OPTIMISING PHA PRODUCTION BY MICROBIAL CONSORTIA USING LOW COST RENEWABLE SUBSTRATES AND MUTAGENESIS MEDIATED STRAIN IMPROVEMENT

A PROJECT REPORT

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BONA FIDE CERTIFICATE

Certified that this project report titled **'OPTIMIZING PHA PRODUCTION BY MICROBIAL CONSORTIA USING LOW COST RENEWABLE SUBSTRATES AND MUTAGENESIS MEDIATED STRAIN IMPROVEMENT'** is the bona fide work of **Ms. SANGEETHA, H. S. (Reg. No. 1120203002)** who carried out the research under my supervision. Certified further that to the best of my knowledge, the work reported herein does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an earlier occasion.

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ABSTRACT

Polyhydroxyalkonates (PHAs) are polyesters, excess carbon are accumulated by microorganism under the limiting condition of nitrogen and phosphorous. PHAs are natural polymers having properties similar to synthetic plastics and find wider applications in various fields like tissue engineering, drug delivery systems and packaging. The major issue concerned over their production process is its high cost and lower yield of PHA. Several alternative strategies have been exercised to overcome these problems by using low cost renewable substrates and strain improvement strategies via mutation and genetic engineering making alternation in metabolic engineering has also been investigated recently. The present work deals with the study on effect of mutagenic agents (ultraviolet light, temperature and chemical mutagens) on PHA accumulating organisms (*C. nectar, K. intermedia*) and further production kinetics of PHA accumulation was investigated. Mutation in microbial culture was induced by exposing the culture to UV and temperature at various time intervals and chemicals at varying concentration and cultured using hydrolyzed grass (*cyanodon dactylon*) as a substrate. Effect of chemical mutagenic agent on accumulation was studied using two mutagens acriflavin and 5 bromouracil. It was found that higher accumulation was obtained in *C. nectar* at a concentration of 50µg/ ml acriflavin and 5 bromouracil (25µg/ ml). *K. intermedia* showed a higher accumulation at acriflavin concentration of just 25µg/ ml and 5- bromouracil at 50µg/ ml concentraions. It was observed that % PHA accumulation significantly decreased with increase in exposure to UV in both *C. nectar* (17 % - 1.18%) and

K. intermedia (15 % - 7%). Exposure of culture to heat resulted in less PHA accumulation in *C. nectar* (16 % - 11%), *K. intermedia* (17 % - 19 %) compared to their parent strain *C. nectar* (17 %) and *K. intermedia* (25 %). Three strains were selected for PHA production using consortium namely *C. nectar, K. intermedia* and SP1. It was found that the accumulation % in consortium was less (10.5%) compared to cultures grown individually.

Kinetics study on PHA accumulation profile of *K. intermedia* showed that the organism accumulated a higher amount of PHA at N/C ratio of 0.04 providing an optimum nutrient feeding strategy for increased accumulation. FTIR and NMR analysis revealed the presence of mcl-PHA in the obtained sample.

Key words: PHA - hydrolyzed grass – consortium- mutation - UV- temperature- chemical.

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LIST OF ABBREVATIONS

PHA	Polyhydroxyalkanoate
DNS	Dinitro Salicylic acid
P (3HB)	Poly – 3-hydroxy butyrate
P (4HB)	Poly – 4-hydroxy butyrate
P (3HB-co-3HV)	Poly (3 hydroxy butyrate-co-3 hydroxyvalerate)
P (3HB-co-4HB)	Poly (3 hydroxy butyrate-co-4 hydroxyvalerate)
P (3HB-co-4HHX)	Poly (3 hydroxy butyrate-co-4 hydroxyhexonate)
P (3HB-co-4HD)	Poly (3 hydroxy butyrate-co-4 hydroxydeconate)
PLA	Polylactates
FISH	Fluorescence in situ hybridization
PCR	Polymerase chain reaction
HPLC	High performance liquid chromatography
GC	Gas chromatography
DSC	Disc scanning calorimetry
TGA	Thermogravimetric analysis
UV	Ultra-violet
L	Litre
RSM	Response surface methodology
DCW	Dry cell weight
CDW	Cell dry weight
mg	milligram
µg	microgram
ml	milliliter

CHAPTER 1

INTRODUCTION

1. 1 GENERAL INTRODUCTION

Plastics play an indispensable role in our day today life activity. About 180 million tons of plastic are known to be produced annually worldwide (Volova *et al*., 2010). Accumulation of these non-biodegradable materials in landfills has resulted in leaching of toxic chemicals to environment causing lethal damage to biotic and abiotic components of our ecosystem (Thompson *et al.,* 2009 and Moore., 2008). To overcome the above ill effects, today much research is focused on production of biodegradable plastics.

Biodegradable polymers like polyhydroxyalkonates (PHA), polylactates (PLA) possess physical and mechanical properties similar to synthetic plastics (Poirier *et al*., 1995) and are widely used in manufacturing of packaging films, sutures, cardiovascular stents, scaffold and fixation rods etc., (Scholz, 2000). They are naturally biodegradable to carbon dioxide and methane under aerobic conditions. Analysis on life cycle of PHB showed that the energy required for production is 44.4MJ/kg when compared to polyolefin requiring 73-85MJ/kg resulting in low energy consumption (Harding *et al*., 2010).

Polyhydroxyalkanoates (PHAs) are polyesters accumulated by bacteria under nutrient limiting conditions like Nitrogen, Phosphorous and Potassium with excess carbon source. PHA occurs as an intracellular product either as inclusion bodies or as complexes with calcium and polyphosphates in the cytoplasmic membranes and

constitute for about 80% of the dry cell weight. They occur with irregular morphology of about 0.2 to 0.5µm diameter and can be identified by staining with Sudan black B or using Nile blue A under fluorescent microscopy (Ostle *et al*., 1982). They are classified in to three types based on their composition: short-chain-length PHAs (3-5 carbon), medium-chain-length PHAs (more than 6 carbons) and long chain PHAs (17-18 carbons) (Stubbe *et al*., 2005). The monomer composition of PHA produced and its physio-chemical characteristics depends on the substrate and type of microorganism used for fermentation process (Vallapil *et al*., 2007 and Brandl *et al*., 1988).

Polyhydroxybutyrate was the first PHA to be discovered by Maurice Lemoigne in 1926 in *B.megaterium* (Lemoigne., 1926). Later it was found that *Ralstonia eutropha*, a facultative autotroph accumulated about 80 % of its cell dry weight as PHA from simple carbon sources like glucose, fructose and acetic acid and was widely exploited for large scale production (Anderson *et al.,* 1990). Various other genera including *Acenitobacter, Sphingobacterium, Yokenella, Brochotrix, Lactobacillus, Streptococcus, Lactococcus* etc., (Dalal *et al*., 2010 and Yuksekdag., 2003) has also been reported for PHA production.

The major concern related to PHA production is its high cost of raw materials and low productivity restricting its large scale production in industries (Ojumu *et al*., 2004). Various methods have been developed for production of PHA from cheaply available raw materials like molasses, saw dust etc. These raw materials were hydrolyzed to glucose and used for production process (Albuquerque *et al*., 2007 and Silva *et al*., 2007). PHA was also produced by two step fermentation process of microbial acidogenesis and acid polymerization using starchy waste water as carbon source (Jian *et al*., 2001).

Several strategies were developed to increase the production of polyhydroxyalkonates accumulation in microorganism. Optimization techniques have been carried out for determining optimum nutrient concentration using various designs of experiments (Kathiresan *et al*., 2007). Metabolic engineering has provided a new strategy for increasing PHA production by manipulating genes involved in biosynthetic pathway. In cyanobacteria it has resulted in increased accumulation of about 52% of its cell dry weight as PHA using naturally available sources (Akiyama *et al*., 2011). The biosynthetic pathway for PHB production in microorganism is regulated by three genes phb A, B and C and synthesize three enzymes namely β-ketoacyl-CoA thiolase, acetoacetyl CoA reductase and PHB polymerase respectively. PhaA gene catalyses the synthesis of acetoacetyl–CoA from acetyl– CoA, PhaB stereo specifically reduces acetoacetyl–CoA to (R)-3-hydroxybutyryl– CoA and PhaC promotes the incorporation of (R)-3-hydroxybutyryl– CoA (Lee, 1995). Genetic manipulation in these genes results in increased PHA production (Alexander *et al*., 2012). Studies have been carried out in production of PHB in transgenic plants for attaining maximum biopolymer production (Nawrath *et al*., 1994).

Metabolic engineering of genes by mutation has been investigated for higher accumulation of PHA in microorganisms. Mutation can be brought about by exposure to physical and chemical mutagens. Mutation in *Bacillus sp.,* induced by UV exposure and chemical mutagens like acriflavin and 5-bromourasil resulted in higher production in mutant strains compared parent strains and also showed different protein profiles (Katircioglu *et al*., 2003). PHA production can also be increased by mutation in active site of 3-ketoacylACP synthase III (Alexander *et al*., 2012).

