MICROBIAL PRODUCTION OF POLYHYDROXYBUTYRATE

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CERTIFICATE

This is to certify that the work presented in the thesis entitled "**Microbial Production of Polyhydroxybutyrate**" is based on the original research done by **Mrs. Nisha V R** under my guidance and supervision in the Biotechnology Division, National Institute for Interdisciplinary Science and Technology (NIIST), CSIR, Trivandrum - 695 019, and that no part of this work has been submitted previously for award of any degree.

Prof. Ashok Pandey

(Research Supervisor)

DECLARATION

I hereby declare that the work presented in this thesis has been carried out by me under the guidance and supervision of Prof. Ashok Pandey, Deputy Director and Head, Biotechnology Division, National Institute for Interdisciplinary Science and Technology (Formerly Regional Research Laboratory), CSIR, Thiruvananthapuram-695 019, India and this work has not been submitted elsewhere for award of any other degree.

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PREFACE

The thesis entitled “Microbial Production of Polyhydroxybutyrate” has been presented into nine chapters. Chapter 1 gives a detailed review of the impact of synthetic plastics on the earth and the importance of the development of a biodegradable plastic and their properties, production strategies and industrial applications. Chapter 2 describes the general materials and methodology used invariably in every chapter. The production of PHB from *Bacillus sphaericus* NII 0838 has been explored by submerged fermentation and solid-state fermentation. The factors responsible for the PHB production have been investigated by statistical approach for screening of process variables and studied their interaction effects. Chapter 3 describes the statistical optimization of the production in SmF and Chapter 4 elaborates PHB production in SSF where inert polyurethane foam (PUF) impregnated with the basal medium used as the solid support. Chapter 5 explains the influence of parameters such as aeration rate and agitation speed on the PHB production by SmF using INFORS HT (750 ml) parallel fermentor and BIOSTAT B (5L) bioreactors. The chapter 6 deals with the purification and characterization of the extracted biopolymer and their blend preparations and also describes the biodegradation of extracted biopolymer and their blends in various environmental samples. Chapter 7 describes the classical mutagenesis for the strain improvement and its stability for hyper production of PHB during successive generations. The chapter 8 depicts the application studies using extracted PHB as a drug delivery system. The whole work has been summarized in Chapter 9. Annexure include list of equipments, media compositions and list of publications.

Nisha V R

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ABBREVIATIONS

%	**Percentage**
^{0}C	**Degree celcious**
ANOVA	**Analysis of Variance**
$CaCl_2$	**Calcium Chloride**
CB	**Cassava bagasse**
CDW	**Cell dry weight**
CFU/ml	**Colony forming units per millilitre**
cm	**Centimeter**
COC	**Coconut oil cake**
$CuSO_4$	**Copper sulphate**
DNS	**Dinitrosalicylic acid**
DO	**Dissolved oxygen**
DSC	**Differential Scanning Colorimetry**
FTIR	**Fourier Transform Infrared Spectroscopy**
g	**Gram**
GOC	**Groundnut oil cake**
h	**Hour**
H_2SO_4	**Sulphuric acid**
H_3BO_3	**Bromic acid**
HCl	**Hydrochloric acid**
JSP	**Jackfruit seed powder**
KH_2PO_4	**Potassium di hydrogen phosphate**
l	**Liter**
Mg	**Magnesium**
µl	**Microliter**

$MgSO_4.7H_2O$	**Magnesium sulphate heptahydrate**
$MnSO_4.H_2O$	**Manganese sulphate**
min	**Minute**
ml	**Milliliter**
N	**Normal**
Na_2HPO_4	**Disodium hydrogen phosphate**
NaOH	**Sodium hydroxide**
$(NH_4)_6Mo_7O_{24}.4H_2O$	**Ammonium molybdate**
$(NH_4)_2SO_4$	**Ammonium sulphate**
nm	**Nanometer**
NMR	**Nuclear Magnetic Reasonance**
pH	**Hydrogen ion concentration**
PHA	**Polyhydroxyalkonate**
PHB	**Polyhydroxybutyrate**
rpm	**Revolutions per minute**
RSM	**Response Surface Methodology**
SEM	**Scanning electron microscopy**
SmF	**Submerged fermentation**
SSF	**Solid -state fermentation**
TGA	**Thermogravimetric Analysis**
UV	**Ultraviolet**
vvm	**Volume of air per volume of fermenter per minute**
WB	**Wheat bran**

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

CHAPTER 1

Introduction and review of literature

1.1 INTRODUCTION

Plastic has been an integral part of our lives by providing a safe and sustainable life and now it is impossible to imagine our days without plastic. From automobiles to medicine, plastics are utilized in almost every manufacturing industry in the world. The versatile qualities of petrochemical-based plastics made them crucial part of mankind. The plastic industry has successively grown continuously for over 50 years. The plastic production increased from 1.5 million tons in 1950 to 230 million tons in 2009. This growth is around 9% a year on average. This considerable growth in plastic use is due to the extensive beneficial properties like extreme durability, lighter weight, resistance to chemicals and biodegradation, good safety and hygiene properties, excellent thermal and electrical insulation properties and most important is relatively inexpensive to produce.

As we all know, nothing is perfect and the same for plastic also. The difficulty in disposal and the predicted end of oil reserves in close to future along with the high prices make the conventional plastics undesirable. Improper disposal of plastics has threatened natural environment worldwide since long time ago. The accumulation rate of synthetic plastics in environment is 25 million tons per year (Lee et al., 1991). Nature's built in system and self regulation ability cannot tackle novel pollutants like plastics, since nature is unfamiliar to it (Reddy et al., 2003). Plastics have molecular weights ranging from 50 to 1,000 kDa and this excessive molecular size is one of the reasons for their resistance to chemicals and biodegradation and their resultant persistence in soil for

a long time (Atlas, 1993). The disposal methods include source reduction, incineration, recycling, landfills, bio or photo-degradation. Incineration and recycling together divert a substantial fraction of polymer refuse away from landfill. Incineration is expensive and in some cases may produce harmful chemicals like hydrogen chloride and hydrogen cyanide (Johnstone, 1990; Atlas, 1993). Recycling is another option but difficulty in sorting of wide variety of plastic is time consuming and the presence of pigments, coatings, fillers etc limits the use of the recycled materials. Another alternative in practice is the use of landfills which is the safest method. During household uses polymers come into contact with fatty foodstuffs, cleaning products, fecal matter or cosmetics and make polymer to bind chemically to a different substance, and polymer articles spread over large areas, making recovery difficult. For these applications, simple discard after the useful life, ensuring a constant contribution to the landfill problem, or abandon in the environment. But these landfills are now reaching their maximum capacity. The improper disposal pollutes the marine environment also. Several hundred thousand tons of plastics are discarded into marine environment every year and may kill the marine animals, either by choking on plastics they mistake as food or becoming entangled in non-degradable plastics debris. Urbanization has also contributed to the plastic pollution and it is concentrated in cities. Plastic thrown on the land may enter into the drainage lines and choke them resulting into flood in local areas in cities as experienced in 1998 and 2007 in Mumbai, India. Since these plastics are not biodegradable, both our ecosystem and environment are disturbed and polluted by the accumulation of these wastes. In answer to this dilemma, any plastic refuse policy must include solutions other than use of landfills, incineration and recycling feature as parts of such policies. The rationale for the

development and marketing of biodegradable plastics here as the necessity to replace oil-based polymers in view of the problems their after-use persistence pose to our society.

Fig 1.Fur seal caught in plastic debris. Source: http://www.ouramazingplanet.com)

Fig 2. Seabed Pollution. Source:http://coastalcare.org

Hence now the interest is leading towards the development of eco-friendly products such as bioplastic with biodegradable and biocompatible properties and look forward to overcome the crisis.

There is variety of biopolymers with diverse chemical and physical properties. The three types of biodegradable plastics have been synthesized are photodegradable, semi biodegradable, and completely biodegradable. The photodegradable plastics have some light sensitive groups in the backbone of the polymer as additives. The ample ultraviolet radiation (several weeks to months) results in the breakdown of their polymeric structure rendering them open to further bacterial degradation. However the plastics discarded to landfills are still resist biodegradation because the unavailability of sunlight (Kalia et al., 2000). Semi-biodegradable plastics are nothing but the starch-linked plastics where starch acts as cross linking agent to hold the short fragments of polyethylene together. When these are dumped in landfills, bacteria in the soil can disintegrate the starch and release polymer fragments make available for other bacteria for further degradation. In the third type, microorganisms are utilized to form a biopolymer and they include polyhydroxyalkanoates (PHA), polylactides (PLA),

aliphatic polyesters, polysaccharides, copolymers and/or blends of the above. These are the only promising 100% biodegradable polymers (Reddy et al., 2003).

The history of biopolymers goes back at least to Beijerinck in 1888 with the observation of the granules as refractile bodies in bacterial cells under the microscope (Chowdhury, 1963). A French microbiologist Maurice Lemoigne, he was the first who isolated, characterized and described polyhydroxyalkanoates (PHA) as lipid granules in the cytoplasm of a soil *Bacillus* resembling *Bacillus megaterium* in 1925. A reduction in pH was observed when cultures were allowed to autolyse in distilled water. This unknown acid was found to be identical to β-hydroxybutyric acid, which is present in urine of diabetic patients. Subsequently, PHB was identified as the polyester component of the granules (Lemoigne, 1926). About thirty years, interest in PHB was scant and nearly restricted to the description of detection and cell-content estimation methods and to culture conditions that lead to its synthesis and degradation inside *Bacillus* cells. Later in 1958, Macrae and Wilkinson put forward a convincing proposal for a functional role for PHB as storage homopolymer in *Bacillus megaterium* and reported the rapid biodegradability of PHB by *B. cereus* and *B. megaterium* itself (Macrae and Wilkinson, 1958). In the following years, researchers spend much time on PHA, their different forms and their potential applications (Braunegg et al., 1998; Volova, 2004; Scott, 2005; Noda et al., 2005; Pandey et al., 2005; Ren et al., 2005). About 200 microorganisms are able to synthesize PHAs during stationary phase of bacterial growth and accumulated as discrete inclusion bodies within the cytoplasm up to 90% of the cell dry weight. PHA accumulated in response to an imbalanced growth condition such as increased carbon to nitrogen/phosphorous/oxygen/magnesium ratio (Schlegel et al., 1961; Schuster and

Schlegel, 1967; Byrom, 1987; Repaske and Repaske, 1976). When nutrient supplies are imbalanced, it is advantageous for bacteria to store excess nutrients intracellularly, especially as their general fitness is not affected since these inclusion bodies have low solubility and high molecular weight, which exerts negligible osmotic pressure to the bacterial cell (Lara et al., 1999).The number of inclusion bodies found ranged from 8-12 in the cytoplasm of microorganisms under nitrogen limitation (Ballard et al., 1987). The size of inclusion bodies range from 0.2 to 0.5 μm in diameter with a membrane coat about 2 nm thick composed of lipid and protein, representing some 0.5 and 2% respectively, of the granule weight (Boatman, 1964; Lundgren et al., 1964; Wang and Lundgren, 1969; Nuti et al., 1972; Dunlop and Robards, 1973). The polyester, water and fatty acids which are present in the inclusion bodies help prevent crystallization of the PHA within the cell, and proteins (phasins) are involved in the formation and stabilization of PHA inclusions and also function as scaffolding materials (Steinbuechel et al., 1995).These inclusion bodies are generally considered as a sink for carbon and reducing equivalents and thought to function as a redox regulator within the cell. It is also found in yeasts, plants and animals as membrane constituents. These play role in the voltage-gated calcium channels or DNA transport and also protect the bounded macromolecules from degrading enzymes (Dawes and Senior, 1973). Both the polymer synthesizing and degrading enzymes are observed within the cell. Once the limiting nutrient is continue to provide to the cell, these energy storage compounds are degraded and used.

Chemically the PHAs are the monomers of 3-hydroxyalkanoates. An alkanoate is a fatty acid which is a linear molecule containing carbon and hydrogen (an alkane) with

a carboxyl group at one end (making an alkanoate) and a hydroxyl group (OH) at the 3rd carbon (β position), making these beta or 3-hydroxyalkanoates. The hydroxyl group of one monomer is attached to the carboxyl group of another by an ester bond; these plastics are thus polyesters. The monomer units are all in D (-) configuration owing to the stereo specificity of biosynthetic enzymes (Senior et al., 1972; Dawes and Senior, 1973; Oeding and Schlegel, 1973; Wang and Bakken, 1998). General structural formula is given in Fig 1.3. PHA that are of low molecular weight, with its monomer consisting of 4-5 carbon atoms have been designated as short chain length PHA (scl). The name medium chain length PHA is specifying to that members with monomers of 6-16 carbon atoms (mcl PHA) eg. poly (hydroxyoctonate-*co*-hydroxydecanate) or P(HO-*co*-HD) (11-13 carbon atoms). The composition of the resulting PHA may vary as per the growth substrate used (Brandl et al., 1988; Lagaveen et al., 1988; Huisman et al., 1989). The mcl-PHA has much lower level of crystallinity than PHB or P(3HB-3HV) and are more elastic. They have a potentially different range of applications compared to scl-PHA (Gross et al., 1989; Preusting et al., 1990). However the basic structure of PHB is same in all bacteria, *in vivo* the molecular weight varies with growth conditions and the stages in cell cycle (Dunlop and Robards, 1995). The molecular mass of PHAs varies per PHA producer but is generally on the order of 50 to 1,000 kDa. Even though within the cell, PHB exists in a fluid, amorphous state, the extraction procedures using organic solvents make PHB, highly crystalline (Doi, 1995). The main members of the PHA family include PHB, PHV, PLA etc.

