PRODUCTION, DEGRADATION AND OPTIMIZATIONOF POLYHYDROXYALKONATE (PHA) FROM *Kluyvera intermedia* UTILIZING HYDROLYZED GRASS AS SUBSTRATE

PROJECT REPORT

Submitted by

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DEPARTMENT OF BIOTECHNOLOGY

PROJECT WORK

PHASE II

APRIL 2012

This is to certify that the project entitiled

PRODUCTION, DEGRADATION AND OPTIMIZATION OF POLYHYDROXYALKONATE (PHA) FROM *Kluyvera intermedia* UTILIZING HYDROLYZED GRASS AS SUBSTRATE

is the bonafide record of project work done by

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Register No: 1020203011

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

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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 "**PRODUCTION, DEGRADATION AND OPTIMIZATION OF POLYHYDROXYALKONATE (PHA) FROM *Kluyvera intermedia* UTILIZING HYDROLYZED GRASS AS SUBSTRATE**" 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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I certify that the declaration made above by the candidate is true.

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(R.PREETHI)

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ABSTRACT

This study deals with the isolation and screening of PHA producing microorganism from soil and best isolate (SP-Y1) was characterized for the determination of morphological, biochemical properties and later identified as *Kluyvera intermedia.* Even though the biologically derived plastics are becoming a better alternative, it has a major disadvantage in the cost of substrate influencing PHA production. Hence the feasibility of a novel and easily available agricultural based cheap substrate, Bermuda grass (*Cyanidon dactylon*) was explored as a major growth component in various combinations of media and was also screened under optimization studies like Plackette-Burman and Box-Behnken. To check the efficiency of PHA production by the best isolate, it was compared with a reference strain *Ralstonia eutropha.* It was found that the % PHA accumulation of the isolate was 26.76 while the % PHA accumulation of *R.eutropha* was 28.97 utilizing medium containing (Glucose + hydrolyzed grass) as substrate which shows a comparative yield than reference strain. After the optimization of media components a final yield of 4.38 g/L of PHA can be obtained which was 6.15 fold increase in PHA yeild than non-optimized condition. The functional group of the sample was identified by FTIR analysis. Finally, the degradation studies were carried out both intracellularly and extracellularly inorder to confirm the degradable nature of produced PHA.

***Keywords*:** Polyhydroxyalkanoate, *Ralstonia eutropha*, *Kluyvera intermedia*, Grass, FTIR

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LIST OF ABBREVATIONS AND SYMBOLS

PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
DNS	Di-nitro Salicylic acid
HCl	Hydrochloric Acid
$K_2Cr_2O_7$	Potassium Di Chromate
$MgSO_4$	Magnesium Sulphate
KH_2PO_4	Di-Potassium hydrogen phosphate
$(NH_2)_4SO_4$	Ammonium sulphate
NaOH	Sodium Hydroxide
KCT	Kumaraguru College of Technology
MTCC	Microbial Type Culture Collection
FTIR	Fourier Transform infrared spectroscopy
NMR	Nuclear magnetic resonance spectroscopy
DSC	Differential scanning calorimetry
GPC	Gel permeation chromatography
DCW	Dry cell weight
CWW	Cell wet weight
CDW	Cell dry weight
MSM	Mineral salt medium
HB	Hydroxybutyrate

HV	Hydroxyvalerate
L	Litre
µg	Microgram
g/L	gram/litre

CHAPTER 1

INTRODUCTION

Plastic materials have become an integral part of contemporary life because of their many desirable properties including durability. But they possess the property of being resistant to degradation, toxicity after incineration and massive waste accumulation into the landfills and the marine environment. These non-degradable plastics accumulate in the environment for millions tons per year. So, the problems concerning the global environment and solid waste management have created much interest in the development of biodegradable plastics that still retain the desired physical and chemical properties of conventional synthetic plastics (Anderson *et al*., 1990).

1.1 Polyhydroxyalkonates (PHA)

In alternative to the conventional petroleum derived plastic, biologically derived Polyhyroxyalkanotes (PHA) were produced. Lemoigne, (1926) was the first person who described a bioplastic-poly (3-hydroxybutyrate) (PHB) in *Bacillus megaterium*. It was noticed by him that when cultures of *Bacillus subtilis* were allowed to autolyse in distilled water, the pH value decreased due to formation of an unknown acid. This unknown acid was found to be identical to β-hydroxybutyric acid, which was present in urine of diabetic patients. Subsequently, PHB was identified as the monomer component of the granules (Lemoigne *et al*., 1926).

1.1.1 Classification of PHA

The PHAs are classified by the number of carbon atoms in their monomers as "short-chain length" PHAs, such as polyhydroxybutyrate (PHB) and polyhydroxyvalerate are there with carbon numbers of monomers are 3 to 5. Conversely, carbon numbers in medium chain-length PHA monomers range from 6 to 16. In addition to PHB, more than 140 different PHAs have been identified. Polyhydroxybutyrate (PHB), a lipid-like polymer of 3-hydroxybutyrate, is the most common PHA stored by bacteria, but polymers of other hydroxyacids can also be found (Madigan *et al*., 2000).

1.1.2 Organism producing PHA

PHA are biodegradable thermoplastics consisting of monomers of d (−) configuration. Depending on PHA accumulation kinetics, bacteria can be divided in two groups. The first group is formed by bacteria that require the limitation of some nutrients. *Ralstonia eutropha* or *Pseudomonas oleovorans* belong to this group. Bacteria of the second group do not depend on nutritional limitation as they accumulate PHA during cell growth. Some examples are *Alcaligenes latus*, *A. vinelandii*, *Pseudomonas putida*, *Pseudomonas aeruginosa* 47T2 and r-*E. coli* (Madison *et al*., 1999).

1.1.3 Production of PHA

The production of biodegradable plastics on a large scale is limited because of the relative expense of the substrate and low polymer production. According to Yamane, (1993), the higher production costs, especially raw material costs, make it difficult for PHA plastics to compete with conventional petroleum-based plastics in the commercial market place. Hence PHA production costs could be reduced by several means by using cheaper substrates such as starch, whey (Kim *et al.,* 1994) or enhancement of product yield e.g. by using recombinant *E. coli* (Lee, 1996). There have been some investigations on the possibility of producing PHB in transgenic plants (Lee, 1996; Nawrath *et al.,* 1994).They are produced also through fermentation strategies which includes batch culture, fed-batch culture, continuous culture and two-stage fermentation. Two-stage fermentation is currently the most common method of producing PHAs. In the first stage of the proposed process, biomass is increased to the level needed for PHB production. In the second stage, nutrients are limited in order to stimulate PHB synthesis by bacteria.

1.1.4 Optimization of PHA

The important factor preventing the industrial and commercial production of PHB is its high price of production as compare to synthetic plastic. Therefore, improved cultivation medium and conditions are required for reducing the cost (Khanna and Srivastava, 2004). Since the amount of polymer obtained using agro-industrial wastes is noticeably lower than that obtained using purified carbon substrates, it is important to develop more efficient and appropriate bioprocess strategies for the production of PHA from renewable carbon sources. Thus, it is necessary to optimize the fermentation medium as has been carried out for a long time to enhance the yield and productivity of many strategic biotechnological processes (Goswami *et al*., 2009). The statistical design of experiments offers advantages over traditional methods of bioprocess optimization. One of these is that it allows the interaction of several variables to be tracked simultaneously, compared to only one factor at a time in conventional models (Lakshman *et al*., 2004). The response surface methodology (RSM) selected is a useful statistical technique frequently used in the design of experiments, analysis of results, and to achieve optimal conditions in a limited number of experiments (Kathiresan *et al*., 2007). 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 bacterial fermentations (Preetha *et al*., 2007). Culture media and PHA production have also been optimized using RSM methodology (Sangkharak *et al*., 2007) .

1.1.5 Degradation of PHA

There is increasing interest regarding the degradation of PHAs in recent years, due to the biomedical industry's need for biodegradable polymer implants and controlled drug release systems (Zinn *et al*., 2001). Moreover, actual understanding of the PHA degradation is very important for the eco-friendly management of polymer wastes (Kim and Rhee, 2003; Steinbutchel and Eversloh, 2003). Even though PHAs are considered to be quite resistant to degradation in the animal body, both enzymatic and non-enzymatic processes can occur simultaneously under normal conditions. It is, therefore, important to understand both processes (Marois *et al*., 1999). It is generally accepted that the rates of degradation are influenced by the characteristics of polymer, such as chemical composition, structure, crystallinity and molecular weight (Abe and Doi, 2002; Ren- stad *et al*., 1999). The nature of the monomer units also has been found to affect degradation. Copolymers containing PHB monomer units have been found to be degraded more rapidly than either PHB or 3HB-co-3HV copolymers. Microorganisms secrete enzymes that break down the polymer into its molecular building blocks, called hydroxyacids, which are utilized as a carbon source for growth. The principal enzyme for the degradation of PHB and oligomers derived from the polymer is PHB depolymerase. Studies on the extracellular PHB depolymerase of *Alcaligenes faecalis* have indicated it to be an endotype

hydrolase. Other prominent organisms in which PHB depolymerase has been identified and worked upon are *Rhodospirillum rubrum*, *B. megaterium*, *A. beijerinckii*, and *Pseudomonas lemoignei* (Reddy *et al*., 2003). Intracelluar degradation studies were not found to explore yet. Biodegradation of PHA under aerobic conditions results in carbon dioxide and water, whereas in anaerobic conditions the degradation products are carbon dioxide and methane. PHA are compostable over a wide range of temperatures, even at a maximum of around 60^{o}C with moisture levels at 55%. Studies have shown that 85% of PHA was degraded in seven weeks (Johnstone, 1990; Flechter, 1993). PHA have been reported to degrade in aquatic environments (Lake Lugano, Switzerland) within 254 days even at temperatures not exceeding 60^{o}C (Johnstone, 1990).