Since metabolic engineering provide a positive strategy for higher PHA accumulation, present work is focused on studying the effect of physical (heat, ultraviolet light) and

chemical mutagens on two major PHA accumulating organisms *C. nectar* and *Kluyvera intermedia*. A study on kinetic analysis profile for PHB production has also been carried out for determining nutrient feeding strategy for maximum PHB accumulation.

1. 2 OBJECTIVES

The main objectives of the study are the following:

1. To study the effect of induced mutation by UV, heat and chemical mutagens on PHA accumulating organisms
2. To determine the production of PHA using consortium of microorganism.
3. To characterize the obtained PHA by analytical methods.
4. To study the kinetics of PHA accumulation by microorganisms.

1. 3 REVIEW OF LITERATURE

Polyhydroxyalkonate (PHAs) are polymers of hydroxyalkanoic acids that are accumulated by microorganism (Eubacteria and Archae) as inclusion bodies or intracellular cytoplasmic granules as carbon and energy reserves or reducing power storage materials (Steinbuchel *et al*., 2003) under excess carbon source and limiting condition of one essential nutrient like nitrogen, phosphorus or iron etc (Anderson and Dawes, 1990). About 150 types of hydrocarboxylic acid have been identified as components of PHA and have attained much commercial interest in production process as hydrolysis of these PHA result in optically pure (R) - form of carboxylic acids which are used in the manufacture of vitamins, antibiotics, perfumes etc., (Lee *et al*., 1995).

1. 3. 1 Structure of PHA

Polyhydroxyalkonate are polyester of various hydroxyalkonates and are hydroxylated at position 3, 4, 5, 6 all of which are (R) chiral molecules that are synthesized by both gram positive and gram negative bacteria (Park *et al*., 2005). Poly (3- Hydroxyl Butyrate) was the first polyhydroxyalkonate that was identified in *Bacillus* sp., (Lemoigne, 1926) and is the most widely studied and characterized PHA.

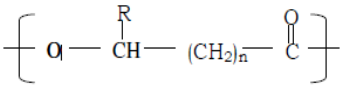


Fig 1. 3. 1 Structure of PHA (Lee, 1995)

n varies from 600 to 35000

R = hydrogen Poly (3-hydroxypropionate)

R = methyl Poly (3-hydroxybutyrate)

R = ethyl Poly (3-hydroxyvalerate)

R = pentyl Poly (3-hydroxyoctanoate)

R = nonyl Poly (3-hydroxydodecanote.

PHA are classified in to various classes based on the number of carbon atoms present in the monomeric unit and are typed as follows

i) Short chain length PHAs (scl – PHAs) contain 3-5 carbon atoms
ii) Medium chain length PHAs (mcl- PHAs) contain 6-14 carbon atoms
iii) Long chain length PHAs (lcl- PHAs) contain 17 and 18 carbon atoms

The specificity of PHA molecule produced is based on the nature and concentration of carbon source used for fermentation process. PHA production using propionate and butyrate yield short chain polymers while oils result in medium chain polymers of different specificity in *Pseudomonas* sp., (Queiroz *et al*., 2009).

1. 3. 2 Properties of PHA

PHB has properties similar to polypropylene with three unique features namely thermoplastic processability, resistance to water and 100% biodegradability. The family of PHAs exhibits a wide variety of mechanical properties from hard crystalline to elastic, depending on composition of monomer units which broadens its application area.

Table 1. 3. 1 Properties of major PHAs

Polymer	Melting temperature (˚C)	Glass transition temperature (˚C)	Young's modulus (GPa)	Elongation to break (%)	Tensile strength (MPa)
P(3HB)	180	4	3.5	5	40
P(4HB)	53	-48	149	1000	104
P(3HB-co-3HV)	145	-1	1.2	50	20
P(3HB-co-4HB)	150	-7	-	444	26
P(3HB-co-3HHx)	127	-1	-	400	21
P(3HB-co-3HD)	130	-8	-	680	17

1. 3. 3 Synthesis of PHA

PHA is produced by both biological means and chemical synthesis in vitro. In microorganism PHA is synthesized by four different pathways depending on the microorganism. In *Alcaligenes eutropus* the synthesis is regulated by three genes in which phaA produce β-ketotiolase that brings out condensation of two acetyl CoA molecules resulting in acetoacetyl-CoA. An NADPH – dependent acetoacetyl - CoA reductase enzyme produced by phaB gene then carries on the conversion of molecule to hydroxylbutryl-CoA. The final polymerization is brought about by PHB synthase or PHB polymerase to form poly (3- hydroxyl butyrate) (Anderson *et al*., 1990). In *Rhodopseudomonas rubrum*, acetoacetyl-Co A thus formed is reduced to

L-(+)-3-hydroxybutryl- CoA which is then converted in to D- (-)-3- hydroxyl butryl CoA by two enoyl CoA hydratases.

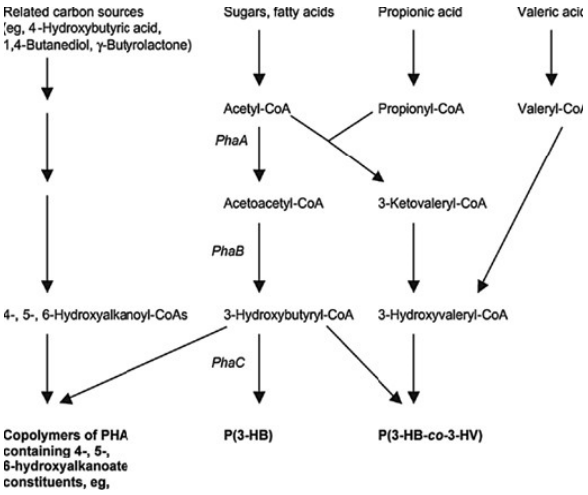


Fig 1. 3. 2 Pathway for biosynthesis of PHA

(Fig 1. 3. 2 Adopted from Lee, S.Y. 1995)

Psuedomonas sp., like *P. oleovarans* exhibit a different pathway by accumulating PHA consisting of 3- hydroxyl alkonoic acids when grown on alkanes, alkanols or alkanoic acids (Lagaveen *et al*., 1988). The fourth type of pathway is found in all *Pseudomonas sp*., accumulate copolyesters consisting of medium chain length polymers from acetyl-CoA.

PHA production has also been reported using ionic liquids with C*andida antartica* lipase B enzyme that bring about ring opening polymerization of lactone (β Propiolactone, δ- Valerolactone and Caprolactone) to poly (6- hydroxyl hexanoate, 3HB) etc.

1. 3. 4 Microorganism producing PHA

Microorganism obtained from oil contaminated soil is known to produce large amount of PHA co polymer like 3- hydroxyl deconate, 3-hydroxyoctanate, 3-hydroxytetra deconate with 49-74 mol% accumulation using sodium gluconate as carbon source. It is produced by microorganism of various genera including *Pseudomonas, Sphingobacterium, Acenitobacter, Brochotrix* and *Yokenella sp*., (Dalal *et al*., 2010). PHA accumulation by thermophilic and mesophilic lactic acid bacteria has also been reported by (Yusekdag *et al*., 2003) and was found that various *lactobacillus sp*., accumulated (0.52 to 25.55%) higher amount of PHB than *lactococcus sp*., (0.61 to 14.81%) and *streptococcus sp* (1.26 to 13.69%). PHB is also synthesized by eukaryotic species such as yeast and are found to be present in complex with polyphosphates in transport membranes (Leaf *et al*., 1996).

1. 3. 5 Methods for screening PHA accumulating organisms

Various simple staining techniques have been developed for easy identification of phosphate accumulating organisms. PHA storage inclusion occurs as refractive granules and can be easily differentiated by staining with lipophilic dyes. Sudan black dye stains all lipophilic granules and stain PHB polymer with black blue color in a clear or light pink background and used for identification of PHB storage polymer in microorganism (Serafim *et al*., 2002). Another dye Nile blue A, an oxazine dye more specific than Sudan black stain intracellular PHA granules specifically and is widely used for screening of PHA accumulating organisms. The oxazine forms of dye is oxidized to its oxazone form (Nile pink) spontaneously in aqueous solution and up on

staining it emit an orange fluorescence with excitation wavelength at 460 nm (Ostle and Holt, 1982). The fluorescence response of Nile blue increases with increasing PHA concentration and used to evaluate variation in PHA accumulation in aerobic / anaerobic experiments.

A rapid method for identification of PHA granules in intact cells can be done by FTIR. The dried biomass is mounted on a KRS-5 window and subjected for analysis. The presence of characteristic peaks around 1728 cm^{-1} and 2925 cm^{-1} in FTIR spectrum provide information about the type and characterization of PHA molecule (Hong *et al*.,1999). FISH along with staining techniques also helps in identification of PHA granules insitu in many microorganisms (Liu *et al*., 2001).