A list of biodegradable polyesters which are commercially developed and progressing in development are shown below.

PHA – polyhydroxyalkonate

PHB– polyhydroxybutyrate

PHH – polyhydroxyhaxanoate

PLA – polylactic acid

PCL– polyhydroxy caprolactone

PET– polyethylene terephthalate

PBS– polyhybutylene succinate

PHV– polyhydroxyvalerate

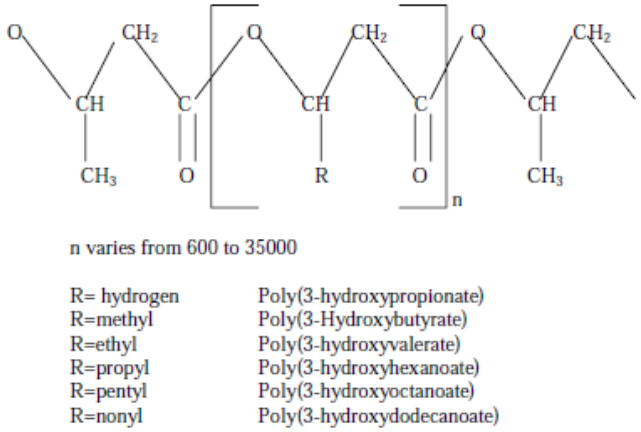


Fig 1.3 PHAs with the general structural formula (Lee, 1995)

1.2 Objectives of the study

- Bioprocess development for the production of polyhydroxybutyrate under submerged and solid-state fermentation
- Studies on polyhydroxybutyrate production in bioreactors
- Purification and characterization of the biopolymer and to improve the physical properties by preparing blends
- Studies on the improvement of *Bacillus sphaericus* NII 0838 by classical mutagenesis
- Studies on application of biopolymer

1.3 Review of literature

1.3.1 Polyhydroxybutyrate

1.3.1.1 Physiological properties

Polyhydroxybutyrate **(**PHB**)** is the best studied and characterized polyhydroxyalkonate and the ability of bacteria to accumulate PHB is often used as a taxonomic characteristic because of its stability (Haward, 1959). Apart from an energy reserve material, PHB supplies energy source for sporulation in *Bacillus* (Slepecky and Law, 1961), to oxidizable substrate for respiratory protection in *Azotobacteria* (Senior and Dawes, 1971). PHB is also present in eukaryotes in a complex form in too minute amounts but its biological function is still in controversy (Reusch, 1992; Seebach et al., 1994). These inclusion bodies are localized in the cell cytoplasm and can be visualized with a phase contrast light microscope when stained with Nile blue A or Sudan Black B. The number of PHB granules found to be remained constant at 8-12 during cell growth under nitrogen starvation. The polymerization of soluble intermediates to insoluble molecules prevents the cell from undergoing alterations of its osmotic state and leakage of these valuable compounds out of the cell. Consequently, the nutrient stores will remain available at a relatively low maintenance cost and with a secured return on investment (Dawes and Senior, 1973; Oeding and Schlegel, 1973; Senior et al., 1972, Senior and Dawes, 1973; Wang and Bakken, 1998). PHB is hydrophobic hence able to resist hydrolytic degradation and shows good oxygen permeability. This fascinating property made them different from other currently available biodegradable plastics which are either water-soluble or moisture sensitive. It posses good ultra-violet resistance but has meager resistance to acids and bases. PHB is soluble in organic solvents such as

chloroform and other chlorinated hydrocarbons. The polymer chains generally form helices, and each granule probably contains a minimum of 1000 molecules. In the solid state, PHB exist as a compact right-handed helix with a two-fold screw axis (i.e. two monomer units complete one turn of the helix) and a fiber repeat of 0.596 nm (Marchessault et al., 1988). The forces underlying this conformation are mainly van der Waals interactions between the carbonyl oxygen and the methyl groups. The stereo regularity of PHB makes it a highly crystalline material. PHB with biological origin are optically active, with the chiral carbon always in the R absolute configuration. Studies of solution-state NMR techniques led the belief that PHB *in vivo* is not crystalline but a mobile amorphous polymer. This property ultimately leads to the rapid and irreversible loss of its degradability by the intracellular depolymerizing enzyme during centrifugation (Barnard and Sanders, 1988). It is biocompatible and non toxic and hence is suitable for medical applications.

1.3.1.2 Mechanical properties

The pure form of PHB is brittle. PHB is a highly stiff, crystalline and relatively brittle thermoplastic. PHB has tensile strength 40 MPa that is close to that of polypropylene, a synthetic plastic (Table 1.1). PHB sinks in water while polypropylene floats. But sinking of PHB facilitates its anaerobic biodegradation in sediments. It possesses a glass transition temperature (Tg) of 5^0C and a high molecular weight. PHB has a melting point (Tm = 175^0C) is only slightly lower than the temperature at which it starts degradation to crotonic acid (approximately 185^0C), making processing difficult. To overcome the poor physical properties of PHB homopolymer, extensive efforts are

being directed towards the synthesis of copolymers that have better properties than PHAs. Incorporation of 3- or 5-carbon monomers into a polymer consisting mainly of 3-hydroxybutyrate leads to a decrease in crystallinity and melting point compared to PHB homopolymer for example in the case of P(3HB-co-3HV). Studies in recombinant strain of *E. coli* harboring the PHA genes of *R. eutropha*, revealed that the accumulated PHB was in a quasi-crystalline form, possibly due to hydrogen bonding to other molecules or cations (Hahn et al., 1995).

Table 1.1 Comparison of properties of different polymers

Polymer	Tm(^{0}C)	Tg(^{0}C)	Tensile strength (MPa)	Crystallinity (%)	Elongation to break (%)
PHB	175	5	40	60	**5**
P(3HB-co-20%3HV)	145	-1	32	56	**50**
P(3HB-co-16%4HB)	150	-7	26	45	**444**
P(3HO-3HH)	61	-36	10	30	**300**
PP	**176**	**-10**	**34.5**	**60**	**400**

1.3.1.3 PHB synthesis pathway

The PHB production involves the following steps (Fig 1.4). The first step is the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by the reversible binding of β-ketoacyl- CoA thiolase (encoded by *phbA*) and is

competitively inhibited by high concentrations of CoASH, which is released by the entering of acetyl-CoA into the TCA cycle (Doi et al., 1988). The second step is the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by *phbB*). Finally, the (*R*)-3-hydroxybutyryl-CoA monomers are polymerized into poly (3-hydroxybutyrate) by PHB polymerase (encoded by *phbC*). In this pathway some microorganisms show some deviation from the main pathway. In *Rhodopsuedomonas rubrum*, the pathway deviated after the second step where the acetoacetyl-CoA 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. The PHB biosynthetic thiolase (acetyl-CoA:acetyl-CoA-acetyl transferase; EC 2.3.1.9) is a member of the family of enzymes involved in the thiolytic cleavage of substrates into acetyl-CoA plus acetyl-CoA. In most *Pseudomonas* sp. belonging to rRNA homology group I, a third type of PHB biosynthesis pathway is operating. They are producing 3-hydroxyalkanoic acid of mcl if the medium is provided with alkanes, alkanoic acids or alkanols (Lagaveen et al., 1988, Brandl et al., 1988, De Smet et al., 1983). The *Pseudomonas* sp. belonging to rRNA homology group II exhibits a fourth type of PHA synthesis. In which co-polyester consisting of mcl 3HAs synthesized from acetyl-CoA is taking place.

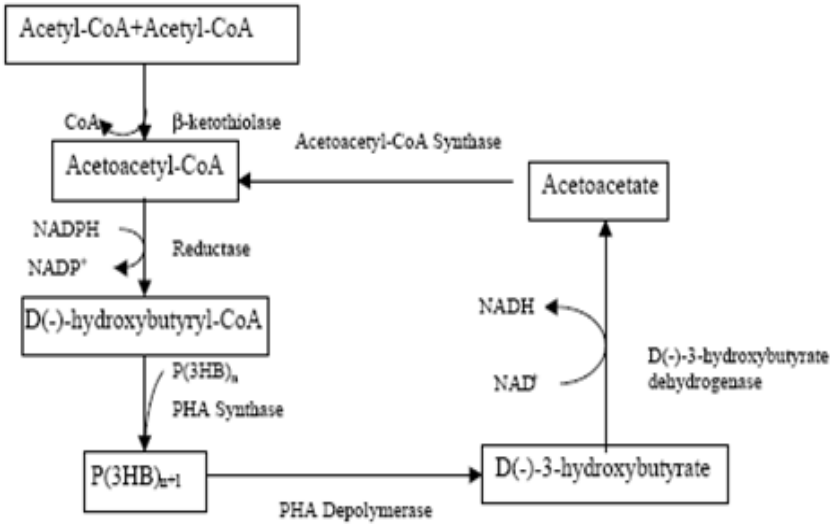


Fig 1.4 Metabolic pathway involved in the synthesis and break down of PHB (Lee, 1995)

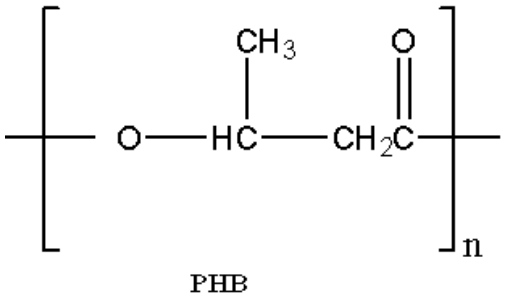


Fig 1.5 Structure of PHB

1.3.1.4 Industry

Although, PHBs have advantages over the conventional plastics, the high production costs and brittle nature have made its widespread use unrealistic. The high production cost relay on the cost of substrate used, and the downstream processing (Lee,

1996).One of the limiting factors in the commercial success of PHB and other PHAs production schemes, is the cost of the sugar substrate used for the biopolymer production It has been calculated that three tons of glucose must be used for each ton of polymer production (Collins, 1987). To reduce the production cost we must pay attention on the selection of better bacterial strain, cheaper raw materials (waste from food processing and agricultural residues) and effective bioprocess development and recovery processes. In 1970s, Imperial Chemical Industries (ICI, UK) started producing PHAs under the trade name BIOPOL. The patents were later sold to Zeneca, then to Monsanto, and are currently property of Metabolix Inc. (USA). BIOPOL® sells at about seventeen times the price of synthetic plastics (Braunegg et al., 1998).

1.3.1.5 Agro residues as potent substrates for fermentative production of PHA

Several naturally occurring agro industrial carbohydrate rich by-products from hydrolysis of starch, and sugar production (molasses) or oily compounds such as soy bean oil, coconut oil, palm oil and glycerol (from biodiesel production) or animal fats have been used to replace expensive glucose substitutes or co-substrates for biopolymer production (Braunegg et al., 1998). If fatty acids supplemented, the products of β-oxidation serves as intermediates for PHA formation. Copolymers with elastic properties are formed by the use of mixed saturated and unsaturated fatty acids in the production medium (Kim and Lenz, 2001). The fermented fruit and vegetable residues (consisting mainly of fatty acids) also can be supplemented as a carbon source for the microorganisms, to reduce the production cost (Ganzeveld et al., 1999; Nonato et al., 2001). A process for the production of PHB and P3HBco-3HV from sugarcane

carbohydrates was developed in Brazil and the technology was transferred to the company PHB Industrial S/A. The use of renewable resources in the production of PHA is advantageous because these biological feed stocks are derived from CO_2 and water and after their conversion to biodegradable PHA; these are ultimately biodegrade to CO_2 and water (Shirai, 1994).

Potato Waste

Starch is an inexpensive, fast biodegradable and abundant product available in nature. The bacterium *Alcaligenes eutrophus* is able to synthesize the biopolymer polyhydroxybutyrate when the enzymatically hydrolyzed potato processing waste used as a fermentable substrate. This is totally degradable in a wide variety of environment and also can used in the development of biodegradable products. Degradation or incineration of starch products recycles the atmospheric CO_2 trapped by starch producing plants during their growth, thus closing the biological carbon cycle (Bastioli, 1998).

Hydrolyzed corn starch

Hydrolyzed corn starch has oligosaccharide and polysaccharide content up to 80% and these can serve as the energy source for bacteria. If glucose ($0.5/kg) is replaced by hydrolysed corn starch ($0.22/kg) for the production of PHB, the cost can be reduced by 24% to $3.72/kg which is $1.19 lower than that obtained with glucose.