1.1.6 Applications of PHA

PHA have a wide range of applications, such as in the manufacture of bottles, packaging materials, films for agriculture and also in medical applications (Philip *et al*., 2007). PHAs can be used to fabricate three-dimensional, porous, biodegradable heart valve scaffold (Sodian *et al.,* 2000), bone fracture fixation (Galego *et al.,* 2000), manufacture of surgical pins, sutures, staples, swabs, fixation rods and cardiovascular stents (Scholz, 2000). PHAs can be used as carriers for long term slow release of drugs, insecticides, herbicides and fertilizers and in wound dressing (Lee, 1996). The PHA types, such as polyhydroxybutyrate (PHB), polyhydroxybutrate -co-hydroxyvalerate (PHBV) poly (hydroxybutyrate -co- hydroxyhexanoate) (PHBHHx) and polyhydroxyoctanoate (PHO) are frequently studied for biomedical applications including tissue regeneration devices, repair devices, repair patches and sutures (Chen, 2009). In drug delivery systems PHA shows a faster drug release than PLA (Poly Lactic Acid).

1.2 *Ralstonia eutropha*

Ralstonia eutropha, a facultative autotroph is a well known bacterium which can accumulate large amount of PHB from simple carbon sources, for example, glucose, fructose and acetic acid. for producing PHB (Reinecke *et al*., 2009). It can accumulate PHB to more than 80% of its cell dry weight (Anderson *et al*., 1990). *R. eutropha* has been re-classified several times in the past. The history of classification includes the genera *Hydrogenomonas, Alcaligenes, Ralstonia, Wautersia* and recently *Cupriavidus necator*. *R. eutropha* H16 has been shown to enhance the production of PHB and its copolymer P (3HB-co-3HV) when given oxidative growth conditions. Cultivation in the presence of hydrogen peroxide and methyl viologen was shown to stimulate the flux of NADPH to the PHA biosynthetic biosynthetic pathway (Jung *et al*., 2000). Since, these are the some of the features processed by the organism which made to choose as a reference strain.

Since the production cost of PHA is quite high compared with that of synthetic non biodegradable one (Yamane, 1993), an attempt was taken in this study to make this process economically more feasible, i.e., 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. So, soil micro-organisms were initially isolated and screened for their accumulating capacity of PHA by Nile Blue method (Ostle *et al*., 1982). The choice of substrate was the Bermuda grass which was added in the mineral salt medium for the growth of screened best isolate for their production of PHA and their PHA accumulating efficiency were compared with the reference strain (*R. eutropha*). In-order to maximize the PHA production, the production media components was optimized and the relative concentrations of the best influencing factors were found out. Then the extracted PHA was analyzed for its structural properties through FTIR analysis and compared with the standard (PHA) peaks. Finally, the isolate was tested for its degradation ability through undergoing intracellular and extracellular degradation studies.

1.3 Objectives

1. To isolate, screen and characterize the PHA producing microorganism from soil.
2. To hydrolyze cellulose present in selected carbon source.
3. To extract PHA and compare the efficiency of the isolate with that of the reference Organism (*Ralstonia eutropha*).
4. To identify and optimize the concentration of best influencing media components on microbial growth and maximizing PHA production through Response Surface Methodology (Box-Behnken).
5. To analyze the extracted PHA through FTIR analysis.
6. To analyze the degradation capacity of the isolate and produced PHA through methods of intracellular and extracellular degradation methods respectively

CHAPTER 2
LITERATURE REVIEW

PHA are the family of polyhydroxyesters synthesized by numerous bacteria as an intracellular carbon and energy storage compound under nutrient-limiting conditions with excess carbon. Once the limiting nutrient is provided to the cell, these energy storage compounds are degraded and used. The molecular weight of these compounds range changes depending on the micro-organism and the growth conditions (Byrom *et al*., 1997). They actually exist in the cell cytoplasm as 0.2 to 0.5 Am granules surrounded by a membrane (Sudesh *et al*., 2000) in the cell. PHA is more observed as hydrophobic inclusions in the cytoplasm of B*acillus megaterium* and in many gram negative and gram-positive bacteria (Brandl *et al*., 1988).

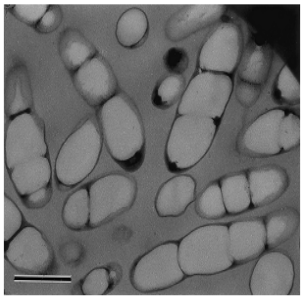
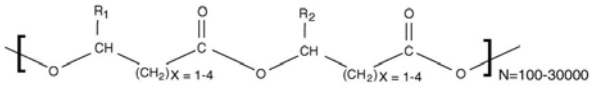


Figure 2.1Transmission electron micrograph of thin sections of recombinant *R. eutropha* producing PHB in large amounts (90% of the dry cell weight) (Sudesh *et al*., 2000).

2.1 Structure of PHA



R1 and R2 are alkyl groups (C1–C13) (Lee, 1995).

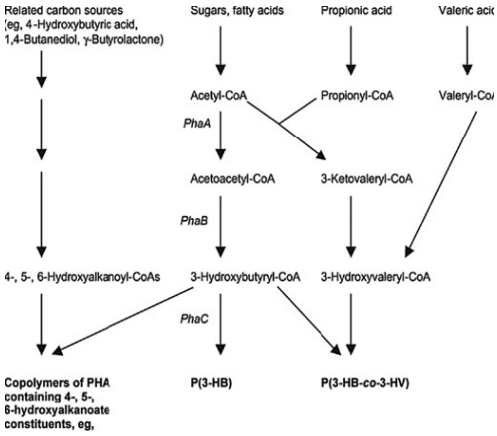
2.2 Properties of PHA

Table 2.1 Physical properties of major PHA's (Lee (1996)).

Property	PHB
Melting point(^{0}c)	175
Glass-transition temp(^{0}c)	15
Crystalline (%)	80
Young's modulus	3.5
Tensile strength (MPa)	40
Elongation to Break (%)	6
Impact strength (v/m)	50

2.3 Synthesis of PHA

There are four different pathways for the synthesis of PHAs in *A. eutrophus*, β-ketothiolase carries out the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA. An NADPH-dependent acetoacetyl-CoA reductase then carries out its conversion to 3-hydroxybutyryl-CoA.The third and the final step is the polymerisation reaction catalysed by PHB synthase (Anderson *et al*., 1990). In *Rhodopsuedomonas rubrum*, the pathway differs after the second step where the acetoacetyl-CoA formed by β-ketothiolase is reduced by a NADH dependent reductase to l-(+)-3-hydroxybutyryl-CoA which is then converted to d-(−)-3-hydroxybutyryl-CoA by two enoyl-CoA hydratases.



P (3HB) is synthesized by the successive action of b-ketoacyl-CoA thiolase (phbA),acetoacetyl-CoA reductase (phbB) and PHB polymerase (phbC) in a three-step pathway.

Figure 2.2 Pathway for the biosynthesis of PHA (Lee, 1995).

A third type of PHA biosynthetic pathway is found in most *Psuedomonas* species belonging to rRNA homology group I. *P. oleovorans* and other *Psuedomonas* species accumulate PHA consisting of 3-hydroxyalkanoic acid of MCL if cells are cultivated on alkanes, alkanols or alkanoic acids (Lagaveen *et al*.,1988.). The fourth type of PHA biosynthetic pathway is present in almost all *Psuedomonas* species belonging to rRNA homology group II. This pathway involves the synthesis of copolyesters consisting of MCL 3HAs from acetyl-CoA.

2.4 Micro-organism producing PHA

Various micro-organisms are capable of producing PHA of which one of it are the several recombinant organisms (other bacteria, yeasts or transgenic plants) have the capacity of PHA production (Snell *et al*., 2002). Some organisms in activated sludge have the ability to accumulate PHA (Gujer *et al*., 1999). Some organisms have the property of enhanced biological phosphorus removal to convert volatile fatty acids (VFAs) to PHA under anaerobic conditions for later use under aerobic conditions (Mino *et al*., 1998). PHB is also synthesized by eukaryotic species that is yeast and other species. They actually contain lower molecular mass of PHB, which functions as complexes with polyphosphates in membrane transport (Leaf *et al*., 1996). PHB was produced by *R. eutropha* DSM 11348 when it was grown in a culture medium containing 20–30 g/l casein peptone or casamino acids as a sole N source (Bormann *et al.,* 1998). *Pseudomonas cepacia* ATCC 17759 grow on xylose produce 60% (w/w) PHB in shake flasks on ammonium-limited mineral salts culture medium. *Bacillus megaterium* was grown on various carbon sources such as date syrup, beet molasses, fructose, lactose, sucrose, glucose in mineral salts medium which also produces PHA. Synthesis of PHA in plants was first demonstrated in 1992 by the accumulation of PHB in the cytoplasm of cells of *Arabidopsis thaliana* (Poirier *et al*., 1992). Ren *et al*., (2010) studied the Influence of growth stage on activities of Polyhydroxyalkanoate (PHA) polymerase and PHA depolymerase in *Pseudomonas putida*.

