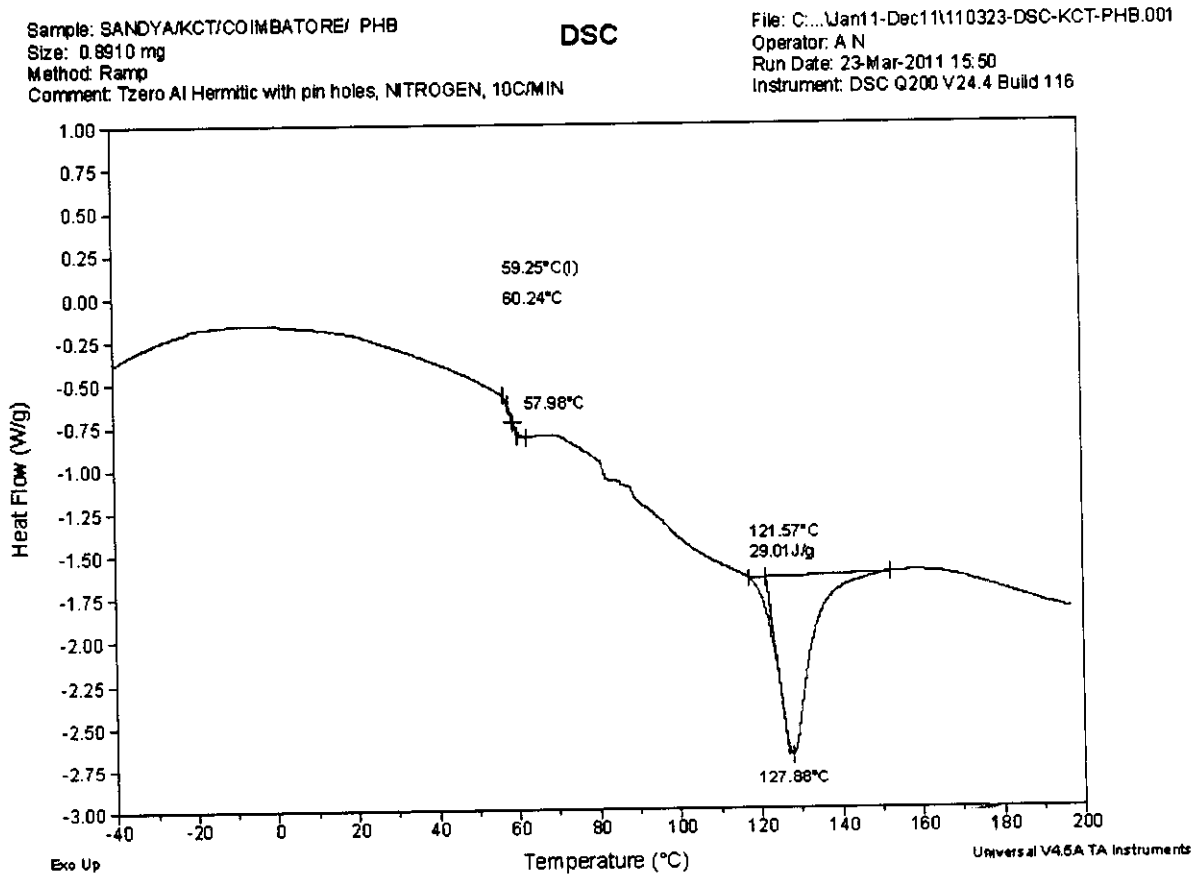
- Glass Transition Temperature T_g: 10-15 °C,
- Crystallisation Temperature T_c: 57.98 °C,
- Melting Temperature T_m: 127.88 °C

Glass Transition Temperature is the one at which the polymer starts to deteriorate under thermal treatment, changing its phase. This would be usually in the range of 9-15°C for

Polyhydroxyalkanoates. (Oliveira *et. al.*, 2007). The temperature of crystallization is where the material starts to form crystals upon heating or cooling. The melting temperature denotes the actual temperature at which the crystallised structure melts.

Hence in comparison with earlier studies, these data suggest that the sample subjected to DSC was a multicomponent PHA, since the melting point of pure polyhydroxy-3-butyrate would be in the region of 170-180° C. In presence of other alkanoate components, the melting temperature has been observed to decrease.

Figure 4.9.1: Differential Scanning Calorimetric curve of extracted PHA



CONCLUSION

CHAPTER 5

CONCLUSION

Thus from these studies it was inferred that the paddy straw can be utilized as a cheap and efficient source of carbon in the Mineral Salt Media, for the bacteria to grow and accumulate Polyhydroxyalkanoates (PHA). The methods adopted for straw hydrolysis are cheap and less time consuming than the costlier and elongated enzymatic methods. Also the carbon content in the hydrolysed straw was proved to be up taken by the bacteria for growth. This kind of approach to utilize the carbon content for bacterial growth and PHA production was a maiden attempt. If done on a large scale, it can be bring about an evident change in the industrial PHA production, whereby the need of high investments can be reduced significantly. An optimized method for the production of Polyhydroxyalkanoates using Straw can be devised further by the Response Surface Methodology, taking into account the significant factors identified from Plackett Burman Design. This would give an idea of the much needed, low cost methodology for PHA production. Thus the objective of the project, to produce Polyhydroxyalkanoates from *Ralstonia eutropha* using Straw as a cheap substrate was achieved.

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