Table 1.2 Effect of substrate cost and PHB production

Substarte	Approximate price (US$/kg)	PHB yield % (g PHB/g substarte)	Substrate cost (US$ /kg/) PHB
Glucose	0.493	38	1.30
Sucrose	0.290	40	0.72
Methanol	0.180	43	0.42
Acetic acid	0.595	38	1.56
Ethanol	0.502	50	1.00
Cane molasses	0.220	42	0.52
Cheese whey	0.071	33	0.22
Hemicellulose hydrolysate	0.069	20	0.34

Jackfruit seed

The largest tree borne fruit in the world, jackfruits can sometimes weigh over 75 lbs. Average sized fruits are 1-2 feet long, and 9-12" wide. It is an excellent example of a food prized in some areas of the world and allowed to go to waste in others. There may be 100 or up to 500 seeds in a single fruit. Surplus jackfruit rind is considered a good stock food. In some areas, the jackfruit is fed to cattle. In South India, the jackfruit is a popular food ranking next to the mango and banana in total annual production. There are more than 100,000 trees in backyards and grown for shade in coffee, pepper and cardamom plantations. The total area planted to jackfruit in all India is calculated as

14,826 acres (26,000 ha). Government horticulturists promote the planting of jackfruit trees along highways, waterways and railroads to add to the country's food supply. The composition of jackfruit seed powder is shown in Table 1.3.

Table 1.3 Composition and some physicochemical characteristics of jackfruit flour (% dry weight basis, except moisture) (Tulyathana et al., 2002)

Determination	% Dry weight basis	
	With brown spermoderm	**Without brown spermoderm**
Moisture	7.70±0.20	8.57±0.25
Crude protein	11.02 ±0.46	11.17±0.21
Crude lipid	1.01±0.12	0.99±0.08
Crude fiber	2.36±0.04	1.67±0.11
Ash	3.97±0.04	3.92±0.03
Total carbohydrate	81.64	82.25
Total starch		77.76±0.96
Amylose content of starch		32.05±1.20
Protein content		1.84
pH		5.68
Water absorption capacity (%)		205
Oil absorption capacity (%)		92.6

Cassava bagasse

Cassava (*Manihot esculenta* Crantz)is a root crop of tropical American origin and the fourth most important staple crop in the tropics. Cassava bagasse, which is a fibrous material, is the by-product of the cassava-processing industry. It contains about 30- 50% starch on dry weight basis. Its starchy roots produce more raw starch per unit of land than any other staple crop. Cassava starch is composed of unbranched amylose (20- 5%) and branched amylopectin (80- 5%). Cassava fibrous residue (CFR) contains about 10–15% crude fibre, 55–65% starch and very low ash content (1–1.2%) (Dry weight basis) (Jyothi et al., 2005). Because of its low ash content, CFR could offer numerous advantages in comparison to other crop residues such as rice straw and wheat straw, which have 17.5% and 11.0% ash contents, respectively, for uses in bioconversion processes using microbial cultures (Pandey et al., 2000a, Pandey et al., 2000b).

Wheat bran

Bran is the hard outer layer of grain produced as a by-product of milling in the production of refined grains. Bran is particularly rich in dietary fiber and essential fatty acids and contains significant quantities of starch, protein, vitamins and dietary minerals.

Oil cakes

It is the solid residue that are usually extracted from various types of oily seeds like soya bean, ground nuts, linseed, sesame, cotton seed, coconut, cotton seed and sunflower by being pressed and removing the oil. They are valued for being rich in

minerals and protein. They are rich in fiber and have high concentration of non-starch polysaccharides (NSP). Their chemical composition varies due to the differences in the extraction methods of oil. They are obtained by extraction of oil by means of a solvent from the expeller pressed oil cake. Palm oil industry expelled out palm oil mill effluent (POME) wastewater and that possess high carbon content (BOD higher than 20g/l) with low nitrogen content around 0.2 and 0.5 g/l as ammonia nitrogen and total nitrogen (Ma et al., 2001; Vijayaraghavan and Ahmad, 2006). It has been reported that the use of POME, containing 100 g/l organic acids, as substrate for fed-batch PHA production (Hassan et al., 1997). Sesame is a flowering plant in the genus *Sesamum* and is grown primarily for its oil-rich seeds. The seeds are exceptionally rich in iron, magnesium, manganese, copper, and calcium and contain vitamin B_1 (thiamine) and vitamin E (tocopherol). Ground nut is a species in the legume or "bean" family (*Fabaceae*). The cultivated peanut was probably first domesticated in the valleys of Peru. It is an annual herbaceous plant growing 30 to 50 cm (0.98 to 1.6 ft) tall.

1.3.2 Strategies for PHA production

Selection of fermentation strategies for PHA production depends on bacteria and can be divided into two groups based on the culture conditions required for PHA synthesis. The limitation of an essential nutrient such as nitrogen, phosphorous, magnesium or sulphur is a possible criterion for the synthesis of PHA from an excess carbon source. The bacteria included in this group are *A. eutrophus*, *Protomonas extorquens*, and *Protomonas oleovorans*. The other group of bacteria, which include *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii*, and recombinant *E. coli*, do

not require nutrient limitation for PHA synthesis and can accumulate polymer during growth. These characteristics have to be taken into consideration while production of PHA. The fermentation strategies such as fed-batch or continuous fermentation facilitates the progress of PHA production. For fed-batch culture of bacteria belonging to the first group, a two-step cultivation method is most often employed. A desired concentration of biomass is obtained without nutrient limitation in the first stage after which an essential nutrient is kept in limiting concentration in the second stage, to allow efficient PHA synthesis. During nutrient limitation (second stage), the residual cell concentration remains almost constant and the cell concentration increases only because of the intracellular accumulation of PHA. For the fed-batch culture of bacteria in second group, a nutrient feeding strategy is vital to obtain a high yield of PHAs since PHA synthesis is not dependent on nutrient limitation in these bacteria. A new fed-batch fermentation strategy using a cell recycle membrane system has been developed for the efficient productivity of 4.6g PHB/l/h. In a two-stage fed-batch method, two different micro-organisms growing on two substrates in complex medium to produce PHB was reported by Tanaka et al (1993). In the first stage, a strain of *Lactococcus lactis* was used to convert the pentose xylose to a mixture of lactic and acetic acids. After the removal of cells by (presumably aseptic) centrifugation, *R. eutropha* was used to inoculate the supernatant in the same bioreactor. No nutrient deficiency was present to favor polymer synthesis, but the cells accumulated PHB to up to 55% of their CDW during growth on lactate. In 24 h, 4.7 g/l homopolymer was produced. With such a strategy for PHA production, the extent to which the advantages of the use of an inexpensive substrate like xylose are offset by the additional procedures needed to separate the two microorganisms

must be carefully calculated. New metabolic engineering approaches have been used to expand the spectrum of utilizable substrates and to improve PHA production. These advances expected to lower the price of PHA from the current market price of ca. US$ 16/kg. Kim's group have used on-line glucose control to obtain high-cell-density cultures of *R. eutropha* with high concentrations of PHB and P(3HB-*co*-3HV) (Kim et al., 1991). The glucose concentration was set between 10 and 20 g/l, dissolved oxygen at 20% and nitrogen limitation was used as the inducing factor for polyester storage. Close monitoring of the glucose concentration in the mineral salts medium was achieved by either exit-gas analysis by mass spectrometry and stochiometric deduction of glucose content from CO_2 evolution rate, or with automatic glucose assay of filtered broth samples. By this they could achieve a cell dry weight of 164 g/l with 121 g PHB /l (74%) in 50 h.

The majority (80%) of excess sludge from wastewater treatment plants in Japan is disposed in landfill. The production of biodegradable plastics using excess sludge has been proposed as a substitute. It has been demonstrated that the sludge developed in an anaerobic/aerobic biological phosphorus removal process can produce PHA with a high yield. High-level production of PHB from whey could be achieved by fed-batch culture is a best option for the production of PHB. It has been reported the use of technical oleic acid, waste frying oil, waste-free fatty acids from soybean oil and glucose for growth and PHA accumulation by a new strain *P. aeruginosa* 42A2 . A better growth rate was found with the entire substrates but the PHA accumulation and composition of polymer varied with the substrate supplied. About 66.1% of PHA accumulation can be found when waste-free fatty acids from soybean oil (WFFA) were used as carbon substrate, 29.4%

with waste frying oil (WFO) and 16.8% when glucose was used. The main saturated or unsaturated components of the polymer were C10:0, C12:0 and C8:0 or C12:1 and C14:1, respectively. Depending on the substrate supplied, a wide range of components was observed.

A new method for PHA production was developed in which the nitrogen-deficient paper and pulp wastewater allowed to pass through an aerobic treatment system. Then the dissolved oxygen content level was kept constant in both stages of activated sludge plants ie.14% and 5% in the first and second stages respectively. In both stages, the mean total production of PHA was 41 g/kg, but the PHA from the first stage had a HB/HV ratio of 79:21, whereas for the second stage, the ratio was 55:45 (Asrar et al., 1999).

1.3.3 Biodegradation of PHA

One of the commercially attractive features of PHAs is their degradation in the natural environment. Biodegradability is defined as the capacity to be broken down, especially into innocuous products, by the action of living things—as microorganisms. In the natural environment this task is taking care by bacteria and fungi. They acquire precursors for cell components and energy for their own energy-requiring biological processes from this break down. Biodegradation is thus nothing more than catabolism. Unlike conventional plastic which needs hundreds of years to be fully degraded, bioplastic can be degraded in a relatively short time. The biodegradable thermoplastic properties of PHB have captured attention for more than 20 years, and patents were originally filed in the United States by J. N. Baptist in 1962, the first industrial production of PHB and PHA did not occur until 1982, when ICI plc marketed them under the trade

name BIOPOL. Being the PHAs are bacterial origin, make these polyesters a natural material, and, indeed, many microorganisms have evolved the ability to degrade these macromolecules. Besides its biodegradability, PHAs are recyclable like the petrochemical thermoplastics. PHAs can be completely degraded to carbon dioxide and water and this property makes PHA more reliable. The biodegradability of plastics depends on the raw materials and the chemical composition and structure of the final product, as well as on the environment under which the product is expected to biodegrade. Song et al (2003) has predicted the number of PHBV degrading microorganisms in the soil around $4.3x10^5$/g of dry garden soil, 5.06 $x10^5$/g of dry paddy-field soil and $3.87x10^5$ /g river- bank soil. The PHBV degrading capacity of soil is directly proportional to number of degraders in the soil. The experiments have shown that glucose has also a main role in inhibiting and accelerating the different phases and finally accelerating the biodegradation in soil suspension. PHB and P(3HB-*co*-3HV)s are biodegrading irrespective of aerobic or anaerobic environments (Luzier, 1992). The parameters that influence the biodegradation include monomer composition, crystallinity, additives, surface area, temperature, moist, pH, and nutrient supply (Abe and Doi, 2002). In another report, the maximum biodegradation rates were observed at a moisture level of 55% and temperatures of around 60^0C. A maximum biodegradation can be obtained and up to 85% of the samples degraded within 7 weeks. PHA coated paper was rapidly degraded and incorporated into the compost.

The biodegradation process can be achieved by two different method of bacterial attack on PHB *viz*, intracellular and extracellular. Relatively little has been reported in the literature concerning the intracellular degradation of PHB. In intracellular biodegradation

pathway the first step is the depolymerization of PHB by PHB depolymerases to R-3-hydroxybutyric acid or R-3HB oligomers and further depolymerized to R-3HB monomer by oligomer hydrolase. This monomer is dehydrogenated with NAD^+ into acetoacetic acid. This acetic acid is then esterified with CoA-SH to form acetoacetyl-CoA by the action of acetoacetyl-CoA synthase using ATP .This acetoacetyl-CoA is then degraded into acetyl-CoA by ketothiolase. This enters TCA cycle to transform carbon dioxide and water under aerobic conditions.

In the extracellular degradation pathway the extracellular depolymerases convert PHB into oligomers and a few amount of monomer. The hydsrolase degrade oligomers into the R-3HB monomer (Tanaka et al., 1981). The degradation rate of a piece of PHB is typically on the order of a few months (in anaerobic sewage) to years (in seawater).