2.5 Screening methods of PHB producing organism

While isolating PHB-accumulating bacteria from nature, it is necessary to screen rapidly a wide collection of bacteria in a short time. Viable colony staining technique has been suggested as a method for rapid screening of PHB accumulating bacteria. Native PHA inclusions can be stained with Sudan Black B (Burdon *et al*., 1946) indicating that they are of a lipid nature (Williamson *et al*., 1958, Kallio *et al*., 1960). Besides Sudan Black B, PHA is more specifically stained by the oxazine dye Nile Blue A, exhibiting a strong orange fluorescence at an excitation wavelength of 460 nm (Ostle *et al*., 1982). While staining methods can be used to identify the presence of PHA, chemical analysis is often required to determine their monomeric compositions. The colony screening and selection systems employed to analyze the production of PHAs in *R. capsulatus*. Screening with Nile red dissolved in acetone distinguished between PHA producers and non producers (Kranz *et al.,* 1997). Viable colony screening method is used for the rapid detection and isolation of PHB producing exopolysaccharide deficient mutants from wild type of *Rhizobium meliloti* (Juan *et al*., 1998).

Colony PCR and semi-nested PCR techniques used for screening Polyhydroxyalkanoate (PHA) producers that were isolated from different environments, in which the three degenerate primers were designed based on multiple sequence alignment results and were used as PCR primers to detect PHA synthase genes.

2.6 Various utilizable substrates

Various substrates are used for the PHA production, especially plant oils, e.g. soybean oil, palm oil, olive oil, sunflower oil and coconut oil (kahar *et al*., 2004) and found to be excellent carbon sources for PHA production. Using municipal wastewater for enrichment, (Chua *et al*., 2003) achieved 20% PHA of sludge dry mass after incubation with a synthetic acetate substrate. Chua and Yu, (1999) achieved 39% PHA when using a chemical process wastewater containing xenobiotic branched carboxylic acids and ketones. Acidogenic fermentation of an olive oil mill effluent and conversion of the produced VFAs to PHA by a culture enriched with synthetic substrate has also been studied (Dionisi *et al*., 2005).

2.7 Media containing inexpensive carbon substrates

The choice of media is important not only to supply optimal conditions for production of a variety of PHAs in different bacteria but also to do so with high volumetric productivity to provide a final product that is economically competitive with the traditional plastics. This proves challenging for the production of commodity products. As the major cost in production of PHA is the medium efforts are focused on finding cheap media. Significant reduction in cost will be achieved if cheap media are found with the necessary requirements for production of PHAs with high productivity (Ojumu *et al*., 2004).

Table 2.2 PHAs biosynthesis from inexpensive carbon sources (Titian *et al*., 2008)

Bacterial strain (s)	Carbon source (s)	Polymer (s) produced	Reference
Aeromonas hydrophila	Lauric acid, oleic acid	mcl-PHAs	Lee *et al*. 2000
Alcaligenes latus	Malt, soy waste, milk waste.	PHB	Wong *et al.* 2004
Bacillus cereus	Glucose, ε-caprolactone,	PHB, terpolymer	Labuzek and Radecka 2001
Bacillus spp.	Nutrient broth, glucose, alkanoates,	PHB, PHBV, copolymers	Katircioglu *et al.* 2003
Burkholderia sacchari sp. nov.	Adonitol, arabinose, arabitol,	PHB, PHBV	Brämer *et al.* 2001
Cupriavidus necator H16	Hydrogen, carbon dioxide	PHB	Pohlmann *et al.* 2006

2.8 Recovery of PHA

PHB and other PHAs are readily extracted from microorganisms by chlorinated hydrocarbons. Refluxing with chloroform has been extensively used; the resulting solution is filtered to remove debris and concentrated, and the polymer is precipitated with methanol or ethanol, leaving low-molecular- weight lipids in solution. Longer-side-chain PHAs show a less restricted solubility than PHB and are soluble in acetone (Brandl *et al*., 1987). Ethylene carbonate and propylene carbonate have been used to extract PHB from biomass. *R. eutropha* shows a recovery in the method of dispersion with sodium hypochlorite and chloroform. This method removes most of the non-PHA cellular materials during sodium hypochlorite digestion, which facilitated the separation of PHA from the cells (Hahn *et al.,* 1994). Due to the high cost of solvent extraction, the enzymatic digestion method which involves the process of 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 were used. Finally, concentrated PHA from centrifugation was bleached with hydrogen peroxide.

2.9 Quantification and analysis of PHA

The analytical method was the first method which is used to determine PHB accumulation in bacteria and it was based on the degradation of PHB with sulfuric acid to crotonic acid, and measurement of the reaction at 235 nm by spectroscopy. GC was also used for the analysis of PHA after methanolyzing the polymer in sulfuric acid and methanol, by the use of benzoic acid as internal standard PHB concentration were measured by comparing PHB peaks. The infrared (IR) spectra of polymer samples were analyzed for its purity. The methyl esters of hydroxyalkanoic acids were analyzed using a gas chromotograph (GC) mass spectrometer (MS) (Dalal *et al*., 2010), Fourier-transform infrared spectroscopy (Hong *et al.,* 1999), staining with the lipid coloring agent Nile red followed by spectrofluorometry (Degelau *et al*., 1995), and flow cytometry (Vidal *et al*., 2001). The thermal properties of the PHA sample can be tested through thermogravimetric analysis (Zakaria *et al*., 2010), differential scanning calorimetry (Oliveira *et al*., 2007).

2.10 Effect of mixed culture and mixed substrate on the production of PHA

In order to lower the production costs, alternative strategies for bacterial PHA production with open mixed cultures have been also proposed. Mixed culture PHA production relies on a selection pressure induced on the microbial population that favors growth of organisms with PHA storage capacity (Majone *et al*., 1996). The utilization of open mixed microbial cultures facilitates the use of mixed substrates since the microbial population can adapt continuously to changes in substrate (Reis *et al*., 2003). Most of the published papers on PHA production by mixed cultures use acetate as the single carbon source. Only a few studies consider other volatile fatty acids (VFAs) like propionate, butyrate or their mixtures (Beccari *et al*., (1998) and even less analyze the effects of other carbon compounds like lactate, succinate, pyruvate and malate (Satoh *et al*., 1992), ethanol, aspartate and glutamate (Satoh *et al*., 1994) and glucose (Dircks *et al*., 2001). A mixture of propionate and acetate leads to the production of a copolymer composed by hydroxybutyrate (HB) and hydroxyvalerate (HV) units with improved characteristics in relation to the homo-polymer of HB. The final composition of the polymer can be defined by adjusting the relative concentrations of acetic and propionic acid (Takabatake *et al*., 2000).

2.11 Optimization methods of PHA

Boonsawang *et al*., (2008) studied nutrient optimization of polyhydroxyalkanoate production from palm oil fiber by *Ralstonia eutropha* TISTR 1095 using response surface methodology in which he studied the effects of propionic acid, butyric acid, $(NH4)_2SO_4$ and K_2HPO_4 upon PHA production were examined. The result showed that the nutrient optimum for PHA production was fermented broth with nutrient addition (2.50 g/L- propionic acid, 6.53 g/L- butyric acid, 1.5 g/L- $(NH_4)_2SO_4$, and 0.03 g/L K_2HPO_4) for which the cell concentration, PHA concentration, and PHA content were 1.53 g/L, 0.70 g/L and 46.5%, respectively.

Halo tolerant bacteria were reported to produce high amounts of PHAs (40- 60% DCW) under the starvation condition (Chen *et al*. 2006). Recently, the highest PHAs production was obtained from *R. sphaeroides* strain 14F when cultivated in modified GM medium showed 3.5 g/L PHA, 60% DCW where malate was substituted by 5 g/L fructose under two-stage aerobic dark condition (Lorrungruang *et al*., 2006).

2.12 Degradation of PHA

The property that distinguishes PHA from petroleum based plastics is their biodegradability. Biodegradation of PHA under aerobic conditions results in CO_2 and H_2O, whereas in anaerobic conditions, the degradation products are CO_2 and CH_4. The degradation of PHA by *R. eutropha* could occur simultaneously with its biosynthesis under nitrogen limitation. This observation is called "cyclic nature of PHA metabolism" (Doi, 1990). PHB depolymerization was tested in plates containing a PHB containing medium which were inoculated with isolates from the samples. The degradation activity was detected by the formation of a clear zone below and around the fungal colony (Lee *et al.,* 1994).

2.12.1 Extracellular degradation

Ghanem *et al*., (2005) studied degradation of polyesters by a novel marine *Nocardiopsis aegyptia sp*. and the application of Plackett- Burman experimental design for the improvement of PHB depolymerase activity, in which he states the strain excreted an extracellular PHB depolymerase and grew efficiently on PHB or its copolymers as the sole carbon sources. The degradation activity was detectable by the formation of a transparent clearing zone around the colony on an agar Petri plate after 25 days, or a clearing depth under the colony in test tubes within 3 weeks. The previous techniques proved that the bacterium was able to assimilate the monomeric components of the shorter alkyl groups of the polymers. Nocardiopsis aegyptia hydrolyzed copolymers 10–20% PHBV more rapidly than the homopolymer PHB. The bacterial degradation of the naturally occurring sheets of poly (3-hydroxybutyrate), and its copolymer poly (3-hydroxybutyrate-co-3-hydroxyvalerate) was observed by scanning electron microscopy (SEM). The samples were degraded at the surface and preceded to the inner part of the materials. Clear morphological alterations of the polymers were noticed, indicating the degradative capability of the bacterium. Plackett- Burman statistical experimental design has been employed to optimize culture conditions for maximal enzyme activity.

Bonartsev *et al*., (2007) studied on biosynthesis, biodegradation, and application of poly (3-hydroxybutyrate) and its copolymers - natural polyesters produced by diazotrophic bacteria in which an effective technology for production of PHB and PHBV of different molecular weight (from 200 to 1500 kDa) by diazotrophic bacteria of *Azotobacter* and *Rhizobium* genus has been developed. A number of medical devices on basis of PHB: surgical meshes, screws and plates for bone fixation, periodontal membranes, and wound dressing are developed. High biocompatibility of PHB films and medical devices implanted in animal tissues has been demonstrated.