1. 3. 6 Recovery and purification of PHA

Recovery of PHA granule from the culture cells can be done by both solvent extraction process and enzymatic digestion. Enzymatic recovery of PHB was studied by Kapritchkoff *et al*., (2006). He found that the treatment of *R. eutropha* cells with pancreatin yielded 90% purity when compared to bromelain without any degradation of accumulated polymer. Enzymatic recovery resulted in high production cost and making the process not feasible for large scale production industries.

PHAs can be easily extracted from microorganism using chlorinated hydrocarbons like sodium hypochlorite, chloroform, methanol and diethylether. Refluxing with chloroform is the most common method used for recovery in which the culture is filtered, concentrated and finally obtained granules are precipitated using methanol or acetone (Brandl *et al*., 1987). Another method involving sodium hypochlorite treatment removes all cell components by digestion resulting in only PHA molecule that is later obtained using solvent extraction (Hahn *et al*., 1994). Acetone extraction of medium chain length polymer has been reported in *Pseudomonas sp*., using methanol as a solvent resulting in higher PHA recovery efficiency (Jiang *et al*., 2006).

1. 3. 7 Quantification of polyhydroxyalkonate

Gravimetric method was the first PHA quantification analytical method that was developed by (lemoigne, 1926), which involved chloroform extraction of PHA from lyophilized biomass followed by precipitation with diethyl ether or acetone. Later in 1958, another method was developed in which all cell components was digested on treatment with sodium hypochlorite (Williamson and Wilkinson, 1958) and were quantified. Further development on this method was done by Law and Slepcky in 1969 in which the extracted PHA molecule was converted in to crotonoic acid and measured at 260nm in UV spectrophotometer.

PHA detection using IR spectroscopy was described by Juttner *et al*., (1975) but require lipid removal before analysis. Gas chromatography can also be used for PHA component analysis which require the sample to be methanolysed under mild acid or alkaline condition to form hydroxyalkonate methyl ester which are then analyzed by GC. Other methods for PHA quantification include HPLC (Karr *et al*., 1983), ionic chromatography and enzymatic determination (Hesselman *et al*., 1999). Two dimensional fluorescence spectroscopy and flow cytometry have also been proposed for quantification of PHA inside intact cells by staining with Nile blue (Degelau *et al*., 1995 and Gorenflo *et al*., 1999).

1. 3. 8 PHA production by microbial consortium

Use of mixed culture in PHA production provides a low cost alternative strategy for increased PHA production. The effectiveness of mixed culture for production relies on the selection of microorganisms and conditions imposed on the reactor (Johnson *et al*., 2009). Mixed cultures are grown under Aerobic Dynamic feeding (ADF) conditions were there is excess of external substrate (feast phase) and limitation (famine) in a reactor (Majone *et al*., 1996). Temperature operating conditions for mixed culture in sequential batch reactor was studied by (Johnson *et al*., 2009) and found that at higher temperature (30˚C) PHB storage capacity was high (84 %) when

compared to low temperature (15˚C and 20˚C) determining an important factor to be monitored in designing a mixed culture production system.

Aerobic consortia obtained from sludge reported higher yield of PHA accumulation in anoxic environment with fermented food waste showed a larger yield (39.6%) compared to unfermented waste (35.6%) in a reactor (Reddy *et al*., 2012).

1. 3. 9 Production of PHA from renewable resources

Since PHA production cost is high, several strategies have been developed for production from various renewable low cost substrates and agro industrial wastes to reduce production cost and to make process economical.

Table 1. 3. 2 PHA production from low cost renewable substrate

Organism used	Substrate	Monomer	Reference
B. cereus	Fructose, Sucrose, Gluconate,	3-hydroxy butyrate, 3-hydroxy valerate,	Vallapil *et al*., 2007;
R. eutropha	Hydrolyzed paddy Straw	Poly (3- HB)	Sandhya *et al*., 2012
A. eutropus	Starchy waste	PHB, P(HB co HV)	Jian *et al*., 2001
B. vesicularis,	Saw dust	PHA	Silva *et al*., 2007
S. macrogoltabida	Beet molasses	(3HB-Co-HV)	Page *et al*., 1992
Azotobacter vinelandii	Whey	P(3HB)	Lee *et al*., 1997

1. 3. 10 Mutational studies

Mutation is any hereditary change in the genetic material of the organism and may be either spontaneous or induced by mutagenic agents like heat, chemicals and ultraviolet light. Exposure to these agents has resulted in both loss and gain of function in many microorganism and mammalian cells. High mutation frequency with maximum synergistic effect was seen when *E. coli* cells exposed to sub lethal temperature of 50˚C for 30 sec along with low dose of irradiation (Sideropolus *et al*., 1968).

Effect of UV exposure on *C. nectar* for production of extracellular R- 3 hydroxybutyric acid was studied by (Ugwu *et al*., 2008) and found that UV caused phaB gene knockout resulting in two mutants that produced 18% and 14% higher extracellular R 3-HB than the wild strain when acetoacetate used as a carbon source. Polyhydroxybutyrate accumulation in *B. thuringiensis* (IAM 12077) was also studied on exposure to UV and treatment with acridine orange, were one strain from UV treated produced 1.54 fold increase in production than wild strain while chemical treatment produced two strain with increased yield, 6 strain with decrease in yield and three with no change (Adwitya *et al*., 2008). Thus induced mutations by these agents provide a way for strain improvement strategies in cultures.

1. 3. 11 Chemical characterization of PHA

The PHA obtained from cultures can be quantified and characterized by various analytical techniques as follows

1. 3. 11. 1 FTIR

Fourier transform infrared spectroscopy allows rapid quantification and structure determination of polyhydroxyalkonate (PHA) in samples including mixed cultures there by reducing analytical time for PHA quantification. The presence of strong absorption bands at 1728 cm^{-1} and 1282 cm^{-1} in FTIR spectrum are the main

characteristic of PHB indicating the presence of ester carbonyl group and -CH group in the biopolymer (Vallapil *et al*., 2007). Further absorption bands at 2900- 2850 cm^{-1} contribute to the aliphatic -CH stretch of ester carbonyl group and signals at 1000-1500 cm^{-1} are due to bendings of CH_3 and CH_2 streches of –C-C and C-O (Sanchez *et al*., 2003). FTIR can also be used to find the nature of PHA biopolymer obtained whether crystalline or amorphous by examining the spectra with absorption spectra at 1710-1760 with maximum crest at 1725 and 17 35-1740 respectively (Hernandez *et al*., 2010).

1. 3. 11. 2 Xray diffraction

Xray diffraction study is used to determine the crystalinity of the PHA biopolymer and performed in a x ray generator with Ni filter to provide CuKα radiation (λ=0.1542 nm). The sample is allowed to scan in range of 2θ=10-40˚C at a scan speed of 3˚/min at same scan speed. The crystalline percentage are calculated by integration of semi crystalline and amorphous polymer area in diffractogram (Sanchez *et al*., 2003)

1. 3. 11. 3 HPLC

Karr *et al*., 1983 developed a rapid method for compositional analysis of PHB with sulphuric acid treatment. The samples were treated at 100 ˚C and converted to crotonoic acid and subjected to HPLC with an UV detector at 210nm. It was found that the PHB peak was detected at 12.5min in the spectrum. The above method can be used only for PHB and not for other PHAs because alkanoic acids greater than 3HB cannot be converted in to their respective saturated fatty acids. So, Watanabe *et al*., (2012) developed a new method by treating sample with 1N NaOH and later subjected for analysis at 210 nm. This method resulted in identification of both PHB and medium chain length polymers.

1. 3. 11. 4 DSC

Differential scanning calorimetry can be used to determine the thermal properties of 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 (Tg), melting temperature (Tm), and crystallization temperature on heating (Thc) are obtained. The crystallization temperature on cooling (Tcc) was obtained from the second cooling. The degree of crystallinity (XC) was determined from the ratio of the melting enthalpy of the sample (ΔHm) and the melting enthalpy of 100% crystalline PHB (ΔH°m =146 J/g) (Oliveira *et al*., 2007 and Sankhla *et al*., 2010).

1. 3. 11. 5 NMR

Nuclear magnetic resonance can be used for structure determination of PHA. The samples are dissolved in deuterated chloroform and operated at 400MHz for H^1 and 100 MHz for C^{13}. The chemical shifts are represented in ppm with relative to the signal in the spectrum and used for prediction of monomer composition of PHA (Sanchez *et al*., 2003).