1.3.4 Applications

Biomer in Germany now possesses the ownership of the PHB production and processing technology. The PHB from *Alcaligenes latus* is using to produce combs, pens and bullets etc. It is used as packaging films (for food packages), such as bags, containers, paper coatings, film including over wrap, mulch film and used as biodegradable carrier for long term dosage of drugs, medicines, insecticides, herbicides, insecticides or fertilizers (Baptist,1963a, 1963b; Webb, 1990). These films can also be used to make laminates with other polymers such as polyvinyl alcohol (Marchessault, 1996). Diaper back sheet materials and other materials for manufacturing biodegradable or compostable personal hygiene articles from PHB copolymers other than P(3HB-3HV) have been described (Noda, 1996a and 1996b; Shiotani and Kobayashi, 1994). PHAs

have also been processed into fibers which then were used to construct materials such as nonwoven fabrics (Steel et al., 1986). PHB and P(3HB-3HV) have been described as hot-melt adhesives (Kauffman et al., 1992). PHAs with longer-side-chain hydroxyacids have been used in pressure-sensitive adhesive formulations (Rutherford et al., 1997). PHAs can also be used to replace petrochemical polymers in toner and developer compositions (Fuller et al., 1991) or as ion-conducting polymers (Reusch et al., 1993, 1996). PHAs can be used as latex, for instance for paper-coating applications (Marchessault et al., 1995), or can be used to produce dairy cream substitutes (Yalpani, 1993a) or flavor delivery agents in foods (Yalpani, 1993b.). These can be used as a preliminary material for chiral compounds. Medical applications - Surgical pins, sutures, staples, swabs, wound dressings, bone replacements & plates and blood vessel replacements, Stimulation of bone growth by piezoelectric properties. In Japan a new mobile phone with bioplastic casing has gone for sale. The Foma N70liECO phone uses PLA resin reinforced with kenaf fibres to replace oil-based plastic for the case.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

Materials and methods

2.1 MATERIALS

2.1.1 Raw materials

Seven agro-industrial residues, *viz.*, wheat bran (WB), potato starch, sesame oil cake (SOC), groundnut oil cake (GOC), cassava bagasse (CB), jackfruit seed powder (JSP) and corn flour (CF) were assessed for the selection of best substrate for PHB production. Polyurethane foam (PUF) was used as an inert support in solid-state fermentation (SSF) medium. All these were procured locally except cassava bagasse (CB), which was procured from Varalakshmi Starch Industries, Salem).

2.1.2 Chemicals and Reagents

Most of the constituents of the culture medium such as beef extract, yeast extract, saccharides such as fructose, starch, salts such as KH_2PO_4, $CaCl_2$, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$ solvents like ethanol, methanol, acetone, sodium hypochlorite, DMSO, dichloromethane, conc H_2SO_4 and chloroform were procured from Hi-media Laboratories, Merck (India), and SRL Pvt. Ltd. Chemicals for various analytical methods such as 3,5-dinitrosalicylic acid, phenol, sodium sulphite, sodium potassium tartarate, Na_2CO_3, NaOH, $CuSO_4$, Folin Ciocalteau reagent, were obtained from Hi-media Laboratories, SRL Pvt. Ltd., Qualigens and Sd Fine Chemicals. Surfactants such as Tween-80 and polyvinyl alcohol were purchased from Hi-media Laboratories and Sigma Chemical Co., USA, respectively.

2.1.3 Software

The software Design Expert (Version 6.0.6, Stat-Ease Inc., USA) and MINITAB® (version 15, USA) were used for the statistical optimizations.

2.2 GENERAL METHODOLOGY

2.2.1 Microorganism and its maintenance

Bacillus firmus NII 0830, *Bacillus firmus* NII 0829, *Bacillus sphaericus* NII 0838 and *Pseudomonas putida* NII 0914 were used in this study. These were grown on Luria Bertani agar (Annexure I) slants at 30^0C for 24 h. The slants were stored at 4^0C and were sub-cultured every two weeks. The cultures were preserved for longer duration at -80^0C in 80% glycerol.

2.2.2 Preparation of inoculum

A loop full of cells from a 24 h old slant were transferred to50 ml Luria Bertani broth taken in 250 ml Erlenmeyer flasks and kept at 30^0C at 200 rpm. After 18 h incubation, one ml of this culture was used as the inoculum. By serial dilution and plating the number of viable colonies in the inoculum was found to be $8x10^8$ CFU/ml.

2.2.3 Fermentation

The production of polyhydroxybutyrate (PHB) was carried out in submerged fermentation (SmF) and solid-state fermentation (SSF). These have been described in detail in chapter 3 and chapter 4, respectively. The PHB production in SmF was scaled up

to 750 ml using parallel bioreactor and 5L using BIOSTAT B bioreactor as described in chapter 5.

2.2.4 Analytical methods

2.2.4.1 Determination of dry cell weight

For the estimation of dry cell weight, a known quantity of culture broth was taken in a pre-weighed tube and centrifuged at 8,000xg for 15 min. The pellet was re-suspended in distilled water and centrifuged as above. The pellet was lyophilized (Operon, Korea) and then the weight was determined.

2.2.4.2 Estimation of moisture content

The solid substrates used in the study were dried at 80°C for 12 h and the weight of the dried material was recorded. The percent moisture content was calculated as follows.

$$\text{Moisture content of solid substrate} = \frac{(\text{Weight of moist substrate} - \text{Dry weight}) \times 100}{\text{Weight of moist substrate}}$$

2.2.4.3 Estimation of reducing sugar

The reducing sugar was quantitatively measured by the method of Miller (1959).

Reagents

Dinitrosalicylic acid reagent - The reagent was prepared by adding 10 g sodium hydroxide, 10 g 3,5-dinitrosalicylic acid, 2.0 g phenol and 0.5 sodium sulphite in 1000 ml distilled water.

Method

The test samples were suitably diluted and from this 1.0 ml was taken to which 3.0 ml of DNS reagent was added and kept in a boiling water bath for 10 min. To this, 1.0 ml 40% sodium potassium tartarate was added immediately and then the contents were cooled. The color developed was read using a Shimadzu UV-160A spectrophotometer at 575 nm against a reagent blank. Glucose was used as the standard.

2.2.4.4 Estimation of total protein by Folin Lowry method

The quantitative estimation of total soluble protein was done by Folin Lowry method (Lowry et al., 1951).

Reagents

Reagent A – 2% Na_2CO_3 in 0.1 N NaOH

Reagent B – 0.5% $CuSO_4$ in 1% Potassium sodium tartarate

Reagent C – Reagent A and Reagent B in the ratio of 50:1 (v/v)

Regent D – 1N Folin Ciocalteau reagent

Method

The test samples were diluted suitably and 1.0 ml was taken, to which 5.0 ml of reagent C was added and mixed well. The mixture was incubated at 30^0C for 10 min. To this, 0.5 ml of reagent D was added, mixed immediately and incubated in dark for 20 min. The absorbance was read at 600 nm. Crystalline serum albumin was used as the standard.

2.2.4.5 Hydrolysis of raw materials

Wheat bran (WB), potato starch (PS), sesame oil cake (SOC), groundnut oil cake (GOC), cassava bagasse (CB), jackfruit seed powder (JS) and corn flour (CF) were gelatinized, liquefied and saccharified as described by John et al (2006). In brief, the gelatinization was carried out at 100^0C for 15 min, followed by liquefaction with alpha amylase (Novo Termamyl, 5000 IU/ml) at 85^0C, pH 5.0 for 30 min and then saccharification with glucoamylase (Novo AMG, 2000 IU/ml) at 60^0C for 70 min. The hydrolyzate obtained was filtered through a muslin cloth and the clear hydrolyzate containing reducing sugar was used as the sole carbon source for the PHB production.

2.2.4.6 Assay of polyhydroxybutyrate

The assay of polyhydroxybutyrate was done by the method of Slepecky and Law (1960). The pellet was collected by centrifugation at 8,000xg for 20 min and lyophilized. The lyophilized pellet was digested with 30% sodium hypochlorite solution at 37^0C for 20 min. The residue was collected by centrifugation at 8,000xg for 20 min and performed a series of washing steps using distilled water, acetone and finally ethanol. The polymer

was dissolved in chloroform and kept at 30^0C for complete evaporation of chloroform. To this, 5.0 ml conc H_2SO_4 was added and heated at 100^0C for 40 min in a water bath. The resultant crotonic acid was measured at 235 nm against conc H_2SO_4 as blank in a spectrophotometer (Shimadzu UV-160A spectrophotometer).

2.2.4.7 Scanning electron microscopy

The polyurethane foams (PUF) after fermentation and after each washing step were subjected for SEM analysis. The PUF (five samples), were cut into thin pieces and lyophilized. The thin pieces of PUF were gold coated prior to the analysis and the bacterial cells inside the pores of PUF were observed by scanning electron microscopic studies using JEOL JSM 5600LV,115 (Japan) scanning electron microscope.

CHAPTER 3

Bioprocess development for the production of polyhydroxybutyrate UNDER submerged fermentation

CHAPTER 3

Bioprocess development for the production of polyhydroxybutyrate under submerged fermentation

3.1 INTRODUCTION

The high cost of production makes the bioplastic unrealistic to mankind. Industrial application of bioplastic demands higher yield of PHB. Commercially available bioplastic named BIOPOL, a bacterial product from *Ralstonia eutropha*, is marketing about 17 times the price of synthetic plastics (Braunegg, 1998). We need an improved fermentation strategy and recovery processes for the commercialization of PHB. The production cost of any biotechnological process can be considerably reduced by optimization of the process (Sangkharak and Prasertsan, 2007). The PHB production cost evaluation has reported that the cost of carbon substrate (up to 50%) is the major contributor to the overall cost (Choi and Lee, 1997). The use of readily available cheap agro-industrial residues as the carbon sources may reduce the higher cost. The utilization of agro residues as substrate for fermentation has growing interests as they are inexpensive energy rich resources.

Optimization has a very old history and typical method involves changing one factor or varying several factors at the same time. The method of 'one variable at a time' approach allows resolving the specific requirements for the growth and product formation by systematically adding or deleting the components from the medium, with minimal complicated medium interactions (Zhang et al., 1996). The use of organized statistical

approach like response surface methodology (RSM), which use sequential experimental methods, is more reliable than unplanned experiments (Nikel, 2005). Statistical optimization not only allows quick screening of large experimental domain, but also reflects the role of each of the components. Central composite design (CCD) is the most accepted design among several classes of RSM and it offers information into a great extent and reveals overall experiment error in a least number of runs (Montgomery, 1997). RSM is a statistical method that uses quantitative data from appropriate experiments and simultaneously determines and solves multivarient equations. Unfortunately, statistical experiments are not widely used in the optimization of PHB production (Lee, 1996). Some works have been reported for the use of RSM by different bacterial strains like *Rhodobacter sphaeroides* and *Ralstonia eutropha* for increased PHB production (Sangkharak and Prasertsan, 2007; Shilpi, 2005). Optimization of the fermentation medium for *Bacillus* sp. using corn steep liquor as a nitrogen source is reported earlier (Vijayendra, 2007). Increased PHB production using industrial byproducts by *Azotobacter beijerinckii* has been investigated through RSM (Purushothaman, 2001).

This chapter explains the studies carried out on the optimization of the most important fermentation variables using 'one variable at a time' approach and RSM for enhancing the PHB production by *Bacillus sphaericus* NII 0838. In this study the first optimization step dealt with 'one variable at a time' experiment which is used to determine the effect of medium components on biomass and PHB production. In the second step, the factors that had significant effects on both biomass and PHB production were optimized using a CCD and response surface analysis. Three variables, inoculum

age, initial medium pH and substrate concentration (reducing sugar present in the jackfruit seed hydrolyzate) were selected as the process variables and biomass and PHB productions were the response variables.

3.2 MATERIALS AND METHODS

3.2.1 Microorganism and preparation of inoculum

Microorganism, its maintenance and inoculum preparation has been described in chapter 2.

3.2.2 Screening of the microorganisms for PHB production

3.2.2.1 Nile blue staining

In an attempt to select a best PHB producer, Nile blue staining was done as a preliminary step. For this, 1% aqueous solution of Nile blue (Hi-media) was prepared and filtered. A thin smear of 24 h inoculums, from the production medium, was prepared on a glass slide. The smear was heat fixed and stained with the Nile blue A solution at 55^0C for 10 min in a coplin staining jar. After being stained, the slides were washed with tap water and de-stained with 8% aqueous acetic acid for 1 min. The smear was washed and blotted dry with filter paper, remoistened with tap water, and covered with a glass cover slip. The use of cover slip protects the stained cells from immersion oil otherwise the standard immersion oil may remove some of the fluorescent dye and obscure the field with a general yellow fluorescence. The preparation was examined with an epifluorescent microscope (Leica DM 2500) at an excitation wavelength of 460 nm.

3.2.2.2 Submerged fermentation

SmF was carried out in 250 ml Erlenmeyer flask containing 100 ml of production medium. The chemical composition of cultivation medium as follows (g/l): $(NH_4)_2SO_4$, 2; KH_2PO_4, 2; Na_2HPO_4, 0.6; $MgSO_4.7H_2O$, 0.2;$CaCl_2$, 0.02; Yeast extract, 0.2; Fructose, 10 and 1 ml trace element solution. The trace element solution contained (g/l) H_3BO_3, 0.01; $MnSO_4.H_2O$, 0.02; $CuSO_4$, 01; $ZnSO_4.7H_2O$, 0.1; and $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.02. The fructose and trace element solution were autoclaved separately. The initial medium pH was set at 7.0. The flasks containing cultivation medium was sterilized at 121.5^0C for 20 min, inoculated with 2% (8×10^8 CFU/ml) inoculum and incubated at 30^0C under shaking conditions (200 rpm) for 28 h. After fermentation, the samples were withdrawn as whole flasks in triplicate and centrifuged at 8,000×g for 20 min; then, the pellet was collected and lyophilized.

3.2.2.2.1 Assay of PHB

The amount of PHB produced was determined as described in chapter 2.