2.12.2 Intracellular degradation

Gao *et al.,* (2001) stated that *Paracoccus denitrificans* degraded poly (3-hydroxybutyrate) (PHB) in the cells under carbon source starvation in which Intracellular poly-(3-hydroxyalkanoate) (PHA) depolymerase gene (phaZ) was identified near the PHA synthase gene (phaC) of *P. denitrificans*. Reaction products were thought to be mainly D (3)-3-hydroxybutyrate

(3HB) dimer and 3HB oligomer. Di- isopropylfluorophosphonate and Triton X-100 exhibited an inhibitory effect on the degradation of PHB granules.

Azotobacter chroococcum MAL-201 accumulates poly (3-hydroxybutyric acid) when grown in glucose containing nitrogen-free Stockdale medium. The same medium when supplemented with valerate alone and valerate plus polyethylene glycol (PEG) leads to the accumulation of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) [PHBV] and PEG containing PHBV–PEG polymers, respectively. The intracellular degradation of these polymers as studied in carbon-free Stockdale medium showed a rapid degradation of PHB followed by PHBV, while it was least in case of PHBV–PEG. The rate of such degradation was 44.16, 26.4 and 17.0 mg h^{-1} l^{-1} for PHB, PHBV and PHBV–PEG, respectively. During the course of such PHBV and PHBV–PEG degradation the 3HB mol% of polymers decreased significantly with increase of 3HV mol fraction, the EG mol% in PHBV–PEG, however, remained constant. After 50 h of degradation the decrease in intrinsic viscosity and molecular mass of PHBV–PEG were 37.5 and 43.6%, respectively. These values appeared low compared to PHB and PHBV. Moreover, the increasing EG content of polymer retarded their extent of degradation. Presence of PEG, particularly of low molecular weight PEG was inhibitory to intracellular PHA depolymerise (i-PHA depolymerase) activity and the relative substrate specificity of the i- PHA depolymerase of MAL-201 appeared to be PHB > PHBV > PHBV–PEG (Saha *et al*., 2007).

2.13 Applications of PHA

The PHA used as a source for the synthesis of chiral compounds (enantiomerically pure chemicals) and raw materials for the production of paints (Brandl *et al.,* 1988). PHA could be depolymerized to rich source of optically active pure bifunctional acids. PHB, for instance, is readily hydrolyzed to R-3-hydroxybutyric acid and used in the synthesis of Merck's antigaucoma drug 'truspot' in tandem with R-1,3-butanediol. It is also used in the synthesis of beta lactams. PHAs are biodegraded in soil. Therefore, the use of PHAs in agriculture is very promising. They can be used as biodegradable carriers for long-term dosage of insecticides, herbicides, or fertilizers, seedling containers and plastic sheaths protecting saplings, biodegradable matrix for drug release in veterinary medicine, and tubing for crop irrigation, food packaging (Lafferty *et al.*, 1988).The hydrophobic biopolyesters have been used as coating with polyhydroxyalkanoate granule binding protein PhaP fused with cell adhesion motif RGD which is seen as an improvement of fibroblast growth factor (Dong *et al*., 2010). Yilgor *et al*., (2010) have made drug delivery through polyester nanocapsules.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials and Instruments required

Muslin cloth, Nutrient broth, Nutrient agar, DNS (3, 5, Di- Nitro Salicylic Acid), glucose, Analytical grade chemicals such as hydrochloric acid (Hcl) and sulfuric acid, sodium hydroxide, chloroform, methanol, diethyl ether and acetone, Sodium hypochlorite, H_2O_2, Zinc chloride, Potassium sodium tartarate, PHB standard (Sigma).

UV- Spectrophotometer, Ultracentrifuge, Hot air oven, Incubator with shaker and pH meter.

3.2 Methodology

3.2.1 Isolation of micro-organisms

Soil sample was collected from KCT campus; Coimbatore, India from which organism were isolated and serially diluted. Then the isolated bacterial colonies were picked up, purified and preserved on nutrient agar slants till further use.

3.2.2 Reference strain

The strain *Ralstonia eutropha* (MTCC 1472) which was obtained from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh and the isolates were refrigerated at -20ºC in 80% glycerol stock. *R. eutropha* was used as a reference for the comparison of PHA production efficiency with that of the isolates.

3.2.3 Choice of substrate

Bermuda grass (*Cyanidon dactylon*) was collected from KCT campus; Coimbatore, India shredded into pieces and dried in oven for about 1 week maintained at temperature of 60ºC and was coarsely pulverized**.**

3.2.4 Hydrolyses of Cellulose

The method followed was based on Chen *et al*., 1984, where 25g of grass and 100 ml of 10% sodium hydroxide added along with 1litre of water was boiled and the residue was filtered and washed. To this, 100ml of 10% HCl was added with 1litre of water. Again the mixture was boiled; the residue was filtered and washed. This alkali- acid treatment was repeated for 3-4 times. The washed residue was chlorinated using 50ml of sodium hypochlorite with 1litre of water and kept in dark for 15 min and the procedure was repeated once more and filtered, washed and then to this residue 250 ml of 2% H_2O_2 was added and kept for 5 min at room temperature then the mixture is filtered and washed. The finally converted cellulose is treated by zinc chloride based on the procedure of Chen *et al.*, 1985. Where cellulose residue was treated with 5ml of 80% Zinc Chloride containing 2% HCl after 10 minutes, cellulose gets dissolved, then 5ml of 2% HCl was added to it, after 10 min of heating cellulose gets hydrolyzed to glucose and finally obtained brownish mass can be used as the substrate.

3.2.5 Estimation of Glucose content in hydrolyzed Grass

The glucose content in the sample was estimated by 3, 5, Di- Nitro Salicylic Acid (DNS) assay taking glucose as a standard solution. To the series of standard glucose solutions, concentrations varying from 200-1000µg, DNS solution was added and kept in boiling water bath for 10 min. The reaction was stopped by adding 40% Potassium sodium tartarate. The same procedure was followed to the sample containing hydrolyzed grass. Absorbance was measured at 540nm and from the standard graph; the glucose content in the sample was estimated (Miller, 1972)

3.2.6 Screening of micro-organism

Staining of cultures, grown under PHA accumulation conditions, was performed with Nile blue A (Ostle *et al*., 1982). The procedure involves Nile blue A, dissolved in acetone at the concentration of (0.5µg/ml), and was added to the agar media for viable colony staining. Stained cells were shown as bright orange colonies under a 550 nm ultraviolet lamp (Spiekermann *et al*., 1999).

3.2.7 Characterization of isolates

The screened, PHB producing bacterial isolates were characterized for their morphological identification. The six potent PHB accumulating strains SP1, SP2, SP5, SP6, SP-Y1 and SP-G1 were selected from the initial 9 isolates and were examined for their gram

reaction as per the standard procedures given by Anon (1957) and Barthalomew and Mittewer (1950).

3.2.7.1 Gram staining

A loop full of Fresh culture of isolates 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 per cent 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.

3.2.8 Bacterial Growth Profile

Isolate SP-Y1 was grown in nutrient broth and studied for its growth profile by taking absorbance at 660nm for every hour.

3.2.9 Bacterial growth in production media

The growth of SP-Y1 in production medium was monitored by taking absorbance at 660nm and the corresponding amount of PHA accumulated was determined by incubating it for five consecutive days ((Amirul *et al*., 2008, Du *et al*., 2001 and Yamanka *et al*., 2010).

3.2.10 Production of PHA

The stock cultures (*R. eutropha* and isolate) were initially revived in nutrient broth incubated for 24 hrs then some of the cells from the revived one were inoculated in mineral salt medium (g/L) containing 10g- Hydrolyzed Grass, 5g- Glucose, 5g- Nacl, 5g- Di-potassium Hydrogen phosphate, 1g- Magnesium sulphate, 1g- Ammonium sulphate, 1L- Distilled water in the pH- 7.4± 0.5 and incubated for 48hrs at 30ºC in orbital shaker.

3.2.11 Extraction of PHA

After the cells incubation period of 48hrs the flasks containing cells are centrifuged for 10,000 for 5min the supernatant was discarded leaving the pellet air dried and weighed. Then the extraction procedure is followed based on the method of (Santhanum and Sasidharan, 2010) in which the PHA was extracted using the solvent chloroform, where 3 ml of bacterial culture grown in N-free medium was transferred to glass centrifuge tubes (tubes were washed with acetone and methanol to remove plasticizers if plastic tubes were used) and centrifuged at 10000 rpm for 10 min. The cell pellet was suspended in 1 ml of standard alkaline hypochlorite solution and incubated at 37°C for 1-2 h for complete digestion of cell components except PHA. The mixture was centrifuged to collect PHA granules and the supernatant was discarded. The sediment was washed twice with distilled water and centrifuged. The PHA granules in the sediment were washed twice with three portions of acetone, methanol and diethyl ether (1:1:1), respectively. The polymer granule was dissolved with boiling chloroform and the chloroform was allowed to evaporate.

3.2.12 Quantification of extracted PHA

The polymer granule was dissolved in concentrated Sulfuric acid (1mg/ml) and heated at 100ºC 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 Sulfuric acid as blank in a spectrophotometer. A standard curve was prepared with Pure PHB (Sigma, Alderich), concentrations ranging from 20-100 µg/ml (Law and Slepecky., 1969). The quantity of PHB produced was determined in comparison with the standard.

3.2.13 Identification of PHA granules

The bacterial cells were stained with Nile blue stain and when visualizing 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).

3.2.14 Analytical procedure

3.2.14.1 Determination of Dry cell weight, Residual biomass of PHA

The bacterial culture was centrifuged at 10,000 rpm for 5 min to obtain the cell pellet. The Cell pellet was dried to estimate the dry cell weight (DCW in mg/ml) followed by the procedure of (Du *et al*., 2001). Residual biomass was estimated as the difference of the dry cell weight and dry weight of PHA extracted (Zakaria *et al*., 2010) and is calculated by the formula below.