1. 3. 11. 6 Transmission electron microscopy (TEM)

TEM can be used to study the accumulation of PHA inside the cells in-vivo. In this technique harvested cells are prefixed in prepared in 0.1 M phosphate buffer (pH 7.2) and post-fixed in 1% (v/v) osmium tetroxide prepared in 0.1 M phosphate buffer at room temperature for 1 h. Later, a series of dehydration is done using 50%, 75%, and 95% and 100% ethanol and 100% acetone and embedded in Spurr's resin in flat-embedding moulds. Embedded cells were cut into ultra thin sections (about 90 nm) and placed onto copper grids. Before observation, the copper grids containing ultra thin sections of cells were stained with uranyl acetate and lead

citrate. Stained samples were observed under TEM at an acceleration voltage of 80 kV. PHA containing granules appear as dark spots inside cells (Lau *et al*., 2010).

1. 3. 12 Kinetic modeling for PHA production

Various kinetic and mathematical models have been developed to simulate nutrient feeding strategies for favoring maximum PHB accumulation under culture conditions. Kinetics of biopolymer accumulation in *Alcaligenes eutrophus* was studied by (Raje *et al*., 1997) under various C/N ratios. Maximum production was found at 0.09 C/N ratios resulting in a mathematical model providing a new feed strategy for production process. An unstructured kinetic model has been developed for mixed cultures with *L. delbreuckii* and *R. eutropha* were two stage fermentation was brought about by converting glucose to lactate and lactate is consumed for PHA accumulation. The experimental data was fitted to a mathematical model and found that higher PHB accumulation was obtained with periodic change in dissolved oxygen concentration. Metabolic modeling has also been carried out for mixed substrate uptake to study the carbon substrate consumption on PHA accumulation by (Jiang *et al*., 2010).

1. 3. 13 Degradation of PHA

PHAs are naturally biodegradable under aerobic conditions resulting in carbon dioxide and water and under anaerobic conditions, the degradation products are methane and carbon dioxide. In many microorganisms PHA degrading machinery has been found both intracellular and extracellular. In *R. eutropha* the degradation process is known to occur simultaneously along with its biosynthesis under nitrogen limiting conditions (Doi, 1990). Degradation of PHA has been reported by a novel *Nocardiopsis aegyptia* sp. which produce an extracellular PHA depolymerase enzyme that hydrolyze copolymer more rapidly than homopolymer PHB (Ghanem *et al*., 2005). Intracellular PHA degradation machinery has been found in *Paracoccus denitrificans* by Gao *et al*., (2000) that degrade PHA under carbon source starvation. Under these

conditions the microorganism activate phaZ gene that produce depolymerase enzyme resulting in breakdown of these energy reserves in to simpler compounds that are later utilized for endogenous metabolism.

1. 3. 14 Application of PHA

PHA polymers find a wide range of application in packaging, medical, agricultural and fisheries industry. PHAs can be a replacement for many petrochemical products including paper coatings, foils, films and diaphragms (Akaraonye *et al*., 2010). They play a pivotal in medical industries in synthesis of bone marrow scaffolds, ligament and tendons, bone graft substitutes and heart valve scaffolds (Martin *et al*., 2003 and Sodian *et al*., 2000). PHA play an effective role in generation of tissue adhesive and sealants for replacement of severe burn disorders (Sittinger *et al*, 1996) and provide a promising therapy for formation of drug containing micro and nanospheres for targeted drug delivery system for cancer (Zinn *et al*., 2001). PHA exhibit a versatile application in agricultural industry and used as bio degradable carriers for long term dosage of insecticide, herbicides, seedling containers, plastic sheath protecting saplings and tubing for crop irrigation (Lafferty *et al*., 1988).

CHAPTER 2

MATERIALS AND METHODS

2. 1. Chemicals required

Nutrient broth, Nutrient agar, DNS (3, 5, Di- Nitro Salicylic acid), glucose, sodium hydroxide, chloroform, acetone, diethyl ether, methanol, hydrochloric acid, sulphuric acid, zinc chloride, ammonium sulphate, magnesium sulphate, sodium hypochlorite, hydrogen peroxide, potassium sodium tartarate and PHB standard.

2. 2. Methodology

2. 2. 1 Selection of microorganism

Three microorganism were selected for PHA production as follows

1. *C. nectar* (Reference strain)
2. *Kluyvera intermedia*
3. Sp 1

2. 2. 2 Substrate selection

Cyanodon dactylon (Bermuda grass) was collected from KCT campus; Coimbatore, shredded into pieces and dried in an hot air oven at 60 ˚C for about one week, hydrolyzed and used as a substrate.

2. 2. 3 Hydrolysis of substrate

Hydrolysis of the substrate to glucose was done according to the procedure Chen *et al*., (1984). To 25 g of grass, 1% sodium hydroxide (1 L) was added and was boiled, filtered and washed. The obtained residue was boiled and washed again with 1% hydrochloric acid (1 L). The above procedure was repeated for about 4 times. The obtained residue was chlorinated with 50 ml of sodium hypochlorite solution made up

to 1 L with distilled water and kept in dark for about 15 min. The above treatment was repeated once again and the washed residue was treated again with 250 ml of 2 % hydrogen peroxide and kept at room temperature for 5 min and was filtered and washed. The finally obtained cellulose was converted to glucose by treatment with zinc chloride followed by Chen *et al*., (1985). The cellulose residue was treated with 5 ml of 80 % zinc chloride containing 2 % hydrochloric acid after 10 min cellulose gets dissolved, then 5 ml of 2 % hydrochloric acid was added to it, after 10 min of heating cellulose gets hydrolyzed to glucose resulting in a white mass which can be used as a substrate.

2. 2. 4 Estimation of glucose content in hydrolyzed grass

The glucose content in the sample was estimated by 3, 5, Di- Nitro Salicylic acid assay. To a series of test tubes containing standard glucose concentration in range of 200 - 1000 µg/ml, 3 ml of DNS solution was added and kept in water bath for 10 min. The reaction was then stopped by adding 40 % potassium sodium tartarate and absorbance value was taken at 540nm. The same procedure was repeated for hydrolyzed grass and the amount of glucose in the sample was estimated.

2. 2. 5 Screening of microorganisms

The microorganisms were screened for PHA production using Nile blue staining technique (Ostle *et al*., 1982). Nile blue chloride dye (0.5 µg/ml) was dissolved in acetone and was added to culture plate for viable colony staining. PHA producing organisms produce an orange fluorescence under UV Trans - illuminator at 460 nm.

2. 2. 6 Production of PHA

The stock culture were revived in nutrient broth and incubated in shaker for 24 hrs. Later the revived culture was inoculated in mineral salt media containing hydrolyzed grass (10 g/l), glucose (5 g/l), sodium chloride (5 g /l), di - potassium

hydrogen phosphate (5 g/l), ammonium sulphate (1 g/l), potassium chloride (1g/l), magnesium sulphate (1 g /l) in 1L of distilled water with pH 7.4 ± 0.5 and incubated for 48 hrs at room temperature.

2. 2. 7 Extraction of PHA

After incubating the culture for 48 hrs, the cells were centrifuged at 10,000 rpm for 5 min and the pellet obtained was dried and weighed. Extraction was done using chloroform where 3 ml of the culture was transferred to a glass centrifuge tube (tubes were washed with acetone and methanol if plastic tubes were used to remove plasticizers) and centrifuged at 10000rpm for 10 min. To the pellet obtained 1ml of sodium hypochlorite solution was added and incubated at 37˚C for about 1 – 2 h for complete digestion of cell components except PHA. The mixture was centrifuged and washed twice with distilled water and three portions of acetone, methanol, diethyl ether (1:1:1). Finally the PHA granule was obtained using boiling chloroform and the solvent was allowed to evaporate (Sasidharan and Santhanum, 2010).

2. 2. 8 Quantification of extracted PHA

Quantification of PHA was done according to Law and Slepecky, (1969). The obtained polymer granule (1mg/ml) was dissolved in concentric sulphuric acid and heated at 100 ˚C for about 10 min and converted to crotonoic acid, which was brown colored. The solution was then read at 260 nm in UV spectrophotometer against concentrated sulphuric acid as a blank. A standard curve was prepared with pure PHB (Sigma Aldrich) concentration ranging from 20 – 100 µg/ml and the amount of PHB produced was determined with the standard.

2. 2. 9 Determination of cell dry weight, residual biomass of PHA

The bacterial culture was centrifuged at 10000 rpm for 5 min to obtain the cell pellet, which was dried to estimate the cell dry weight (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) and can be calculated by the formula as below

Residual biomass (mg/ml) = Dry cell weight (mg/ml) – dry weight of extracted PHA (mg/ml).

2. 2. 10 Determination of % PHA accumulation and PHA concentration

The intracellular PHA accumulation was estimated as ratio of dry weight of PHA extracted to cell dry weight (Sangkharak and Prasertsan, 2008).

$$\textbf{PHA accumulation} = \frac{\text{Dry weight of PHA extracted (mg/ ml)}}{\text{Dry cell weight (mg/ml)}} \times 100$$

PHA mass = Concentration obtained from standard PHB curve (mg/ml).