3.2.3 Process development for culture parameters

3.2.3.1 Identifying the significant variables using 'one variable at a time experiment'

Seven agro-industrial residues, *viz.*, wheat bran, potato starch, sesame oil cake, groundnut oil cake, cassava powder, jackfruit seed powder and corn flour were assessed for selecting the best substrate for PHB production. These were gelatinized, liquefied and saccharified as described in chapter 2.The hydrolyzate obtained was filtered through muslin cloth, and the clear hydrolyzate containing reducing sugar was used as the sole

carbon source for PHB production. The concentration of reducing sugar in the hydrolyzate was adjusted to 1%. The reducing sugar in the hydrolyzate was estimated by dinitrosalicylic acid method using glucose as standard.

Several cultural parameters were evaluated to determine their effect on biomass and PHB production in submerged fermentation. The optimized value for each parameter was selected and kept constant for further experiments. The main strategy used in this regard is medium engineering for which the optimal operating condition of a parameter is optimized by changing one parameter at a time and keeping the others at a constant level (Liu and Tzeng, 1998). The time course (0-72 h) and temperature optimization (25, 30, 35 and 40^0C) studies have been carried out with the production medium containing jackfruit seed hydrolyzates with 1% reducing sugar. The optimization of inoculum age ranging from 12 to 24 h with an interval of 4 h was carried out. The effect of inoculum size on PHB production was studied by using different volumes (1, 2, 3, 4 and 5 ml) of 16 h culture. Effect of initial medium pH on biomass and PHB production was evaluated by varying the pH (4.0, 5.0, 6.0, 7.0 and 8.0) using 0.1N NaOH or HCl. The effect of different inorganic nitrogen sources (2 g/l) (ammonium chloride and urea) were determined and compared with ammonium sulphate (control) and complex sources like corn steep liquor (CSL), peptone and beef extract at a concentration of 2 g/l were studied by replacing yeast extract(control) from the medium. Influence of trace element solution was determined by varying the measure of solution (1 to 10 ml/l) in the fermentation medium. Finally, studies were conducted to determine the effect of concentration of the reducing sugar present in jackfruit seed hydrolyzate for the biomass and PHB production (10 to 40 g/l).

3.2.3.2 Central Composite Design (CCD)

After identifying the potential factors that influenced the bioprocess by "one variable at a time" approach, CCD was selected to resolve their optimum combination. In the present work, the selected variables were inoculum age, pH and reducing sugar concentration present in jackfruit seed hydrolyzate. The CCD of 20 runs with five levels was set using the Design Expert software. All the experiments were done in triplicate, and the average of biomass and PHB production obtained was taken as the dependent variables or responses (Y). The second order polynomial coefficients were calculated and analyzed using the "Design Expert" software (6.0, Stat-Ease Inc., Minneapolis, MN, USA) statistical package. The predicted response can be calculated from the second-degree polynomials, Eq (1), which included all the interaction terms, where Y stands for the response variable, β0 is the offset term, βi represents the coefficient of the linear effect, βii, the coefficient of quadratic effect, and βij the ijth interaction coefficient effect; XiXj are input variables which influence the response variable Y; βi is the ith linear coefficient. Other parameters which have no effect on PHB production were kept constant.

$$Y = \beta_0 + \sum \beta i X\text{i} + \sum \beta ii Xi^2 + \sum \beta ij XiXj \qquad \text{Eq (1)}$$

The experimental design is shown in Table 3.1

Table 3.1 Experimental design for central composite design of response surface methodology

Run	X_1, Inoculum age (h)	X_2, pH	X_3, Substrate (%)
1	12	4	1
2	24	8	1
3	18	6	2.5
4	28	6	2.5
5	12	4	4
6	18	6	2.5
7	18	6	2.5
8	18	6	5
9	12	8	4
10	18	6	2.5
11	18	6	2.5
12	18	6	2.5
13	18	2	2.5
14	18	6	0
15	12	8	1
16	24	8	4
17	24	4	1
18	24	4	4
19	18	9	2.5
20	7	6	2.5

The experimentation was conducted in 250 ml Erlenmeyer flask containing 100 ml of sterile production medium prepared as per the design. The flasks were kept for incubation in an incubator shaker maintained at 30^0C and 200 rpm. Incubation time was ceased at 48 h, and the responses studied were biomass (g/l) and PHB (g/l). All the

experiments were done in triplicates, and the statistical and numerical analyses of the model were executed by means of the analysis of variance (ANOVA). The statistical significance of the model was analyzed by the Fisher's F-test, its associated probability p(F), correlation coefficient R, determination coefficient R^2 which explains the quality of polynomial model. For each variable, the quadratic models were represented as contour plots (3D), and response surface curves were generated.

3.3 RESULTS AND DISCUSSION

3.3.1 Screening of the microorganisms for PHB production

3.3.1.1 Nile blue staining

Nile blue A (Nile blue sulfate, Basic blue 12) is a basic oxazine dye which is soluble in water and ethyl alcohol (Lillie, 1977). Earlier Sudan black B dye was used for the identification of PHB granules inside the cell. Nile blue stain is recognized as superior to Sudan black B. In this study, the PHB granules were fluoresced as bright orange, when stained with Nile blue A and from the Fig 3.1; it was evident that *Bacillus sphaericus* was the best PHB producer with bright orange fluorescence. Bacterial cells which lack PHB granules did not show orange fluorescence. It was reported that cell membranes or other lipid-containing cell components cannot absorb enough of the dye to give a detectable fluorescence (Ostle and Holt, 1982).

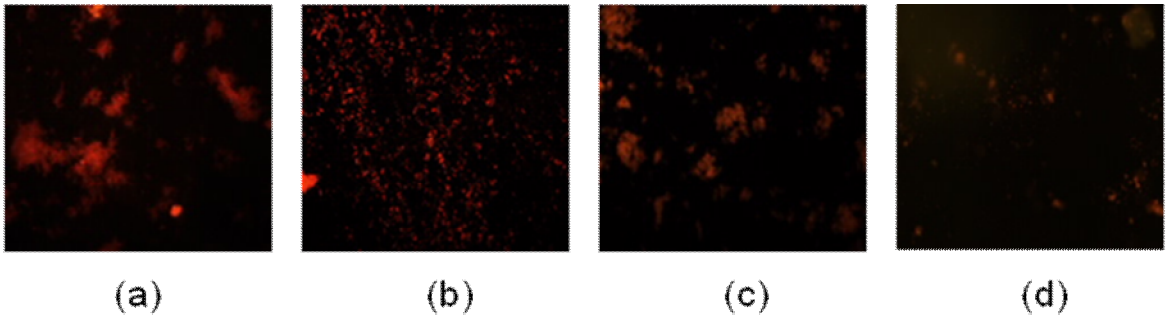


Fig 3.1 PHB granules in (a) *Bacillus firmus* NII 0830 (b) *Bacillus sphaericus* NII 0838 (c) *Pseudomonas putida* NII 0914 (d) *Bacillus firmus* NII 0829 stained with Nile blue A

3.3.1.2 Submerged fermentation

The result of Nile blue staining was confirmed by the submerged fermentation and spectrophotometric assay of PHB. The highest biomass (1.1±0.06 g/l), PHB (0.29±0.004 g/l) and % PHB (25%) was observed in *Bacillus sphaericus* (Fig 3.2). The lowest % PHB according to cell dry weight was in *Bacillus firmus* NII 0829 (7.8%). It was reported that *Bacillus sphaericus* was able to synthesize PHB up to 36.36% (Aslim, 2002). In another study the reported highest value of PHB production by *Bacillus sphaericus* strain was 32.5% (w/v) of cell dry weight (Mercan and Beyatli, 2001).

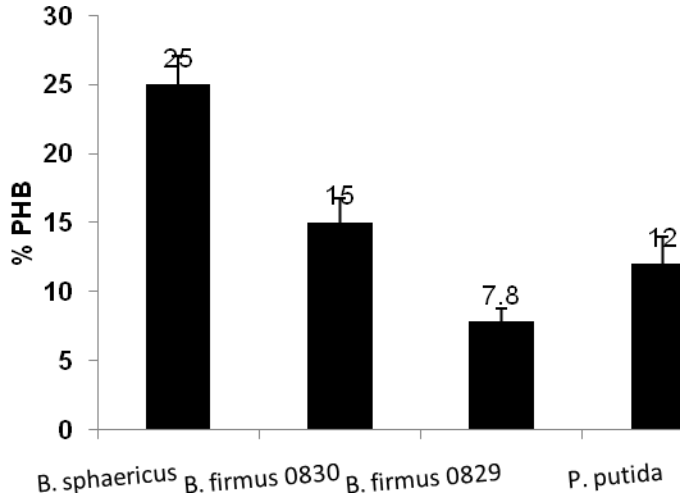


Fig 3.2 Screening of the microorganisms for PHB production in submerged fermentation

3.3.2 Identifying the significant variables using 'one variable at a time experiment'

It is a prerequisite to standardize all the fermentation conditions for the successful implementation of commercial PHB production systems. The "one variable at a time" optimization studies were employed as the first step to confirm the significant factors that influenced the PHB production by *Bacillus sphaericus* NII 0838.

3.3.2.1 Evaluation of agro-industrial residues as the carbon substrate

The use of readily available cheap agro-industrial residues as the carbon sources may reduce the higher production cost. Several studies have shown the utilization of various agro-industrial residues as carbon sources for different bacterial strains. For the effective utilization of agro-industrial residues as fermentation substrates, these should be first subjected for hydrolysis step to release the easily metabolizable sugars. In this preliminary study the maximum biomass production (15.5±0.07 g/l) was observed when

the fructose was replaced by wheat bran hydrolyzate but the PHB concentration was very low (1.06±0.002 g/l). Thuoc et al., (2007) reported that when wheat bran hydrolyzate was used as the carbon source, the PHB concentration was 1.08 g/l, which was almost similar to the result of the present study (1.06±0.002 g/l) (Table 3.2).The higher protein content present in wheat bran and nutrients in yeast extract might facilitated the biomass accumulation. In the present study, there was only slight difference in PHB production in potato starch and jackfruit seed powder. However, jackfruit seed powder was selected as the substrate for further studies due to its relative cheaper cost and also it is a novel substrate for PHB production.

Table 3.2 Screening of for PHB production

Agro -industrial residue	Biomass (g/l)	PHB (g/l)
Corn flour	1.5±0.002	0.049±0.001
Wheat bran	15.5±0.02	1.065±0.002
Cassava bagasse	2.5±0.009	0.161±0.003
Jackfruit seed powder	1.5±0.005	0.69±0.001
Potato starch	1.5±0.004	0.710±0.002
SOC	1.0±0.006	0.146±0.004
GOC	1.5±0.02	0.280±0.007

3.3.2.2. Effect of incubation time on biomass and PHB production

Biomass and PHB production were studied at a range of incubation period from 0-72 h. In the case of *Bacillus* strains it is imperative to determine the optimum

incubation time, because it is believed that till the sporulation stage *Bacillus* can synthesize PHB and then it was consumed and usually sporulation starts at the stationary phase of growth (Benoit, 1990; Nam and Ryu, 1985). From the Fig 3.3 it was evident that PHB production was very low from 0-12 h and after that a drastic increase could be seen. This study revealed that maximum cell density (3.8±0.25 g/l), and PHB (1.68±0.28 g/l) production was at 48 h; hence this was selected as the fermentation time for further studies. A decrease in PHB after 48 h can be explained as a result of PHB utilization by the strain during sporulation.

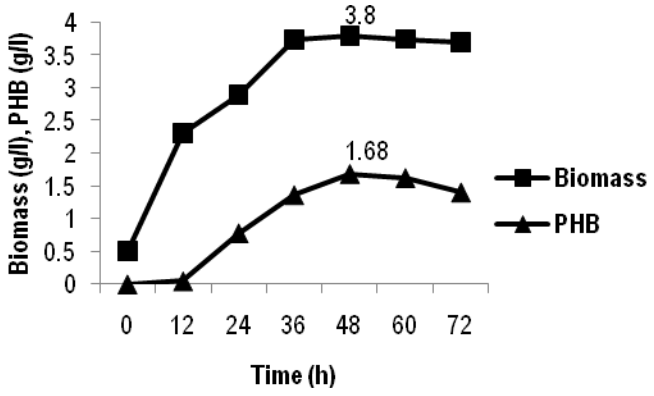


Fig 3.3 Effect of incubation time on biomass and PHB production

3.3.2.3 Effect of incubation temperature on biomass and PHB production

Effect of incubation temperature on biomass and PHB accumulation was studied over a range of temperature 25^0C to 40^0C. The data obtained from the effect of incubation temperature indicated the range of 30–35^0C was suitable for the biomass and PHB production (Fig 3.4). Even though 30 -35^0C range was found to be suitable for PHB

production, 30^0C was selected as optimum temperature for further studies to reduce the production cost. Biomass and PHB production was low at 25^0C and 40^0C.