Residual Biomass (mg/ml) = DCW (mg/ml) - Dry weight of extracted PHA (mg/ml).

3.2.14.2 Determination of % PHA Accumulation and PHA concentration

The intracellular PHA accumulation (%) is estimated as the percentage of PHB mass in the dry weight of extracted PHA (Sangkharak and Prasertsan, 2008).

PHA Accumulation (%) = $\frac{\text{Dry weight of PHA extracted (mg/ml)}}{\text{DCW (mg/ml)}}$ X 100

PHB Mass (µg/ml) = Obtained from Standard PHB assay.

3.2.15 Optimization studies on pH

The isolate SP-Y1 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 supplying glucose as carbon source.

3.2.16 Experimental design and statistical analysis

Statistical experimental design methods provide a systematic and efficient plan for experimentation to achieve highest PHA yield so that many control factors can be simultaneously studied it was carried out by means of the analysis of variance (ANOVA) which includes analysis methods like Plackett- Burman and Response surface methodology by box behken design.

3.2.16.1 Plackett- Burman optimization method

As a first step in the optimization of cell growth conditions and PHB production in shaken flasks, a Plackett- Burman design was used to analyze the most significant component influencing bacterial growth and maximum production of PHA (Stanbury *et al*., 1984). In this study, (n=7) variables (production media components) were considered, one dummy variable (potassium chloride) were assigned and (n+1 = 8) trails were conducted and finally selected best influencing variables were taken over to the experimental design of response surface methodology for finding its optimum concentration. Here, the culture flasks of different high and low combinations of the media components were made to run in duplicate with potassium chloride taken as dummy variable which were kept in shaker for three days and the PHA was extracted by the same procedure (Santhanum and Srinivasan, 2010).

The dry weight of PHA was taken as yield factor. The yields of each trial were determined. The summation of yields corresponding to all high and low value runs of each trial was calculated.

Table 3.1 Plackett- Burman design containing media components

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 obtained results are subjected to the following formulas:-

- The difference between these two levels of a variable:
  Difference (D) = (Σ (H) – Σ (L))
- The effect of each variable was calculated as**:**
  Effect = $\frac{(\Sigma (H) - \Sigma (L))}{4}$
- The factor mean square of each variable was calculated as,
  Factor Mean Square = $\frac{(\Sigma (H) - \Sigma (L))^2}{8}$
- Finally the f- test values were obtained as
  f- Test= Factor Mean Square
  Error Mean Square

Finally from the data obtained through calculating these formulas, the most significant component for bacterial growth and PHA accumulation can be determined.

3.2.16.2 Response Surface Methodology

A response experimental design called response surface method (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes which allows us to find the optimal formulation for the experiment. It is used to examine the relationship between one or more response variables and a set of quantitative experimental variables or factors. Furthermore, Responses obtained from trial experiments were compared with the predicted regression models. Furthermore, the fitted polynomial equation was presented in counter plot response surface plots that expressed the interaction between the response and the experimental levels of each factor used in the experiments (Lakshman *et al*., 2004). In this study the best three media components influencing PHA production were selected from Plackett- Burman design which were the hydrolyzed substrate, K_2HPO_4, $(NH_4)_2SO_4$ used for the optimization and other components are taken in a constant proportion (table 3.2). All of the computational analyses were carried out using statistical software, called Design-Expert 8.0.7.1. Box- Behken design (Deepak *et al*., 2008).

Table 3.2 The media components and their concentrations taken for Response Surface Methodology

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		

3.2.17 Analysis of PHA

The PHA extracted from the organism was analyzed by FT-IR spectroscopy by adding the sample with kBr and evaporated (Oliveira *et al*., 2007 and Pandiyan *et al*., 2010). It was used under the following conditions: spectral range, 4000-400 cm^{-1} to confirm the functional groups of the extracted polymer.

3.2.18 Degradation studies

3.2.18.1 Extracellular degradation

Penicillium and *Aspergillus Sp* were inoculated to the Erlenmeyer flask containing 100 ml of the mineral salt medium and PHB as the sole carbon source and incubated at 30^{o}C with shaking (150 rpm min-1) for about 10 days. The composition of MSM are (8g/L- K_2HPO_4, 1g/L- KH_2PO_4, 0.5g/L- $(NH_4)_2SO_4$, 0.2 g/L- $MgSO_4$, 0.1g/L- NaCl, 0.02g/L- $FeSO_4.7H_2O$, 0.05 g/L- Na_2MoO_4, 0.05g/L- $MnSO_4$, 0.05g/L- $CuSO_4$, 0.05g/L- $ZnSO_4$, 0.5g/L- $CaCO_3$, Agar- 15 and pH- 7) (Nishida and Tokiwa, 1993). After the growth of fungi, the culture broth obtained through enrichment technique was tested for the growth of fungi in PHB emulsified mineral salt agar plates (Nishida and Tokiwa *et al*., 1993).

3.2.18.2 Intracellular degradation

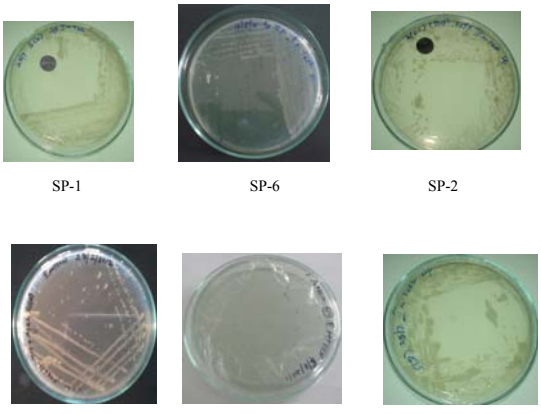
The medium for degradation is maintained with the same condition as for the PHA production but incubated for more than 2 days (Reddy *et al*., 2008). For which, the cell wet weight and dry weight of PHA produced was noted for each 24 hrs altogether for about 8 days.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation and screening of Micro-organism

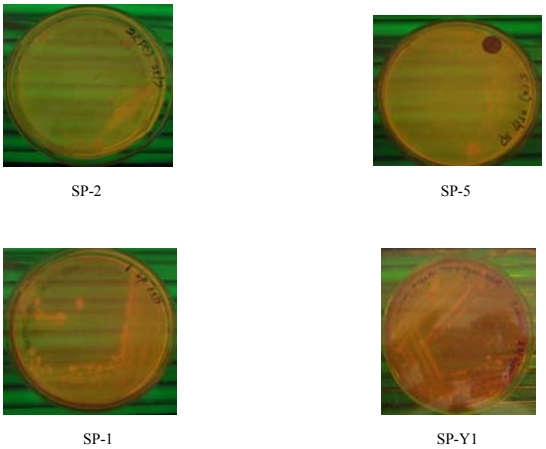
Several micro- organisms were isolated from soil and serially diluted. From that the isolates, SP-1, 2, 5, 6, Y1, G1 (figure 4.1) organism were selected through Nile blue screening method. The organisms which emit orange fluorescence while staining with Nile blue dye when seen under UV-light of 460nm are shown in figure 4.2 which confirms the ability of PHA production. The results obtained were similar to the results stated by Cortes *et al*., (2008).



SP-1 SP-6 SP-2

SP-Y1 SP-G1 SP-5

Figure 4.1 Isolation of organisms from soil



SP-2 SP-5

SP-1 SP-Y1

Figure 4.2 Screening of the Organism containing intracellular PHA

4.2 Characterization of PHA producing bacterial isolates

Six PHA producing bacterial strains were characterized by Gram staining as shown in (Table 4.3).

Table 4.1Characterization of bacterial isolates

Isolated organism	Gram staining	Colony morphology
SP1	Gram-ve	Cocci
SP2	Gram -ve	Small rod
SP5	Gram +ve	Cocci
SP6	Gram -ve	Dispersed rods
SP-Y1	Gram -ve	Small rod
SP-G1	Gram +ve	Small rod

4.3 Comparison of effeciencies of isolates of the PHA accumulation utilizing glucose as a sole carbon source

The isolates were compared with the reference strain *R.eutropha* for their production of PHA utilizing glucose as a sole carbon source in their growth medium and from the figure 4.3 obtained it was inferred that the *Kluyvera intermedia* is selected for the further studies by considering the weight of PHA produced comparitively higher than other isolates.

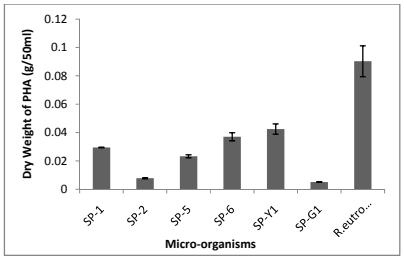


Figure 4.3 Comparison of weight of extracted PHA from the isolates.

4.4 Cellulose Hydrolysis

The hydrolysis of grass into glucose without dergradation of glucose was done based on zinc chloride method where the end product was used as substrate as shown in figure 4.4.

Grass (Before hydrolysis)

Glucose (substrate) – After hydrolysis

Figure 4.4 Cellulose in substrate Hydrolyzed to glucose

4.5 Determination of Glucose By DNS method

The hydrolysed sample was subjected to estimate the glucose content in it. From the standard curve obtained, it was inferred that the absorbance of the 0.2 ml of sample was 2.05 and the concentration of the glucose was found to be 4100 µg/ml. The amount of glucose liberated fromt the hydrolysed sample were comparison with those stated by chen *et al*.,(1984).