2. 2. 11 Selection of microbial consortium

The production of PHA using microbial consortium was done by inoculating mixed culture in 250 ml conical flask containing 50 ml of mineral salt medium with glucose as a carbon source. The culture was incubated for 48 h at 30 ˚C and the % PHA accumulation was determined.

The consortium used for culture is as follows

i) *C. nectar*+ *K. intermedia*
ii) *C. nectar* + SP 1
iii) *C. nectar* + *K. intermedia* + SP 1

2. 2. 12 PHA production using hydrolyzed paddy straw as a substrate

PHA was produced by consortium culture using hydrolyzed paddy straw as a substrate. Time period for maximum PHA accumulation was determined by incubating

the culture for 96 h in a shaker at room temperature and samples were withdrawn at specific time intervals and % accumulation was calculated.

2. 3 Effect of various mutagenic agents on PHA production

2. 3. 1 Effect of UV exposure on PHA production

The effect of UV light on PHA production was studied by exposing the culture to UV at various time intervals as followed by Kato *et al*., (1976). Fresh 24 h inoculated culture was spread plated on a sterile petri plate and incubated at 37 ˚C for 24 h in an incubator. Later the plates were transferred to UV chamber and placed at a height of 40 cm from the light source and exposed to UV at different time interval ranging from 5 min to 30 min. The plates after irradiation were immediately transferred to a black box to avoid photo - reactivation by light. The plates were kept inside for a period of 2 h and then the culture was inoculated in production medium containing hydrolyzed grass as a substrate.

2. 3. 2 Heat induced mutation

Mutation in culture can be induced by exposing the culture to high temperature as followed by Sideropoulos *et al*., (1968). One ml of the culture was taken in a sterile test tube and incubated at 60 ˚C for various time interval ranging from 1 min to 4 min and then was allowed to cool at room temperature. The cultures were then inoculated in mineral salt media containing hydrolyzed grass as a carbon source and incubated for 48 hrs and the % PHA accumulation was determined for each time of exposure.

2. 3. 3 Effect of chemicals mutagens on PHA production

Chemical mutagens like acriflavin and 5 bromouracil were used to study their effect on PHA accumulation. One ml of 24 hr culture was collected in a eppendorf tube and centrifuged at 10000 rpm for 5 min. To the pellet obtained, mutagens were added

at various concentrations ranging from 25µg/ml to 100 µg/ml and left for about one hour. Later the exposed culture was centrifuged at 5000 rpm for 10 min to remove the traces of mutagen and suspended in sterile saline and was inoculated in minimal salt media containing hydrolyzed grass as a substrate. The culture was incubated and PHA accumulation was determined.

2. 4 Kinetic studies

The kinetic profile for PHA production was carried out by growing cultures at various C/N ratios ranging from 0.02 to 0.1. The work was carried out in a 500 ml of Erlenmeyer flask containing 100 ml of minimal salt media inoculated with culture and incubated at room temperature. Biomass concentration, glucose estimation, protein content and PHB concentration was determined for cultures at 2hr time interval.

2. 4. 1. Biomass estimation

Biomass estimation was done by measuring OD at 660 nm using visible spectrophotometer.

2. 4. 2. Glucose concentration

The amount of residual glucose in media was estimated by 3, 5, Di- Nitro Salicylic method. To 0.2 ml of sample supernatant DNS was added and incubated at 90˚ C for 15 min. One ml of Rochelle salt was added and absorbance was measured at 540 nm using visible spectrophotometer.

2. 4. 5. Quantification of PHB

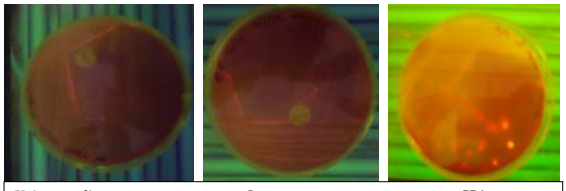
PHB concentration was determined by spectrophotometer by converting it in to crotonoic acid by heating with sulphuric acid according to the procedure Law and Slepecky (1969).

CHAPTER 3

RESULTS AND DISCUSSIONS

3. 1 Screening of microorganism for PHA production

PHA accumulating organisms were screened using Nile blue staining technique and cultures produced an orange fluorescence when viewed under UV- transilluminator at 460 nm as shown in fig 3.1 which confirms theability to produce PHA as reported by (Ostle *et al*., 1982).



K. intermedia *C. nectar* SP1

Fig 3. 1 Nile blue staining of PHA accumulating organisms

3. 2 Cellulose hydrolysis

The hydrolysis of grass in to glucose was done based on zinc chloride method and the finally obtained white mass was used as a substrate.

Grass (Before hydrolysis) Glucose substrate (After hydrolysis)

Fig 3. 2 Hydrolysis of grass

3. 3 Determination of glucose by DNS method

The amount of glucose in the hydrolyzed sample was estimated from the standard glucose curve and the concentration was found to be 3700 µg/ml.

3. 4 Extraction of PHA

PHA was obtained using chloroform extraction method using sodium hypochlorite digestion of cell components resulting in PHA granule (figure 3. 3). The PHA granule thus obtained was ivory white colored powder.

Fig 3. 3 PHA granules obtained after extraction

3. 5 PHA production using microbial consortium

The amount of PHA accumulated by microbial consortia was determined using glucose as a carbon source and was found that amount of PHA accumulation was less in consortium (10.55 %) compared to the amount of PHA accumulated by 3 isolates when grown individually (*C. nectar*, *K. intermedia*, SP1) as shown in fig 3. 4. The decrease in production of PHA may be due to the competitive inhibition between the isolates and uptake of glucose for biomass growth rather than for PHA accumulation.

Table 3. 1 PHA production in microbial consortium

Microorganism	Cell dry weight (g/100ml)	Dry weight of PHA extracted (g/100ml)	% PHA accumulation
K. intermedia	0.70 ± 0.014	0.08 ± 0.028	11.47 ± 0.042
C. nectar	2.69 ± 0.028	0.36 ± 0.014	13.38 ± 0.006
SP 1	0.255 ±0.035	0.02 ± 0.01	7.53 ± 0.045
K. intermedia + *C. nectar*	1.65 ± 0.042	0.17 ± 0.014	10.29 ± 0.005
C.nectar + SP1	1.175 ± 0.049	0.115 ± 0.021	9.75 ± 0.013
All three	2.46 ± 0.021	0.26 ± 0.028	10.55 ± 0.012

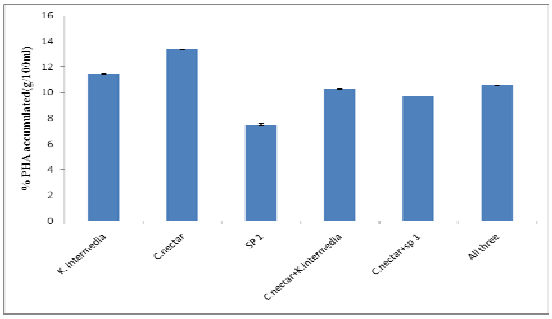


Fig 3. 4 PHA accumulation (%) in microbial consortia

3. 6 PHA accumulation using paddy straw as a substrate

Hydrolyzed paddy straw was used as a substrate for PHA production and was observed that maximum amount of PHA accumulated at 72 h of incubation similar to the result obtained by Sandhya *et al*., (2012).

Table 3. 2 PHA production using paddy straw as substrate

Micro-organism	Incubation time (hrs)	Cell dry weight (g/l)	Dry weight of PHA (g/ l)	%PHA accumulation
All three	48	2.53 ± 0.098	0.6 ± 0.19	23.84 ± 0.06
	72	4.17 ± 0.035	1.36 ± 0.31	32.6 ± 0.011
	96	3.28 ± 0.06	0.85 ± 0.30	25.8 ± 0.05
K.intermedia	48	2.765 ± 0.03	0.6 ± 0.1	21. 66 ± 0.04
	72	5.615 ± 0.035	2.22 ± 0.02	39.53± 0.002
	96	3.82 ± 0.042	0.98 ± 0.04	25.77 ± 0.01

3. 7 Effect of UV exposure on PHA production

The effect of UV on PHA accumulation was studied by exposing culture to UV at various time intervals. It was found that the PHA production decreased with increase in exposure time for *C. nectar* and the same result was also obtained for *K. intermedia* (fig 3. 5 and 3. 6). The decrease in PHA accumulation on UV exposure was similar to the result obtained by (Adwitya *et al*., 2008). The decrease in accumulation may be due

to mutation in the gene involved in PHA biosynthetic machinery resulting in decreased catalytic efficiency of the enzyme to produce polymers of PHA.