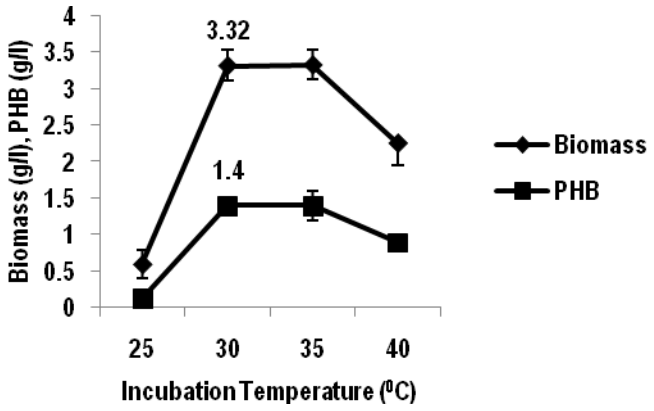


Fig 3.4 Effect of incubation temperature on biomass and PHB production

3.3.2.4 Effect of initial medium pH on biomass and PHB production

It has been reported that initial medium pH significantly influence the PHB production by changing the bioavailability of some of the trace elements (Grothea et al., 1999). Effect of initial pH studies showed that as the pH in the medium increases, PHB production increased up to pH 7.0. *Bacillus sphaericus* showed maximum PHB production (1.82±0.15 g/l) at pH 7.0, and at alkaline pH there was a sharp decrease in both biomass and PHB production (Fig 3.5). It has been reported that pH in the range of 6.0 –7.5 was the best for microbial growth, and the maximum growth and PHB production of *Alcaligenes eutrophus* was reported at optimum pH of 6.9, and the growth declined at pH below 5.4 (Palleroni and Palleroni, 1978).

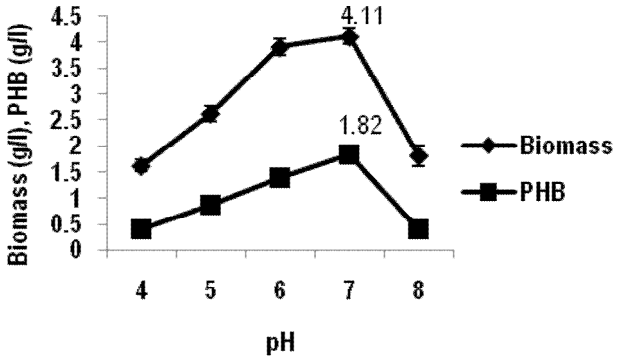


Fig 3.5 Effect of initial medium pH on biomass and PHB production

3.3.2.5 Effect of inoculum age on biomass and PHB production

The effect of inoculum age on growth and PHB production by *Bacillus sphaericus* was studied by inoculating the production medium using 12 -24 h aged inoculum. It is reported that inoculum age affected both the growth rate and PHB accumulation (Kareem et al., 2002). The data represented in Fig 3.6 indicated that the inoculum age of 16 h gave maximum biomass (3.9±0.11 g/l) and PHB production (1.7±0.11 g/l). After 16 h there was an inverse relation for inoculum age and biomass production, since the specific growth rate of older inoculum age was lower and it took longer for the culture to reach stationary phase (Asenjo and Suk, 1986). When these bacterial cells were transferred from actively growing phase to the production medium, the easily assimable carbon source in the medium could facilitated the synthesis of PHB.

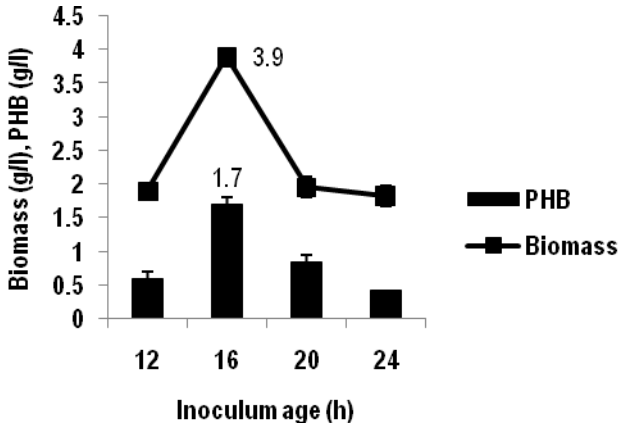


Fig 3.6 Effect of inoculum age on biomass and PHB production

3.3.2.6 Effect of inoculum size on biomass and PHB production

As the effect of inoculum age was studied, it was necessary to study the effect of inoculum size on growth and PHB accumulation. The effect of inoculum size on growth of *Bacillus sphaericus* and PHB production was studied by inoculating the production medium using five increasing size of inoculum of 16 h age, in the production medium. The Fig 3.7 described that 1 ml i.e. $8x10^8$ CFU/ml was responsible for lower production of both the biomass and PHB. This could be due to the lower amount of biomass present in 1 ml inoculums and which might be insufficient to utilize the nutrients present in the production medium. A significant increase was obtained with 2 ml of inoculum size, which gave 3.53±0.24 g/l biomass and 1.63±0.31 g/l (Fig 3.7). Further increase in inoculum size lead to decrease in the biomass accumulation.

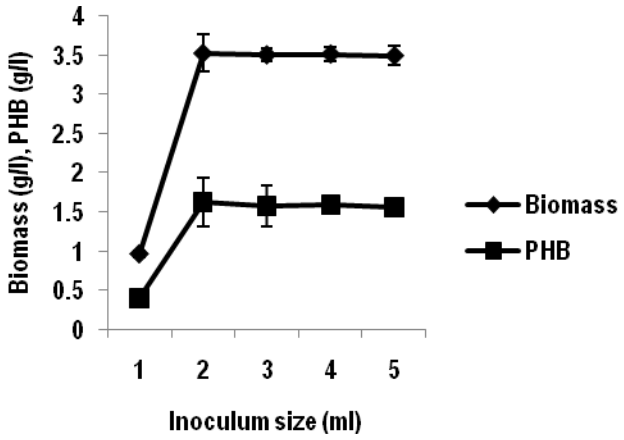


Fig 3.7 Effect of inoculum size on biomass and PHB production

3.3.2.7 Effect of inorganic nitrogen source on biomass and PHB production

In the case of inorganic nitrogen sources, ammonium sulfate (control) gave 3.89±0.11 g/l biomass, and PHB yield was 1.71±0.11 g/l, which was comparable to the result, obtained with *Bacillus megaterium* (Vijayendra et al., 2007). Even though good biomass accumulation was found with ammonium chloride, yield of PHB was not related to the increased growth, which was contradictory to other reports (Gouda et al., 2001). Both the biomass and PHB production was low when ammonium sulphate was replaced by urea; this could be due to the inability of *Bacillus sphaericus* to metabolize urea effectively, as the sole source of nitrogen (Fig 3.8).

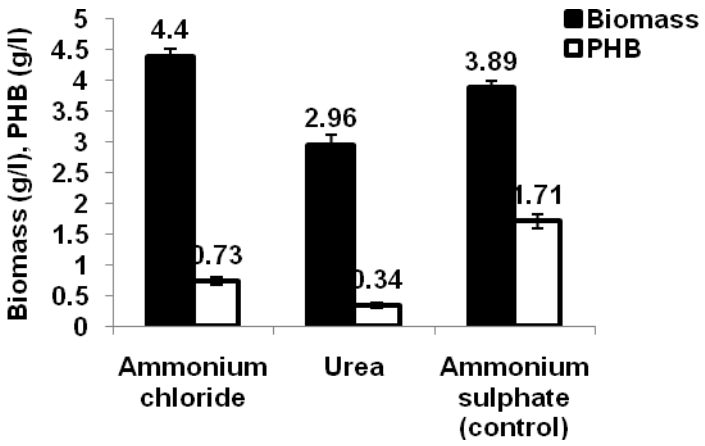


Fig 3.8 Effect of inorganic nitrogen source on biomass and PHB production

3.3.2.8 Effect of complex nitrogen source on biomass and PHB production

Beef extract was found to be the best complex nutrient source and the PHB yield was 1.82±0.21 g/l with biomass 4.83±0.25 g/l compared to the control (yeast extract), where the biomass and PHB production was 3.9±0.12 g/l and 1.7±0.13 g/l, respectively. A low PHB production of 0.51±0.012 g/l was observed with corn steep liquor (Fig 3.9).

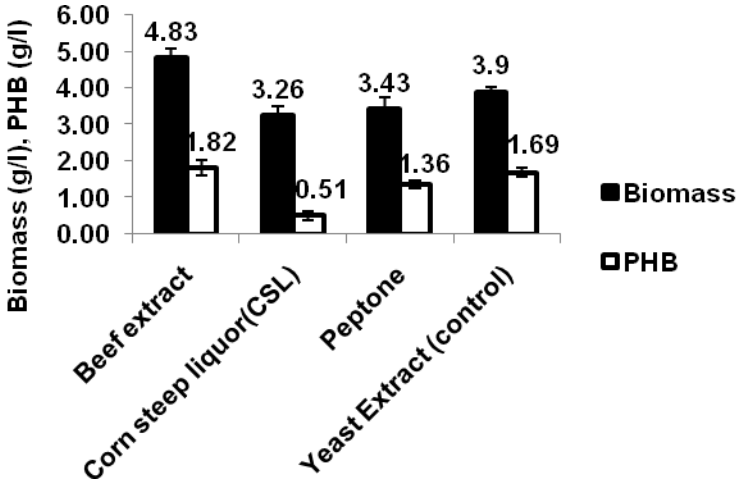


Fig 3.9 Effect of complex nitrogen source on biomass and PHB production

3.3.2.9 Effect of trace element solution on biomass and PHB production

The increasing amount of trace element solution negatively affected the biomass and PHB production (Fig 3.10).

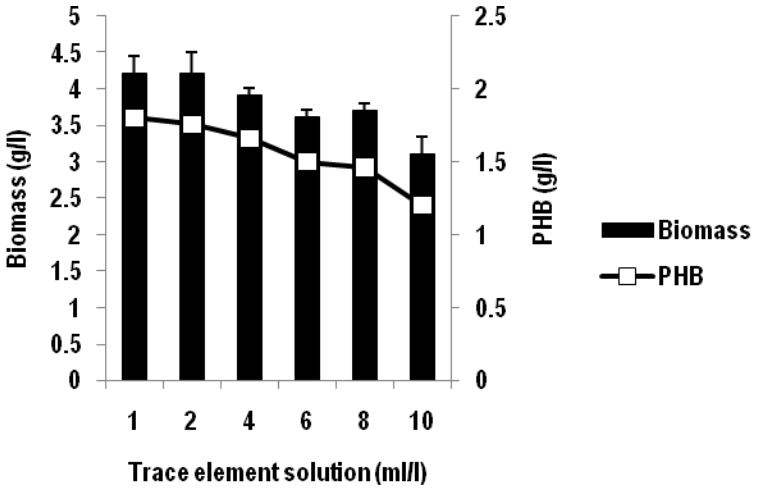


Fig 3.10 Effect of trace element solution on biomass and PHB production

3.3.2.10 Effect of reducing sugar concentration on biomass and PHB production

The analysis of growth and PHB production characteristics of the *Bacillus sphaericus* suggested that jackfruit seed hydrolyzate containing 20 g/l reducing sugar was best for PHB production (1.98± 0.24 g/l) and the biomass was 4.2±0.15 g/l (Fig 3.11).