4.6 Identification and Characterization of best isolate (SP-Y1)

Table 4.2 and 4.3 infers that the strain SP-Y1 was characterized for its morphological, physiological and biochemical properties and finally identified as *Kluyvera intermedia* which was found to be the novel PHA producer. The results of table 4.2, 4.3 were given IMTECH, Chandigargh (Appendix I). From the result (table 4.3), *Kluyvera sp* was found to be stable at temperature ranging (25 to 42^{o}C) and pH ranging from (6 to 9).

Table 4.2 Morphological, Cultural and Biochemical tests for the identification of the strain

Morphological test		Biochemical Tests	
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	-

Table 4.3 Physiological tests and acid production test.

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	-				

4.7 Bacterial growth profile

From the figure 4.5 ,it was inferred that at the 7th hour the log phase starts and corresponding biomass, protein concentration was found to be high. So, this time interval was selected for the subsequent studies.

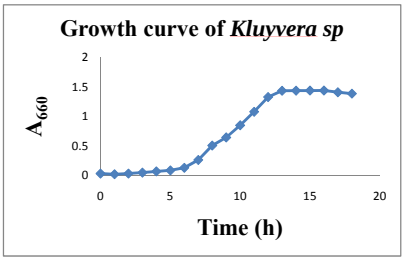


Figure 4.5 Bacterial growth profile

4.8 Bacterial growth in Mineral Salt Media

From table 4.4, when the bacterial culture was incubated for five consequtive days for PHA accumulation, it was inferred that the PHA accumulation was in proportion to the bacterial density and cell weight.

Maximum growth was observed on 2nd day of incubation and similar kind of studies was done for *Ralstonia eutropha* by Amirul *et al*., (2008),Yamanaka *et al*., (2010). The studies of Henderson *et al*., (1997), Du *et al*., (2001) have revealed similar kind of studies, that the PHA accumulation was directly proportional to bacterial density.

Table 4.4 Relationship between bacterial growth, PHA accumulation and PHA concentration

Number of Incubation days	Hydrolysed grass as sole carbon source		
	Absorbance at 660nm	% PHA Accumulation	PHA concentration
1	0.159±0.001	6.346±0.328	17.5±3.535
2	0.245±0.033	16.27±0.382	42.5±3.53
3	0.12±0.004	16.844±0.065	14.5±0.707
4	0.0355±0.005	11.434±0.048	22±1.414
5	0.0205±0.002	10.6735±0.250	18±2.828

4.9 Studies on pH optimization

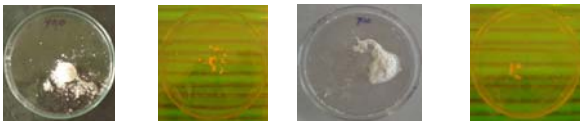
The table 4.5, infer that the optimum pH for *Kluyvera intermedia* was found to be alkine pH (8.5) in which the % PHA acumulation was found to be high which was confirmed by doing duplicate.

Table 4.5 Effect of different PH levels on PHA production

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

4.10 Extraction and screening of PHA

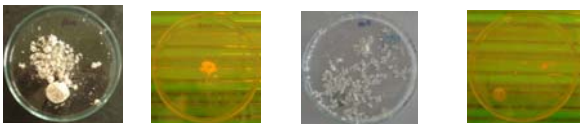
The Reference strain *R.eutropha* (figure 4.6) and the isolate *Kluyvera intermedia* (figure 4.7) was grown in the mineral salt media containing the combination of glucose and hydrolyzed grass, and hydrolyzed grass alone for the production of PHA and is confirmed by staining by Nile blue stain which emits orange fluorescence under UV light absorbance of 460 nm.Similar kind of studies were done earlier by Amirul *et al*., (2008) using fluorescent microscopy for visualizaton of intracellular PHA accumulation.



***K. intermedia* (HG)** **PHA- Stained (HG)** ***K. intermedia* (G+HG)** **PHA-Stained (G+HG)**

G – Glucose HG – Hydroyzed Grass

Figure 4.6 Extraction and screening of PHA produced by isolate *K. intermedia* by utilizing different media composition



***R.eutropha* (HG)** **PHA -Stained (HG)** ***R.eutropha* (G+HG)** **PHA – stained (G+HG)**

G – Glucose HG – Hydroyzed Grass

Figure 4.7 Extraction and screening of PHA produced by the reference strain *R.eutropha* utilizing different media composition

4.11 Comparitive studies of R.eutropha and isolate for their efficiency when mixed substrate was used as carbon source

The extracted PHA of isolate *K.intermedia* and *R.eutropha* were quantified. Their corresponding residual biomass, % PHA accumulation and PHA mass were determined and from the table 4.6, it was inferred that the PHA accumulation was proportional to the well cell weight which was earlier stated by Due *et al*., (2001) and Zakaria *et al*., (2010) and it was the *R.eutropha* showed greater efficiency of PHA production when utilizing hydrolyzed grass alone compared when utilizing combination of glucose and hydrolysed grass. But while comparing the effeciency of utilizing the mixed substrate the kluyvera intermedia found to 8.67% PHA acumulation than R.eutropha acculating PHA of 8.03%. According to the results of Yang *et al*., (2010), the % PHA accumulation increases proportionally with its corresponding PHA mass.

Table 4.6 Comparison of % PHA accumation and PHA mass by isolate and reference strain

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+HG**	0.003±0.002	0.036±0.078	0.033±0.012	8.5	25
	HG	0.043±0.456	0.151±0.004	0.107±0.078	28.97	73
Kluyvera intermedia	**G+HG**	0.006±0.067	0.039±0.007	0.033±0.367	16.03	49
	HG	0.029±0.09	0.106±0.006	0.078±0.023	26.76	52

HG- Hydrolysed Grass G- Glucose HS- Hydrolyzed seed

4.12 PHA optimization using statistical analysis

The production media components were used as variables in Placket- Burman design (table 4.7) for media optimization and the best influencing variables again taken into the response surface design (table 4.8) for identifying their exact concentration which influences more PHA production. The variables were Hydrolyzed substrate, Glucose, Sodium chloride, Potassium chloride, Dipotassium hydrogen phosphate, Ammonium sulphate, Magnesium sulphate.

4.12.1 Plackett- Burman experimental design

From the table (4.11.1), bacterial growth, dry cell weight and PHA accumulation were taken for analysis in the Placket- Burman design. The dry weight of extracted PHA was taken as yield factor, with which the effect and the mean square were calculated for each variable. Considering Potassium chloride as dummy variable, the f-test values were finally calculated to determine the most significant factors for the bacterial growth and PHA accumulation.

When probability tables were examined it was found that the factors A, E, F (Hydrolyzed substrate, K_2HPO_4, $(NH_4)_2SO_4$) showed large effect which were found to be more significant in influencing maximum PHA production. Whereas factors B, C, G (Glucose, NaCl, $MgSO_4$) show very low effect which was not significant and D shows no effect. It was proved that presence of glucose and sodium chloride was not much significant because the substrate alone was able to produce PHA without the influence of glucose which was proved earlier.

From the f-test values (table 4.8), Hydrolyzed Substrate, K_2HPO_4, $(NH_4)_2SO_4$ are the significant factors for the PHA production and so they are been considered for the determining optimum concentrations by RSM. Similar kind of studies was carried out by Cavalheiro *et al*., 2009, Panda *et al*., (2006),Yang *et al*., (2010) and Zakaria *et al*., (2010) which suggested nearly the same factors such as carbon, nitrogen and phosphate sources as the most influencing components for the growth and PHA accumulation. The studies of Panda *et al*., (2006) states that the carbon substrate supplementation was proved to increase PHA accumulation.

Table 4.7 Observations of Placket- Burman design

	A'	B'	C'	D'	E'	F'	G'	Absorbance at 660nm	Dry weight of PHA/Wet cell weight
1	**H**	**H**	**H**	**H**	**H**	**H**	**H**	0.134	0.063
2	**H**	**L**	**L**	**L**	**H**	**L**	**H**	0.140	0.133
3	**L**	**L**	**H**	**L**	**L**	**H**	**H**	1.162	0.073
4	**L**	**H**	**L**	**H**	**L**	**H**	**H**	0.936	0.597
5	**H**	**H**	**H**	**L**	**H**	**H**	**L**	0.077	0.110
6	**H**	**L**	**L**	**H**	**L**	**L**	**L**	0.194	0.035
7	**L**	**H**	**H**	**H**	**H**	**L**	**L**	1.664	0.131
8	**L**	**L**	**L**	**L**	**L**	**L**	**L**	1.122	0.055

Table 4.8 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)	1.139	0.863	0.892	0.704	1.117	0.782	0.745
Σ(L)	0.375	0.651	0.622	0.81	0.397	0.732	0.769
Difference	0.764	0.212	0.27	-0.106	0.72	0.05	-0.024
Factor Mean Square	0.073	$5.618X10^{-3}$	$9.113X10^{-3}$	$1.405X10^{-3}$	0.065	$3.125X10^{-4}$	$7.2X10^{-5}$
f-Test Values	51.96	3.99	6.486	1	46.263	0.223	0.0512

4.12.2 Response Surface Methodology

Based on results of Plackett- Burmann method, three factors $(NH_4)_2SO_4$, KH_2PO_4, Hydrolyzed Grass) that showed positive influence on growth and PHA production were selected and Box-Behnken method of analysis was used to determine the optimum levels of these parameters. A series of 17 experiments were carried out to obtain a quadratic model. As presented in table 4.9, three crucial variables namely; $(NH_4)_2SO_4$, Hydrolyzed Substrate, KH_2PO_4 were taken in three different levels, 17 runs carried out and corresponding response was calculated. Regression analysis was performed to fit the response function with the experimental data done by predicting the optimal point, within experimental constrains. A second-order polynomial function was fitted to the experimental results of PHB production, initially described by Lakshman *et al*., (2004). The equation obtained for dry weight of extracted PHA (Y_{PHA}(g/L)) was

$$Y_{PHA}(g/L) = +1.07859+0.25604*A -4.27691* B -2.17111*C +0.042963* A* B +0.030222* A* C+1.06667 * B*C -8.89778E-003* A^2+3.40494* B^2+0.80800* C^2 \quad (4.1)$$

A– Hydrolyzed Grass, B- $(NH_4)_2SO_4$, C- K_2HPO_4

From the equation 4.1, it was observed that optimum levels of Nitrogen and Phosphorus sources and Maximum level of Carbon source when given in production media increases PHA production. Similar kind of studies was carried out by Duta *et al*., (2006).