Table 3. 3 Effect of UV exposure on % PHA accumulation by *C. nectar*

Time of exposure (min)	Cell dry weight (g/l)	Dry weight of PHA extracted (g/l)	% PHA accumulation
0	4.5 ± 0.014	0.156 ± 0.002	17.33 ± 0.04
5	3.10 ± 0.028	0.088 ± 0.003	14.56 ± 0.11
10	2.45 ±0.063	0.068 ±0.001	13.81 ± 0.15
15	2.3 ±0.021	0.009 ± 0.001	4.17 ± 0.25
20	2.1 ± 0.021	0.004 ±0.0007	2.81 ± 0.05
25	1.6 ±0.014	0.002 ±0.001	2.25 ± 0.35
30	1.7 ± 0.007	0.0015 ± 0.0007	1.18 ± 0.09

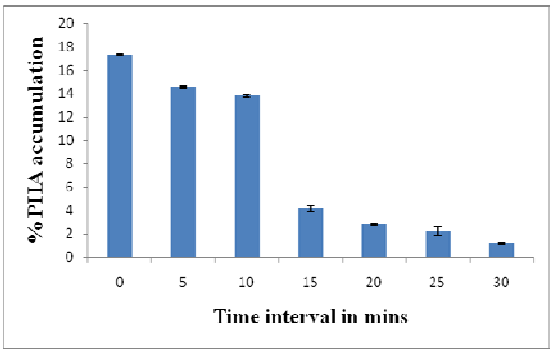


Fig 3. 5 PHA accumulation (%) by *C. nectar* on exposure to UV

Table 3. 4 PHA accumulation by *K. intermedia* on UV exposure

Time of exposure (min)	Cell dry weight (g/l)	Dry weight of PHA extracted (g/l)	% PHA accumulation
0	2.40 ± 0.028	1.56 ± 0.002	15.83 ± 0.3
5	1.2 ± 0.014	0.09 ± 0.0007	6.185 ± 0.67
10	0.8 ± 0.014	0.05 ± 0.001	3.090 ± 0.33
15	1.5 ± 0.007	0.19 ± 0.0021	12.67 ± 0.24
20	0.9 ± 0.007	0.11 ± 0.0007	11.00 ± 1.41
25	0.8 ± 0.01	0.06 ± 0.0014	10 .00 ± 0.0
30	0.7 ± 0.007	0.05 ± 0.0007	7.08 ± 0.59

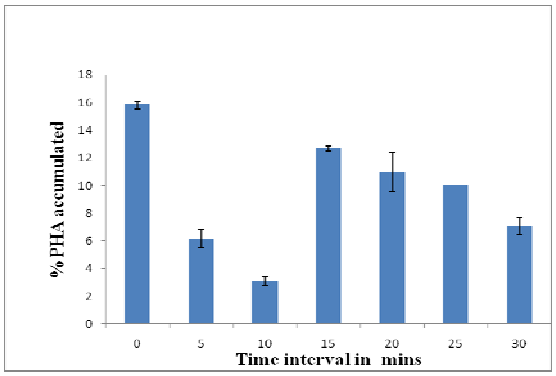


Fig 3. 6. PHA accumulation (%) by *K. intermedia* on exposure to UV

3. 8 Effect of temperature on PHA production

The cultures were exposed to 60 ˚C at various time intervals (0 to 4 min) and then inoculated in mineral salt media with hydrolyzed grass as a substrate. It was found that PHA accumulation in *C. nectar* and *K. intermedia* decreased on exposure to heat at various intervals of time as shown in fig 3. 7 and 3. 8. The exposure of culture to higher temperature for a long period of time would have been resulted in DNA damage resulting in decreased PHA accumulation.

Table 3. 5 Effect of Temperature on % PHA accumulation by *C. nectar*

Time of exposure (min)	Cell dry weight (g/l)	Dry weight of PHA extracted (g/l)	% PHA accumulation
0	3.16 ± 0.007	0.4 ± 0.0035	17.87 ± 0.019
1	0.88 ± 0.006	0.12 ± 0.0042	16.68 ± 0.023
2	1.52 ± 0.005	0.20 ± 0.002	14.79 ± 0.029
3	0.44 ± 0.004	0.06 ± 0.001	11.26 ± 0.010
4	1.98 ± 0.004	0.34 ± 0.002	15.05 ± 0.005

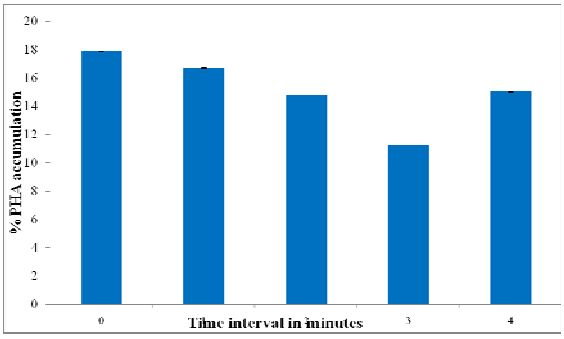


Figure 3. 7 PHA accumulation (%) by *C. nectar* on exposure to temperature

Table 3. 6 PHA accumulation by *K. intermedia* on exposure to temperature

Time of exposure (min)	Cell dry weight (g/l)	Dry weight of PHA extracted (g/l)	% PHA accumulation
0	1.0 ± 0.004	0.28 ± 0.003	25.9 ± 0.04
1	0.64 ± 0.004	0.1 ± 0.001	17.48 ± 0.04
2	0.96 ± 0.009	0.16 ± 0.003	17.14 ± 0.04
3	1.20 ± 0.007	0.12 ± 0.002	20.47 ± 0.09
4	0.24 ± 0.007	0.16 ± 0.001	19.10 ± 0.08

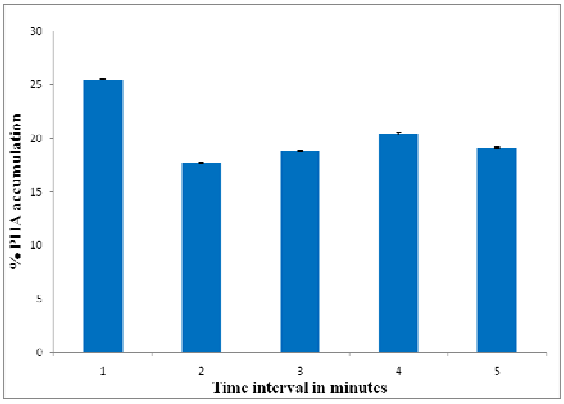


Fig 3. 8 PHA accumulation (%) by *K. intermedia* on exposure to temperature

3. 9 Effect of chemical mutagens on PHA production

Effect of mutagens on PHA accumulation was studied by exposing culture to various mutagenic concentration from 25 µg/ml to 100 µg/ml. It was found that higher PHA accumulation was found in *C. nectar* when chemicals were treated at a concentration of acriflavin (50µg/ ml) and 5 bromouracil (25µg/ ml). *K. intermedia* showed a higher accumulation at acriflavin (25µg/ ml) and 5- bromouracil (50µg/ ml). Increase in chemical concentration after the optimum resulted in decrease in PHA accumulation.

Table 3. 7 Effect of acriflavin on % PHA accumulation by *C. nectar*

Concentration (µg/ ml)	Cell dry weight (g/l)	Dry weight of PHA extracted (g/l)	% PHA accumulation
0	0.33 ± 0.0007	0.05 ± 0.0003	15.25 ± 0.049
25	0.31 ± 0.004	0.06 ± 0.0007	18.85 ± 0.031
50	0.43 ± 0.002	0.15 ± 0.0003	35.20 ± 0.067
75	0.16 ± 0.001	0.03 ± 0.001	18.24 ± 0.056
100	0.13 ± 0.0007	0.02 ± 0.00	15.40 ± 0.016

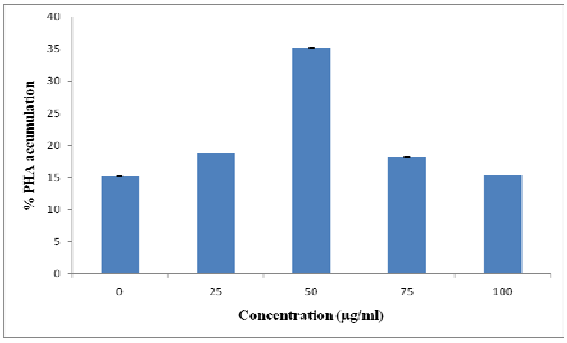


Fig 3. 9 PHA accumulation (%) by *C. nectar* on exposure to acriflavin

Table 3. 8 Effect of acriflavin on % PHA accumulation by *K. intermedia*

Concentration (µg/ ml)	Cell dry weight (g/l)	Dry weight of PHA extracted (g/l)	% PHA accumulation
0	0.16 ± 0.006	0.05 ± 0.001	31.25 ± 0.07
25	0.32 ± 0.001	0.12± 0.0015	37.50 ± 0.055
50	0.23 ± 0.002	0.06 ± 0.0011	26.08 ± 0.07
75	0.22 ± 0.001	0.03 ± 0.0013	13.63 ± 0.082
100	0.16 ± 0.001	0.02 ± 0.0011	12.50 ± 0.022