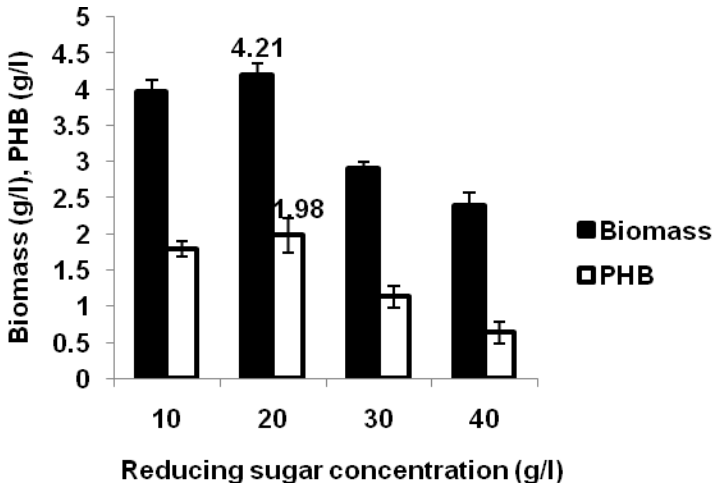


Fig 3.11 Effect of reducing sugar concentration in jackfruit seed hydrolyzate on biomass and PHB production

3.3.3 Central Composite Design (CCD)

From the "one variable at a time" experiment, it was clear that inoculum age, pH, and concentration of reducing sugar in the jackfruit seed hydrolyzate had a positive influence on both biomass accumulation and PHB production. CCD was employed to identify the optimum levels of the variables such that the response is maximized or minimized. The results of CCD are showed in Table 3.3. The run no. 7 exhibited a maximum response of PHB production of 49% (Cell dry weight) with biomass of 4.5±0.04 g/l and PHB concentration of 2.22±0.001 g/l. The lower response was found in run no. 19. From these results, it was clear that *Bacillus sphaericus* was able to synthesize maximum PHB at pH 6 with 2.5% substrate concentration and inoculum age of 18 h. Multiple regression analysis was used to analyze the data and a polynomial equation was derived from the regression analysis as follows:

Biomass (Y) = $4.38 + 0.0059\ X_1 + 0.025\ X_2 + 0.097X_3 - 0.588\ X_1^2 - 0.977\ X^2_2 - 0.977\ X_3 + 0.425\ X_1 X_2 + 0.075\ X_1 X_3 - 0.1\ X_2X_3$ Eq (2)

PHB (Y) = $2.03 + 0.084\ X_1 - 0.045\ X_2 + 0.069\ X_3 - 0.383\ X_1^2 - 0.581\ X_2^2 - 0.565\ X_3^2 + 0.095\ X_1 X_2 + 0.074\ X_1 X_3 - 0.047\ X_2X_3$ Eq (3)

Table 3.3 Observed and predicted responses obtained for CCD

	Biomass (g/l)		Amount of PHB (g/l)	
Run	**Actual value**	**Predicted value**	**Actual value**	**Predicted value**
1	2.8	2.1	0.84	0.5
2	2.2	2.2	0.55	0.5
3	4.1	4.3	1.96	2.0
4	3	2.7	1.2	1.0
5	2.7	2.3	0.783	0.6
6	3.7	4.3	1.66	2.0
7	4.5	4.3	2.22	2.0
8	3.8	3.5	0.8	0.7
9	3	2.5	0.48	0.22
10	4.6	4.3	1.97	2.0
11	4.8	4.3	2.2	2.0
12	4.7	4.3	2.25	2.0
13	1	1.5	0.2	0.46

14	1.3	1.4	0.22	0.31
15	1.5	1.5	0.37	0.32
16	2.1	2.3	0.66	0.8
17	1	1.1	0.28	0.34
18	2	1.6	0.88	0.72
19	1.8	1.6	0.30	0.31
20	2	2.7	0.42	0.8

Where Y was the response variable (Biomass and PHB production), X_1 X_2 and X_3 were the coded value of inoculum age, pH and substrate concentration respectively. Using ANOVA, The model characteristics for both responses, biomass and PHB, were analyzed for the capability and the results are shown in Table 3.4 and 3.5. The models exhibited a high R^2 value, a significant *F*-value, an insignificant lack-of-fit *F*-value and standard deviation less than 10 in all the cases. The calculated R^2 value of 0.910 for biomass production showed an improved correlation between the observed and predicted response. The model F-value of 10.97 implies the model was significant. There was only a 0.04% chance that a 'Model F-value' this large could occur due to noise. The 'Lack of Fit F-value' of 2.37 implies the Lack of Fit was not significant relative to the pure error. There was 18.30% chance that a 'Lack of Fit F-value' this large could occur due to noise. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this study the ratio was 8.473 and 9.190 for the biomass and PHB respectively, which indicated an adequate signal. The regression coefficient (R^2) obtained for the PHB could explain that the variability in the PHB yield could be associated to the experimental

factors to the extent of 92.8%. Here also the F value 14.50 and values of prob> F (<0.05) indicated that the model terms are significant. The 'Lack of Fit F-value' for response PHB was 2.08 explained that the Lack of Fit was not significant relative to the pure error. There was a 21.97% chance that a 'Lack of Fit F-value' this large could occur due to noise. The closer the R^2 is to 1, the stronger the model and the better it predicts the response (Gangadharan et al., 2008). The P values represents the significance of the coefficients and also helpful in understanding the pattern of the mutual interactions between the variables. Value of Prob>F less than 0.05 indicates the model terms were significant. Here, X_1^2, X_2^2, and X_3^2 were significant models terms.

Table 3.4 Analysis of variance (ANOVA) for the response of biomass

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	29.26654	9	3.251838	10.97313	0.0004
A	0.00049	1	0.00049	0.001653	0.9684
B	0.008737	1	0.008737	0.09484	0.8671
C	0.130766	1	0.13076	0.441262	0.5215
A2	4.99625	1	4.99625	16.85954	0.0021
B2	13.77607	1	13.77607	46.4865	< 0.0001
C2	13.77607	1	13.77607	46.4865	< 0.0001
AB	1.445	1	1.445	4.876064	0.057
AC	0.045	1	0.045	0.15185	0.7049
BC	0.08	1	0.08	0.269955	0.6147

Residual	2.963456	10	0.296346		
Lack of Fit	2.083456	5	0.416691	2.367563	0.1830

R^2 – 0.910

Table 3.5 Analysis of variance (ANOVA) for the response of PHB concentration

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	10.10981	9	1.123313	14.50913	0.0001
A	0.096636	1	0.096636	1.248182	0.2900
B	0.027679	1	0.027679	0.357509	0.5632
C	0.066819	1	0.066819	0.863061	0.3748
A2	2.12155	1	2.2155	27.40275	0.0004
B2	4.876004	1	4.876004	62.98032	< 0.0001
C2	4.612916	1	4.612916	59.58218	< 0.0001
AB	0.072771	1	0.072771	0.93994	0.3552
AC	0.044551	1	0.044551	0.575439	0.4656
BC	0.018336	1	0.018336	0.236836	0.6370
Residual	0.774211	10	0.077421		
Lack of Fit	0.523211	5	0.104642	2.084505	0.2197

R^2- 0.928

The response surface and contour plots were generated as graphical representations of the regression equation which helped in the investigation of the interactive effects of variables for biomass and PHB production. The three-dimensional response surface and their corresponding contour plots for the biomass production and concentration of PHB against any two independent variables while keeping the other independent variable at zero levels are presented in Fig 3.12 to 3.17. Six response surfaces were obtained for both responses by considering all the possible combinations. The contour plots help in identify the relation between the response and experimental levels of each variable, and the type of interactions between the test variable in order to deduce the optimum conditions. The optimum value of each variable was identified based on the hump in the three-dimensional plot, or from the central point of the corresponding contour plot. Each contour curve represents an infinitive number of combinations of two test variables with the other one maintained at their respective zero level. The maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram. Elliptical contours are obtained when there is a perfect interaction between the independent variables (Muralidhar et al., 2001).

Fig 3.12 showed the interaction between inoculum age and initial pH on biomass production. It was depicted that increase in inoculum age had not much influence on biomass accumulation. The alkaline pH had a deleterious effect on bacterial growth and consequently slowed down the biomass accumulation.

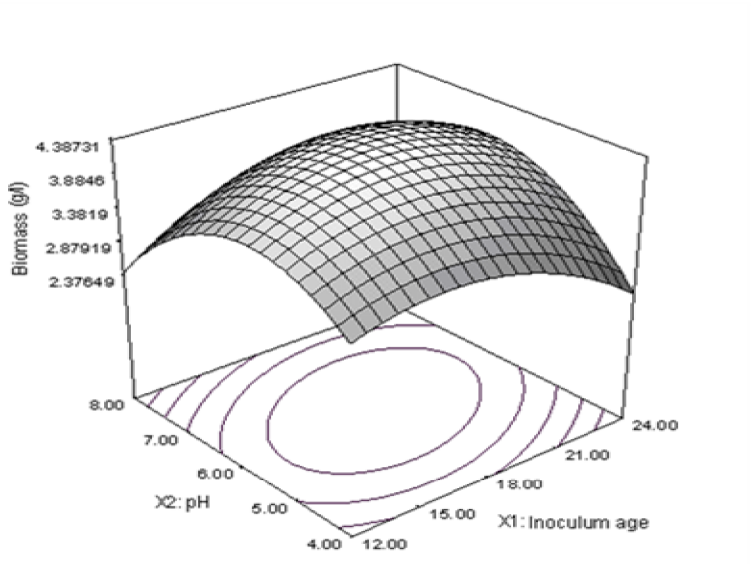


Fig 3.12 Response surface graph showing interaction between inoculum age and initial pH on biomass production

Fig 3.13 represented the three dimensional plot and its respective contour plot showing the response surface from the interaction between the substrate concentration (X_3) and inoculum age (X_2) while keeping other variable at its zero level.

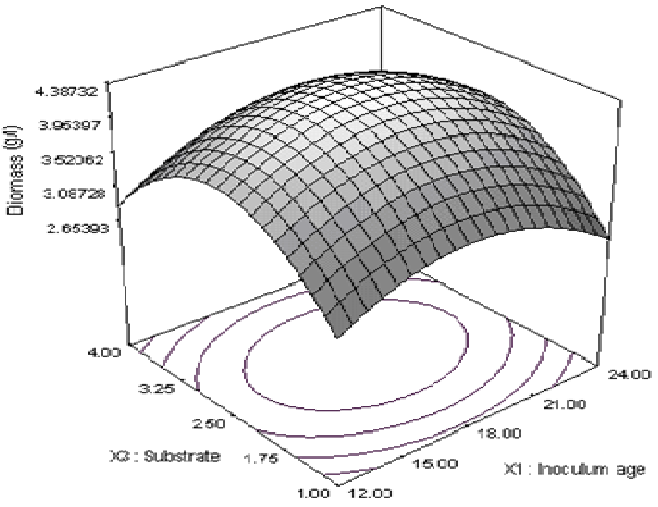


Fig 3.13 Response surface graph showing interaction between inoculum age and substrate concentration on biomass production

From the figure it was evident that biomass production increased gradually with the increasing substrate concentration and above 2.25 g/l a decrease in production could be seen. This might be due to the inhibiting effect of high concentration sugar on bacterial growth. Inoculum age did not show much effect on biomass accumulation.

Fig 3.14 represented the interaction of substrate concentration (X_3) and pH (X_2).The shape of the contour showed a positive interaction between the two variables. The production decreased at the maximum and minimum values of ranges considered in both parameters.

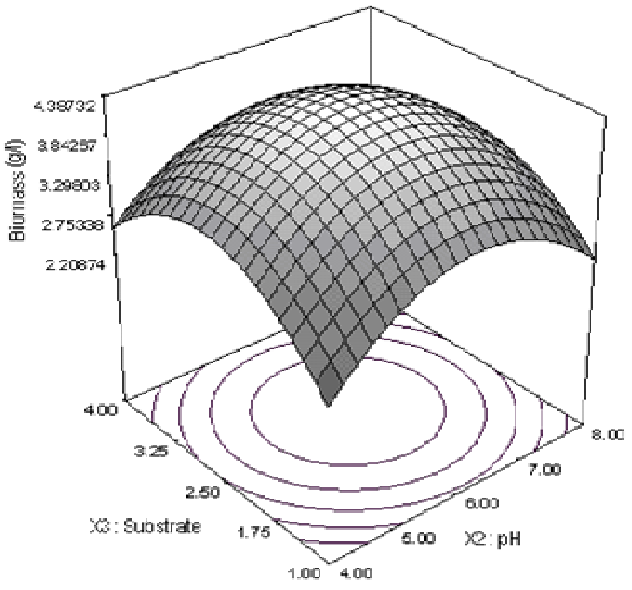


Fig 3.14 Response surface graph showing interaction between initial pH and substrate concentration on biomass production

The interaction effect between initial pH and inoculum age is showed in Fig 3.15. The 18 h inoculum and pH 6.0 was best for maximizing PHB production (2.2±0.04 g/l). The PHB production was increased gradually with the increasing pH and beyond a particular level it is found to be reduced. It has been reported that the inoculum age of 15 h has given maximum PHB production by *Ralstonia eutropha* and above 20 h the productivity found to be decreased (Tabandeh and Farahani, 2003).

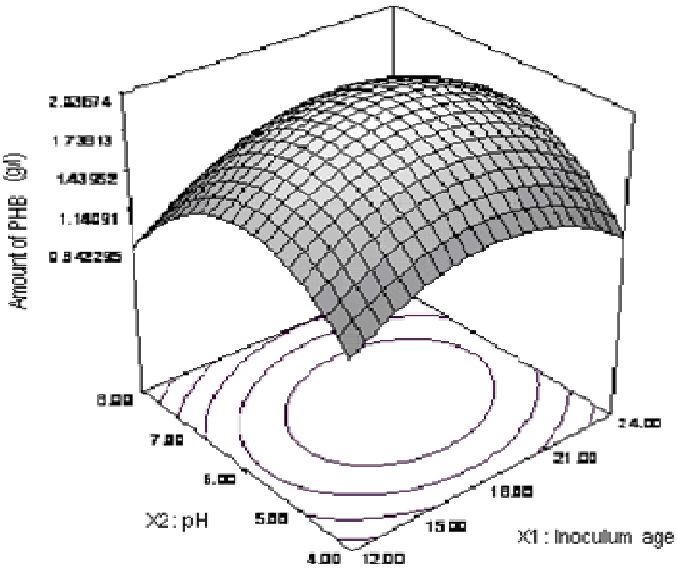


Fig 3.15 Response surface graph showing interaction between inoculum age and initial pH on PHB production

Fig 3.16 represented the three dimensional plot and its respective contour plot showing the response surface from the interaction between the substrate (X_3) and inoculum age (X_1) while keeping other variables at their zero level. The increase in the substrate concentration did not show a positive influence on PHB accumulation. The reduction in the polymer synthesis can be explained by the higher protein concentration

in the hydrolyzate. The response surface graph of substrate concentration and inoculum age for PHB production indicated that the inoculum age and substrate concentration did not effect the PHB synthesis beyond their optimum values. Inoculum age of 18 h ($8x10^8$ CFU/ml) with substrate concentration of 2.5% gave highest PHB concentration.