Besides the linear effect of the media components on maximum amount of PHA production, the response surface method using Box- Behnken method also gives an insight about the parameters quadratic and combined effects. These analyses were done by using both Fisher's F- test and Student t-test statistical tools. The analysis of variance, t-stat and p-value for the three variables indicated that PHA production can be well described by a polynomial model with a relatively high coefficient of determination. When expressed as a percentage, R^2 implies that the total variation of 94.32% for PHA production was attributed to the independent variables and only about 5.68% of the total variation cannot be explained by the model. The R^2 value of 0.9432 (table 4.10) for PHA production indicates the accuracy of the model, which was found greater than R^2 value obtained by Berekaa and Thawadi, (2012) which was 0.932.

The "Lack of Fit F-value" of 0.32 implies the Lack of Fit is not significant relative to the pure error. There is a 81.20% chance that a "Lack of Fit F-value" this large could occur due to noise. The Model F-value of 12.93 implies the model is significant. There is only a 0.14% chance that noise could occur.

Table 4.9 Experimental design by Box- Behnken analysis method and the response for Dry weight of PHA extracted (g/L)

Std	Run	Factor 1: Hydrolyzed Grass (g/L)	Factor 2: $(NH_4)_2SO_4$ (g/L)	Factor 3: K_2HPO_4 (g/L)	Response: PHA
4	**1**	**20**	**1**	**1.25**	**4.38**
12	2	12.5	1	2	3.3
10	3	12.5	1	0.5	2.2
17	4	12.5	0.55	1.25	2.26
6	5	20	0.55	0.5	1.66
2	6	20	0.1	1.25	1.66
16	7	12.5	0.55	1.25	1.16
15	8	12.5	0.55	1.25	1.66
14	9	12.5	0.55	1.25	1.66
8	10	20	0.55	2	2.98
13	11	12.5	0.55	1.25	1.34
1	12	5	0.1	1.25	0.6
5	13	5	0.55	0.5	0.5
7	14	5	0.55	2	1.14
11	15	12.5	0.1	2	2.6
3	16	5	1	1.25	1.66
9	17	12.5	0.1	0.5	1.86

Table 4.10 Design Summary

Source	R^2 value
Linear	0.647323699
2FI	0.694078726
Quadratic	0.943275429
Cubic	**0.954246355**

The objective of response surface methodology is to detect which experimental parameters generate signals, which are large in comparison to do noise. Adequate precision measures signal-to-noise ratio, a ratio greater than 4 is desirable. Here the adequate precision of 14.469 for PHA production indicated as an adequate signal. The Adjusted R-Squared was 0.870344 was in reasonable agreement with the Predicted R-squared value was 0.752975.

According to table 4.10, the R^2 value gives a measure of how much variability in the observed response values can be explained by the experimental parameters and their interactions. The closer the values of R^2 to 1, the better the correlation between the experimental and predicted values and the better the model predicts the response (Haaland, 1989).

Table 4.11 Model Summary Statistics

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio
Y1	PHA	g	17	Polynomial	0.5	4.38	1.919	0.9797569	8.76

The student t-test was used to determine the significance of the parameters regression coefficients. The p-values were used as a tool to check the significance of the interaction effects, which in turn may indicate the patterns of the interactions among the variables. In general, larger magnitudes of t, F values and smaller of p, indicates that the corresponding coefficient term more significant (Myers and Montgomery, 2002) and according to the saying, table 4.12 stands as an evident, in which values of "Prob > F" less than 0.0500 indicate the model terms are significant. In this case, the model is highly significant, as table 4.12 stands as an evident which contains a high model F-value and a very low probability value (P-value- 0.0014) obtained as a result. The coefficients of the quadratic terms of Hydrolyzed Grass, $(NH_4)_2SO_4$, K_2HPO_4 (P = 0.0007, P = 0.0019, P = 0.0018, respectively) appear to be very significant. The interaction effects between of Hydrolyzed Grass, $(NH_4)_2SO_4$, K_2HPO_4 with other medium components (all probability coefficients P < 0.0001) are also significant.

Table 4.12 ANOVA for Response Surface Quadratic Model Analysis of variance table

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	14.48755647	9	1.6097285	12.93370156	0.0014
A-Hydr Grass	4.06125	1	4.06125	32.63096577	0.0007
B-(NH4)2SO4	2.90405	1	2.90405	23.33319942	0.0019
C-K2HPO4	2.9768	1	2.9768	23.91772457	0.0018
AB	0.0841	1	0.0841	0.675719107	0.4382
AC	0.1156	1	0.1156	0.92881247	0.3673
BC	0.5184	1	0.5184	4.165193637	0.0806
A^2	1.054737895	1	1.05473789	8.474513054	0.0226
B^2	2.001727368	1	2.00172737	16.0832988	0.0051
C^2	0.869769474	1	0.86976947	6.988345442	0.0333
Residual	0.87122	7	0.12446		
Lack of Fit	0.1685	3	0.05616667	0.319710079	0.8120
Pure Error	0.70272	4	0.17568		
Cor Total	15.35877647	16			

Table 4.13 Diagnostics Case Statistics

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residual	Externally Studentized Residual	Run Order
1	0.6	0.635	-0.035	0.75	-0.19842	-0.18422	12
2	1.66	1.77	-0.11	0.75	-0.6236	-0.59408	6
3	1.66	1.55	0.11	0.75	0.623602	0.594081	16
4	4.38	4.3325	0.0475	0.75	0.269283	0.250609	2
5	0.5	0.4175	0.0825	0.75	0.467702	0.439936	13
6	1.66	1.5025	0.1575	0.75	0.892885	0.878171	5
7	1.14	1.2975	-0.1575	0.75	-0.89289	-0.87817	14
8	2.98	3.0625	-0.0825	0.75	-0.4677	-0.43994	10
9	1.86	1.9075	-0.0475	0.75	-0.26928	-0.25061	17
10	2.2	2.3925	-0.1925	0.75	-1.0913	-1.10909	3
11	2.6	2.4075	0.1925	0.75	1.091304	1.109095	15
12	3.3	3.265	0.035	0.75	0.198419	0.184219	1
13	1.34	1.616	-0.276	0.2	-0.87468	-0.85804	11
14	1.66	1.616	0.044	0.2	0.139442	0.129278	9
15	1.66	1.616	0.044	0.2	0.139442	0.129278	8
16	1.16	1.616	-0.456	0.2	-1.44512	-1.59723	7
17	2.26	1.616	0.644	0.2	2.04092	2.969287	4

From figure-4.8 the percentage of deviation of actual values from predicted values can be found.

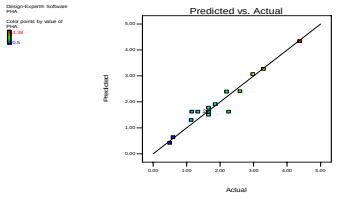


Figure 4.8 Plot showing the distribution of Actual values versus predicted values by the mathematical model of the Y_{PHA}(g/L)

The contour plot described by the regression model was drawn to illustrate the effects of the independent variables, and combined effects of each independent variable upon the response variable. From the figure 4.9 and 4.10, the level of PHA production increases, when the concentration of $(NH_4)_2SO_4$ increases and intermediate level of K_2HPO_4 concentration with respect to increased concentration of Hydrolyzed Grass was given in the production medium. Finally, from figure 4.11, when the level of both the nitrogen and phosphorus increases, the PHA production increases. According to the results of El. Sayed *et al*., (2009), PHA accumulation can also take place during active cell growth, but this ability is limited to only a few microorganisms such as *alcaligenes latus* that can accumulate P [3HB] up to 80% of the dry cell weight without limitation of any nutrient. Similar kind of studies was done by Boonsawang and Wongsuvan, (2010) in which they have stated that when the concentration of nitrogen source increases it increases the PHA production. According to results of Wang *et al*., (2007), Grothe *et al*., (1999) the biomass concentration decreased and PHA content increased with increasing C: N ratio.