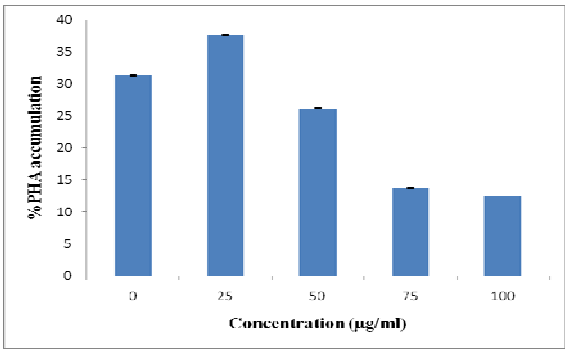


Fig 3. 10 PHA accumulation (%) by *K. intermedia* on exposure to acriflavin

Table 3. 9 Effect 5 bromouracil on % PHA accumulation by *C. nectar*

Concentration (µg/ ml)	Cell dry weight (g/l)	Dry weight of PHA extracted (g/l)	% PHA accumulation
0	0.022 ± 0.01	0.03 ± 0.03	13.63 ± 0.066
25	0.41 ± 0.004	0.07 ± 0.007	17.07 ± 0.068
50	0.23 ± 0.0007	0.03 ± 0.003	13.04 ± 0.043
75	0.23 ± 0.002	0.02 ± 0.0014	12.69 ± 0.069
100	0.58 ± 0.004	0.70 ± 0.003	12.06 ± 0.0007

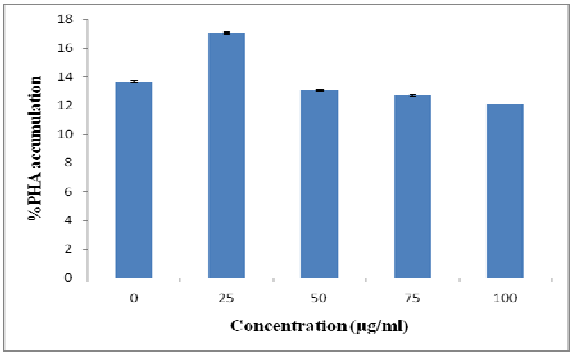


Fig 3. 11 PHA accumulation (%) by *C. nectar* on exposure to 5 bromouracil

Table 3. 10 Effect of 5 bromouracil on % PHA accumulation by *K. intermedia*

Concentration (µg/ ml)	Cell dry weight (g/l)	Dry weight of PHA extracted (g/l)	% PHA accumulation
0	0.13 ± 0.002	0.0015 ± 0.0007	22.50 ± 0.03
25	0.19 ± 0.002	0.002 ± 0.000	21.59 ± 0.048
50	0.38 ± 0.003	0.0045 ± 0.0007	23.12 ± 0.005
75	0.27 ± 0.004	0.0025 ± 0.001	22.22 ± 0.015
100	0.20 ± 0.006	0.0015 ± 0.0007	14.83 ± 0.007

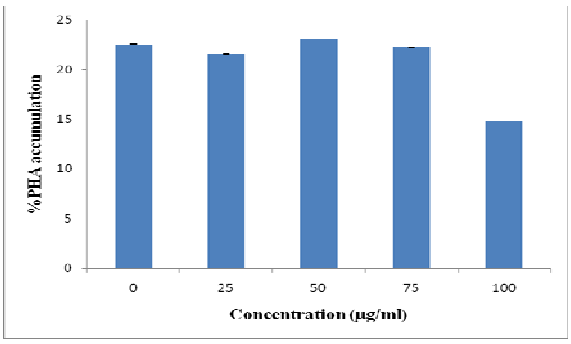


Fig 3. 12 PHA accumulation (%) by *K. intermedia* on exposure to 5 bromouracil

3. 10. Kinetic profile of *K. intermedia* on PHB production

The study was performed with different N/C ratio ranging from 0. 02- 0.1. The culture was inoculated for 48 hrs and sample was withdrawn for every 2 hr time interval and estimated for biomass, glucose and PHA concentration.

3. 10. 1 Biomass concentration

The biomass concentration was measured using spectrophotometer at 660nm. It was found that biomass increased with incubation time and maximum concentration was observed at N/C ratio 0.1(fig 3. 13)

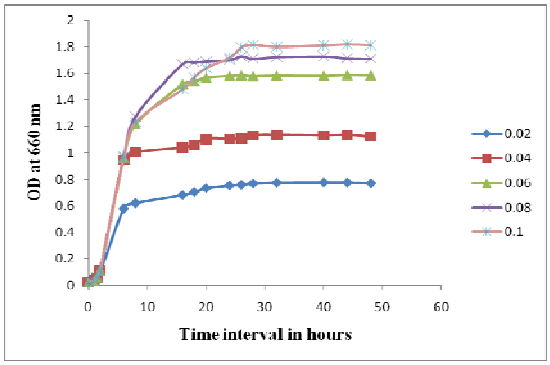


Fig 3. 13 Growth curve of *K. intermedia* at varying N/C ratio

3. 10. 2 Residual glucose estimation

The amount of residual sugar in culture supernatant was estimated using 3, 5 di- nitrosalicylic acid method. It was found that glucose concentration decreased with increase in biomass concentration as it is utilised for bacterial growth (fig 3. 14)

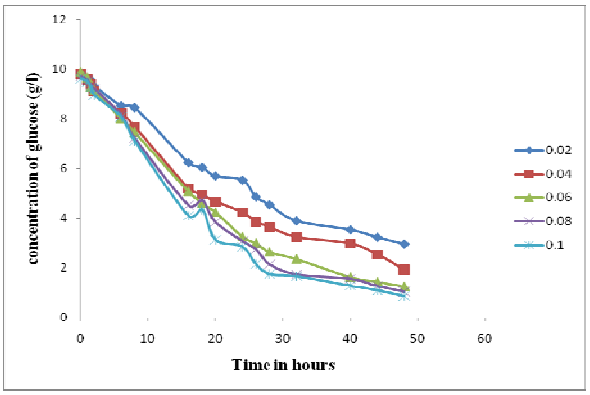


Fig 3. 14 Residual glucose concentration at various time intervals

3. 10. 3 Quantification of PHA

The amount of PHA accumulated at each hour time interval was determined by converting it in to crotonoic acid and measured at 260 nm in spectrophotometer.

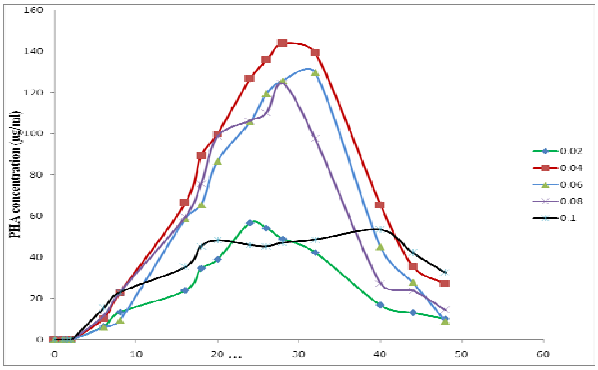


Fig 3. 15 PHA concentration profile at various N/C ratio

From the kinetic profile data, it was found that maximum amount of PHB was accumulated at 28 hr at N/C ratio 0.04. Though maximum biomass was attained at 0.1, the glucose utilized was used for biomass production rather than PHA accumulation as stated by Raje *et al*., (1997). The kinetic profile provide an insight in to the nutrient feeding strategy for maximum PHA accumulation

3. 11 Chemical characterization of PHA

Chemical characterization and structure determination of PHA was carried out by two analytical techniques FTIR and NMR.

3. 11. 1 FTIR

FTIR spectra was recorded in range from 400-4000 cm^{-1}. Fig 3. 16 shows the spectra of standard PHB molecule with a strong signal at 1728 cm^{-1} representing the characteristic peak of PHB. The intense bands at 2980-2850 cm^{-1} correspond to the aliphatic C-H group, medium signals at 1000-1500 cm^{-1} represent the bending due to CH_2 and CH_3, 1280-1050 cm^{-1} due to valence symmetric and asymmetric stretch vibration of C-O-C.

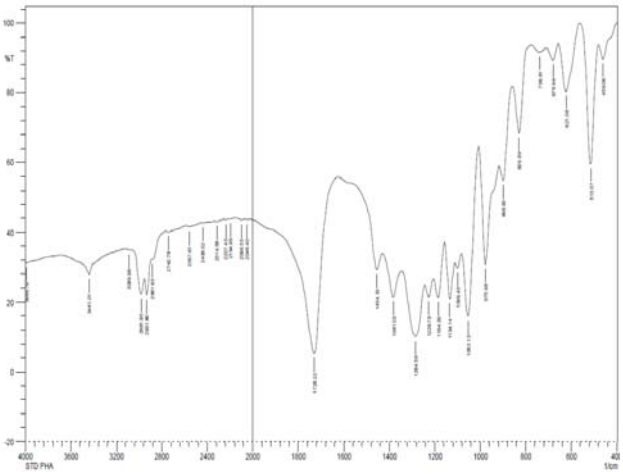


Fig 3. 16 FTIR spectra for standard PHA

Fig 3. 17 and Fig 3. 18 shows the spectra obtained for PHA extracted from *C. nectar* and *K. intermedia.* The absorption bands at 3600-3100 cm^{-1} represent the OH stretch and aliphatic C-H group at 2954 and 2854 cm^{-1} (Sanchez *et al*., 2003) similar to standard PHA. The presence of weak symmetric peaks near 1447- 1380 cm^{-1} suggest the presence of carboxyl group (C=O) in COOH in the polymer representing alkanoic acids in the sample (Noghabi *et al*., 2007). Intense peaks at 1500-1000 cm^{-1} represent the alkene and alkane bendings of C-O stretch.

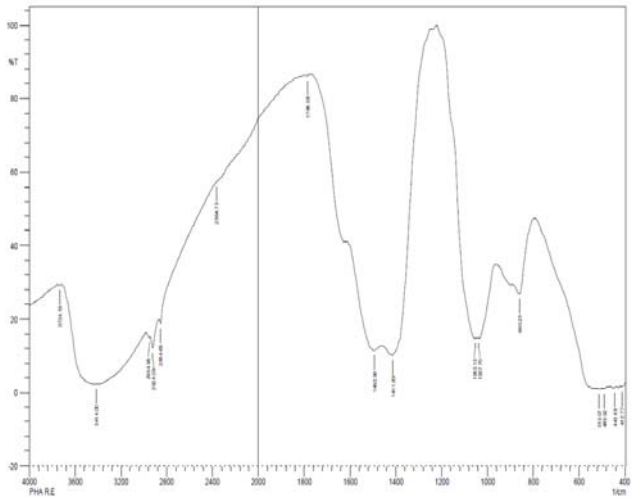


Fig 3. 17 FTIR spectra for PHA isolated from *C. nectar*

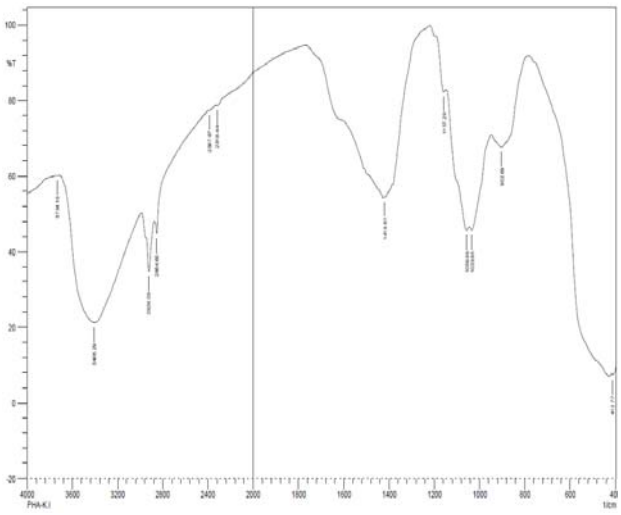


Fig 3. 18 FTIR spectra for PHA isolated from *K. intermedia*

3. 11. 2 NMR

Nuclear Magnetic Resonance spectrum was obtained for H^1proton at 4ooMHz by dissolving in deuterated chloroform. The obtained spectrum was compared with the standard PHB for structure determination.

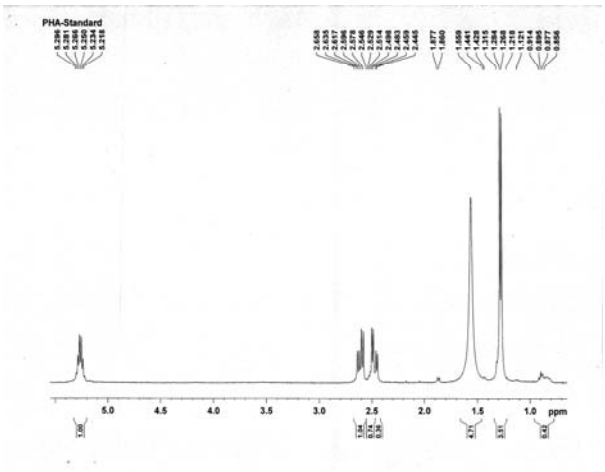


Fig 3. 19 H^1spectra of standard PHB

ppm	Chemical compound	Reference
1.29	CH_3 resonance of PHB	Reddy *et al*., (2012)
1.57	Methylene protons next to methine in side and main chain	Sato *et al*., (2012)
2.54	CH_2 of C_2 carbon (doublet in figure)	Reddy *et al*., (2012)
5.20	Methine (-CH)	Sanchez *et al*., (2003)

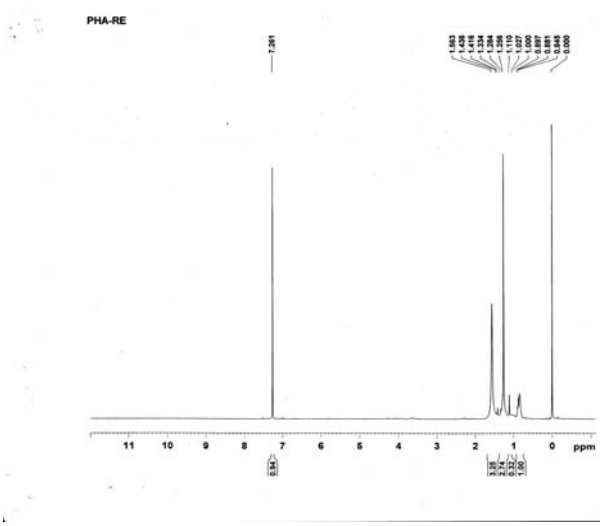


Fig 3. 20 H^1spectra of PHA obtained from *C. nectar*

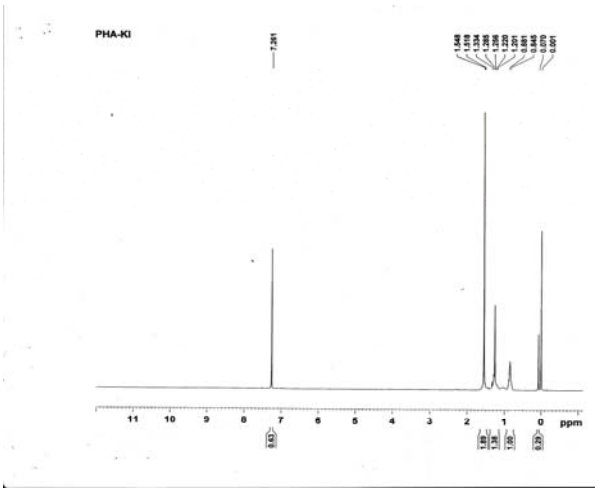


Fig 3. 21 H^1spectra of PHA obtained from *C. nectar*

Fig 3. 20 and 3. 21 represent the spectrum obtained for PHA samples. Both spectra showed characteristic methyl peak of PHB at 1.28 ppm and methylene peaks similar to standard PHB.

CONCLUSION

Effect of mutagenic agents (UV, temperature, chemicals) on PHA accumulation was studied in two microorganism namely *C. nectar* and *K. intermedia* to increase the yeild of PHA. Chemical induced mutation was studied using two chemical mutagens, acriflavin and 5 bromouracil. It was found that *C. nectar* (35. 20%, 17%) and *K. intermedia* (37.5%, 23.12%) treated with acriflavin at 50µg/ ml and bromouracil (25µg/ ml) accumulated a higher amount of PHA compared to their parent strains *C. nectar* (15.25, 13.63%) and *K. intermedia* (31. 25%, 22.50) respectively. Exposure of culture to UV and temperature resulted in decrease in accumulation ratio when compared to their parent strain. Study on kinetic profile of PHA accumulation in *k. intermedia* showed that maximum accumulation was obtained at N/C ratio 0.04. Further increase in N/C ratio resulted in decreased accumulation of PHB were most of the substrate was used for biomass growth rather than for accumulation. FTIR and NMR analysis spectrum revealed the presence of mcl-PHA molecule in sample. Thus treatment of cultures with chemical mutagens and optimum nutrient feeding ratio for increased accumulation provides a new strategy for high yield of PHA production. Further, kinetic analysis of mutant strains will be investigated for high yield of production.

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