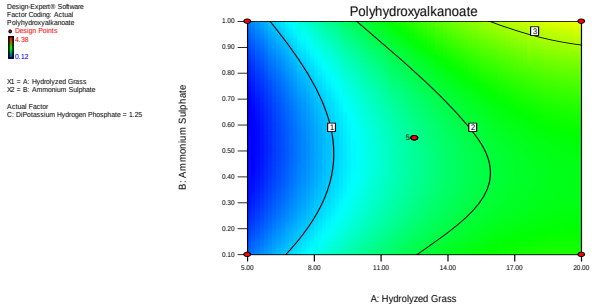


Figure 4.9 Plot between Ammonium sulphate and Hydrolyzed grass on PHA production

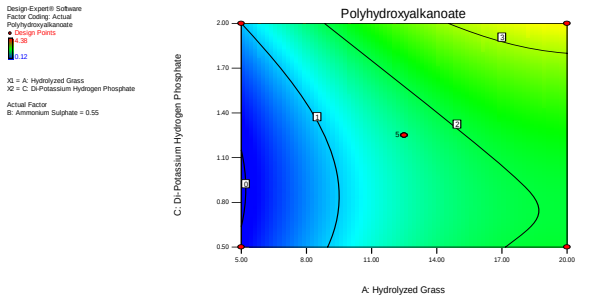


Figure 4.10 Plot between Di-potassium hydrogen phosphate and hydrolyzed grass on PHA production

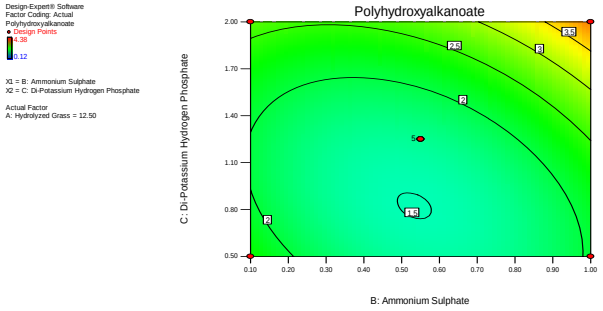


Figure 4.11 Plot between Di-pottasium hydrogen phosphate and Ammonium sulphate on PHA production

4.12.2.1 Model Verification 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. In order to prove, an independent shake flask study was done taking the levels of Hydrolyzed Grass, $(NH_4)_2SO_4$, K_2HPO_4 as 20 (g/L), 1 (g/L), 1.25 (g/L) respectively yielded an average maximum concentration of 4.38 g/L PHA which were compared to predicted response, using the concentrations specified by RSM for the PHA model, of 4.52 g/L of PHA as seen in table 4.14, which has to be optimized further. This concentration of PHA is higher than that obtained in the optimization studies with a wild-type strain of *Rhizobium meliloti* (3.09 g/L PHA) using an excess of sucrose as the carbon source (51.58 g/L) and a limited concentration of phosphate (0.48 g/L K_2HPO_4) (Lakshman *et al*., 2004). Similar results for PHA accumulation were given by Panda *et al*., (2006), who reported PHB content of up to 29% of DCW in *Synechocystis* sp. PCC 6803 by optimizing the nutrient medium composition and Carmona *et al*., (2011), produced PHA concentration of 4.63 g/L by *Pseudomonas aeruginosa* 42A2 (NCIMB 40045) utilizing Agro-industrial residues by optimizing culture medium. Finally, results showed that the predicted and experimental values are not significantly different, indicating that this model is effective.

Table 4.14 Confirmation Report

Response	Prediction	Std Dev	SE (n=1)	95% PI low	95% PI high
PHA	4.51391	0.35278889	0.51570643	3.29445	5.7333573

4.13 Analysis of PHA

The extracted PHA was analyzed for their identification of functional group through FTIR analysis and then was compared with reference sample. FT-IR spectra between 4000–400 cm-1 was done which includes stretching vibrations and deformation of C–H, C=O, N–H, P=O, and C–O–C and which reflect the differences in the biochemical composition of microbial cells i.e., DNA, proteins, polysaccharides and fatty acids (Hong *et al*., 1999; Kansiz *et al*., 1999; Oberreuter *et al*., 2002). From the FTIR spectra (figure 4.12, 4.13) obtained, the band observed in sample found between the spectral range (3570-3200 cm^{-1}), (1055-1000 cm^{-1}/1005-925 cm^{-1}), (1300-1700 cm^{-1}), (1380-1370 cm^{-1}/1470-1430 cm^{-1}), (1120-1300 cm^{-1}) were due to the presence of H-X stretch region, Methylene (>CH_2), C-C vibration, Methyl (C-H) asymmetric/symmetric stretch and carboxylate group respectively (Coates, 2000). According to the results of Berekaa and Thawadi, (2012), the peak found in the sample at 2919.78 corresponds to the presence of aliphatic group (CH_3, CH_2). According to the findings of Coates, (2000) and Sharma *et al*., (2011), the peak found at 1637.43 in the sample corresponds to C=X stretch, similar peak range was obtained by Pandiyan *et al*., (2010) at 1631. 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. According to Sharma *et al*., (2011), the peak found in the sample at 632.55 corresponds to presence of alkene C-H bend. The peak found at 1108.27 in sample was found exactly at the peak range of standard PHA which confirms the presence of PHA.

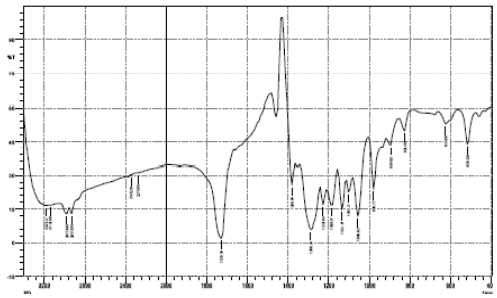
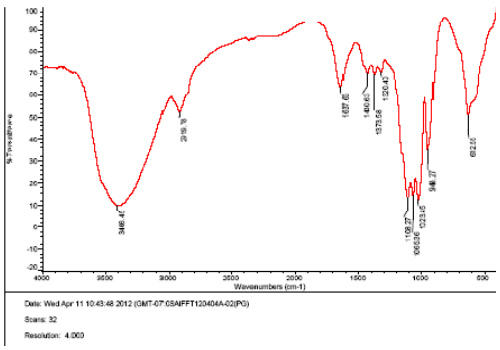


Figure 4.12 FTIR analysis of standard Polyhydroxy-3-Butyric acid



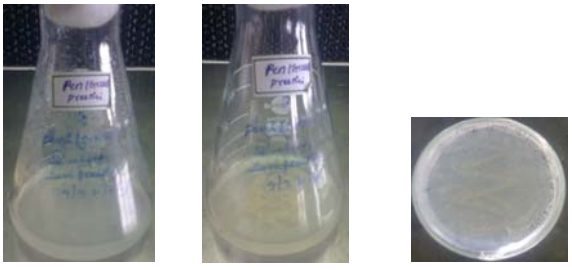
The peaks found were 3406.45, 2919.78, 1637.63, 1430.63, 1373.58, 1320.40, 1108.27, 1065.36, 1023.85 and 948. 27.

Figure 4.13 FTIR analysis of *K. intermedia* produced PHA

4.14 Degradation studies

4.14.1 External degradation

From the figure 4.14, the fungi *Penicillium sp* was made to grow in the liquid media which contains PHA as external carbon source. After 10 days of growth, fungi was then transferred into fresh minimal agar medium containing agar plate and observed for its growth. This confirms the fungi's utilization of PHA as sole carbon source and degrading it. Similar kind of studies was done by Lodhi *et al*., (2005) in which the *Aspergillus* fungi was inoculated in MSM and checked for the degrading capacity through the formation of zone of clearance. Silmilarly the studies of Matavuly and Molitori., (2009) proved that marine isolates as PHA degraders through the same technique.



(A) (B) (C)

(A) Fungi inoculated degradation medium before degradation, (B) fungi inoculated degradation medium after degradation, (C) Observation of growth of *Penicillium* sp. in minimal agar plate.

Figure 4.14 Extracellular degradation studies

4.14.2 Intracellular Degradation

The table 4.15 infers that the degradation happens intracellularly by incubating the PHA production medium with inoculated *Kluyvera intermedia* for more than 2 days, the biomass content and dry weight of PHA extracted from the medium found to be decreased considerably. So, it can be proved that the extracted PHA has the ability to degrade. As similar studies were carried out by Saha *et al*.,(2007), which the degradation studies were carried by calculation % degradation which increases as the mol% of PHA decreases.

Table 4.15 Intracellular degradation studies with changes in wet cell weight and dry weight of PHA

Number of days	Hydrolyzed grass as sole carbon source	
	Wet cell weight(g/50ml)	Dry weight of extracted PHA(g/50ml)
1	1.509±0.230	0.0625±0.067
2	1.8595±0.022	0.3025±0.004
3	1.5205±0.180	0.211±0.032
4	1.1±0.0141	0.07±0.001
5	0.606±0.167	0.038±0.004
6	0.532±0.029	0.015±0.001

CHAPTER 5

CONCLUSION

The efficiency of PHA produced by bacteria depends on the species and media components available in the medium for the bacteria. *Ralstonia eutropha* is taken as a reference strain since it has proved its capacity of converting its 80% of the dry cell weight as PHA.

From the initial 9 isolates, 7 were selected based on PHA accumulation, from which best isolate SP-Y1 (later identified as *Kluyvera intermedia*) was taken for all experimental studies. Using minimal salt media with the supplementation of glucose as carbon source, *Kluyvera intermedia* produces comparatively similar amount of PHA as of reference strain *Ralstonia eutropha* 0.82 mg/L and 1.8 mg/L respectively. In-order to reduce the production cost Bermuda grass was selected as cheaper substrate and hydrolyzed to glucose for the utilization of substrate for microbial growth.

The isolates were compared with *R. eutropha* for their efficiency of maximum PHA production by utilizing the minimal medium containing combination of glucose and hydrolyzed grass and hydrolyzed grass alone. It was found that the % PHA accumulated by *K. intermedia* was 26.76 and reference strain was 28.97%. Media Optimization studies were carried out in-order to maximize the production and finally it was found out the concentration of Hydrolyzed Grass, $(NH_4)_2SO_4$, K_2HPO_4 as 20 (g/L), 1 (g/L), 1.25 (g/L) when added with other constant concentration of other components in the production produced a yield of 4.38g/L PHA. Through FTIR analysis and the extracted PHA's functional group was identified. Then degradation studies were carried out both intracellularly and extracellularly and thus proved that the PHA has the ability to degrade when present in environmental conditions too. Thus, the identified isolate *Kluyver intermedia* utilizing the selected substrate can be used as a better PHA producer under economical and optimized conditions.

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