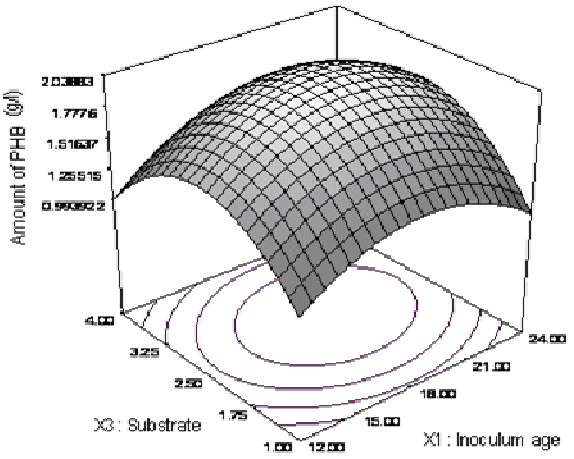


Fig 3.16 Response surface graph showing interaction between inoculum age and substrate concentration on PHB production

Fig 3.17 showed the interaction between initial medium pH and substrate concentration on PHB production. The contour plots showed a good level of interaction. At the optimum pH 6.0 and 2.5% substrate concentration gave maximum response of PHB production. The increase of substrate concentration and initial pH did not increase the yield of PHB. The available nitrogen from the proteins in the hydrolyzate would have shifted the PHB synthesis to biomass accumulation.

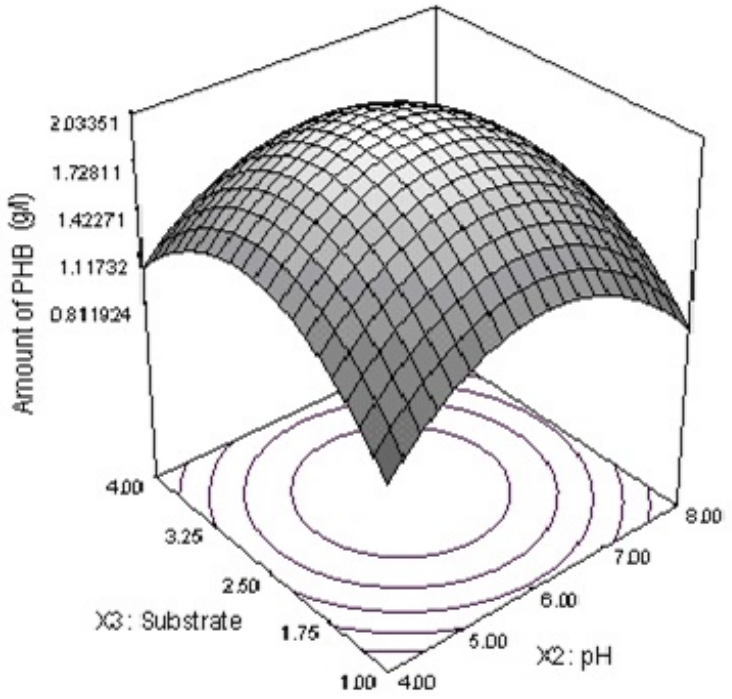


Fig 3.17 Response surface graph showing interaction between initial pH and substrate concentration on PHB production

3.3.4 Validation of the model

Validation of the model was carried out in shake flasks under the conditions predicted by the software. A good correlation could be seen between the experimental and the predicted values, and hence, the model was successfully validated. Validation of the statistical model and regression equation was performed by taking X_1 (18 h), X_2 (6) and X_3 (2.5%) in the experiment. The predicted and the actual (experimental) responses of biomass (4.8 g/l and 5 g/l) and PHB production (2.4 g/l and 2.7 g/l) were comparable. The experimental values were very close to the predicted values, and hence, the model was considered valid.

3.4 CONCLUSION

To the best of our knowledge, there are no reports of optimization of PHB production by *Bacillus sphaericus* using statistical experimental design. The statistical approach showed significant results for optimizing the process parameters for maximal biomass and PHB production under SmF. This study also explained the use of cheap agro-residues as substrate for fermentation, thus contributing to the cost reduction in the bioplastic production. The present study identified three significant variables i.e. substrate concentration, pH, and inoculum age which influenced the PHB production of *Bacillus sphaericus* to a greater extent. The higher similarity between the predicted and experimental results indicated the accuracy and applicability of RSM to optimize the process for PHB production. Considering the results obtained, we can conclude that the *Bacillus sphaericus* offered great potential for further investigation for PHB production.

CHAPTER 4

Bioprocess development for the production of polyhydroxybutyrate UNDER solid-state fermentation using polyurethane foam as inert support

CHAPTER 4

Bioprocess development for the production of polyhydroxybutyrate under solid-state fermentation using polyurethane foam as inert support

4.1 INTRODUCTION

Solid-state fermentation (SSF) is defined as any fermentation process performed on a non-soluble material that acts as physical support and source of nutrients (sometimes only as physical support) in the absence or near-absence of free flowing liquid. SSF has several advantages over SmF, which include simpler fermentation media, higher product titers, better product recovery, lower waste water output, reduced energy requirements, absence of foam formation, smaller fermentation space requirement, easier aeration, reduced bacterial contamination, high reproducibility, lower levels of catabolite repression and end product inhibition and absence of rigorous control of fermentation parameters (Pandey et al., 2000a, Lonsane et al., 1985). The disadvantages followed in SSF are the availability of nutrients for the microorganisms may increase, decrease, or remain relatively constant during the fermentation, while in the SmF, it is readily accessible (Knapp and Howell, 1985). Even though, PHB is an interesting alternative for conventional polymers, until recently, only submerged fermentation processes had been used for its production. The substrates used in SSF should possess the ability to absorb water so the microorganism can use this for growth and metabolic activities. There is a lot of reported data on the effective utilization of agro-industrial residues such as cassava bagasse, sugar cane bagasse, sugar beet pulp, coffee pulp/husk, apple pomace, etc by the

microorganisms. Several processes have been developed that make the microorganisms to utilize these as raw materials for the production of bulk chemicals and value-added fine products such as ethanol, single cell protein (SCP), mushrooms, enzymes, organic acids, amino acids, biologically active secondary metabolites, etc. (Pandey, 1992, 1994; Pandey et al., 1988; Nampoothiri and Pandey, 1996; Pandey and Soccol, 1998). Only very few cases were reported on the production of PHB using SSF as the mode of fermentation. Since the PHB is synthesized as intracellular inclusion bodies, the retrieval of bacterial biomass from the medium, containing agro industrial residues as the substrate, after fermentation is a very difficult task. In this study, the polyurethane foam (PUF) was used as the inert support in SSF medium. However, there were no reports on the production of PHB under SSF using polyurethane foam (PUF) as an inert support.

The PUF has possessed some physical properties such as high porosity, low density and relatively high water absorption capacity. PUF allows cell adsorption to a large extent because it allows a large no of cells to immobilize in a short period (Yang, 2006). Zhu et al has reported that the use of nutritionally inert materials for SSF helps in the designing of media, monitoring of process parameters, scaling-up strategies and various engineering aspects (Zhu et al., 1994). The PUF can provide a continuous homogenous aerobic condition till the end of incubation period (Aidoo et al., 1982).

As stated earlier, response surface methodology (RSM) has been extensively applied for the optimization of fermentation processes (Cui et al., 2006). The single parameter optimization studies do not consider the interaction effects among the variables as any process is influenced by several variables (Silva and Roberto, 2001). Limitations of the single factor optimization can be removed by employing

response surface methodology (RSM) which is used to explain the combined effects of all the factors in a fermentation process (Elibol, 2004). Response surface methodology is a collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables. The production of PHB under SSF was carried out using polyurethane foam as inert support. A statistical approach such as Plackett- Burman design was used for identifying significant variables influencing the biomass and PHB production under SSF by *Bacillus sphaericus.* The levels of the significant variables were further optimized using response surface methodology (RSM).

4.2 MATERIALS AND METHODS

4.2.1 Materials

PUFs were cut into 1cm ^{3} and this was used as the solid support in the SSF medium. It was thoroughly washed using distilled water and dried in an oven at 85^0C for overnight.

4.2.2 Microorganism and preparation of inoculum

Microorganism, its maintenance and inoculum preparation has been described in chapter 2.

4.2.3 Solid-state fermentation

SSF was carried out in 250 ml Erlenmeyer flask containing 1g PUF moistened with10 ml production medium. The chemical composition of cultivation medium as

follows (g/l): $(NH_4)_2SO_4$, 2; KH_2PO_4, 2; Na_2HPO_4, 0.6; $MgSO_4.7H_2O$, 0.2;$CaCl_2$, 0.02; beef extract, 0.2; jackfruit seed hydrolyzate containing 2.5% reducing sugar and 1 ml trace element solution. The trace element solution contained (g/l) H_3BO_3, 0.01; $MnSO_4.H_2O$, 0.02; $CuSO_4$, 01; $ZnSO_4.7H_2O$, 0.1; and $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.02. The jackfruit seed hydrolyzate and trace element solutions were autoclaved separately and mixed with production medium prior to use. The initial medium pH was set at 7.0. The flasks containing PUF was sterilized at 121.5^0C for 20 min, after cooling it was moistened with 10 ml production medium and inoculated with 3 ml (8×10^8 CFU/ml) inoculum and incubated at 30^0C for four days. After fermentation, the samples were withdrawn as whole flasks in triplicate and the fermented solid mass collected from each flask was thoroughly agitated with 50 ml distilled water for 20 min, centrifuged at 8,000xg for 15 min. This process was repeated for four times. The pellet was collected and lyophilized.

4.2.4 Assay of PHB

The amount of PHB produced was determined as described in chapter 2. The reported results are the average values with standard deviation.

4.2.5 Preliminary studies

4.2.5.1 Biomass recovery from fermented PUF

Distilled water was used for the complete extraction of biomass from the fermented PUF. After fermentation, 50 ml distilled water was added to the flask and agitated at 250 rpm for 20 min. This was repeated for four times to ensure the complete

extraction of bacterial cells. The resulting bacterial suspension, which was a pool of the four filtrates, was centrifuged at 8,000xg for 15 min and the pellet was washed twice with distilled water. The biomass separated by centrifugation was lyophilized and took the dry weight and subjected for PHB assay.

4.2.5.2 Scanning electron microscopic study

Scanning electron microscopy (SEM) studies were conducted to verify the effectiveness of washing steps for the recovery of biomass from PUF after fermentation. Five samples were subjected for SEM analysis. These were PUF after fermentation and PUF after each washing step. Samples were prepared as described in chapter 2.

4.2.5.3 Screening of agro industrial residues

The enzymatic hydrolysis of wheat bran, cassava bagasse, jackfruit powder was performed as described in chapter 2. These hydrolyzates were screened individually as well as in combinations at 10 g/l as a sole source of carbon in the SSF medium.

4.2.5.4 Effect of initial moisture content of the medium on the production of PHB

The effect of moisture content (55, 60, 65, 70, 75, 80 and 85%) on the biomass and PHB production was checked by adjusting the volume of medium and distilled water. Moisture content was determined as described in chapter 2.

4.2.5.5 Effect of nitrogen source on the production of PHB

Studies were carried out to evaluate the effect of nitrogen sources on PHB production by using 0.2% (w/v) inorganic nitrogen sources (ammonium nitrate, urea, glycine and ammonium chloride) by replacing ammonium sulphate (control) from the medium. Organic complexes evaluated were beef extract, corn steep liquor and peptone at 0.2% (w/v) concentration by replacing yeast extract (control) from the production medium.

4.2.6 Identifying the significant variables using Plackett Burman design

Plackett-Burman experimental design was employed for the identification of significant parameters that influence the PHB production. The design was generated using MINITAB® version 15 software and it is shown in Table 4.1

Table 4.1 Design for Plackett-Burman experiment for screening of significant process variables affecting biomass and PHB production

Std order	(A) Carbon (g/l)	(B) Inoculum size (ml)	(C) Inoculum age (h)	(D) Temperature (^{0}C)	(E) $(NH_4)_2SO_4$ (%)	(F) Beef extract (%)	(G) pH	(H) Incubation time (h)	Biomass (g/g PUF)	PHB (g/g PUF)
1	60	1	24	25	0.01	0.01	8	120	0.02	0.001
2	60	5	12	35	0.01	0.01	5	120	0.01	0.006
3	10	5	24	25	0.40	0.01	5	72	0.06	0.025
4	60	1	24	35	0.01	0.40	5	72	0.012	0.004
5	60	5	12	35	0.40	0.01	8	72	0.101	0.048
6	60	5	24	25	0.40	0.40	5	120	0.046	0.012

7	10	5	24	35	0.01	0.40	8	72	0.062	0.020
8	10	1	24	35	0.40	0.01	8	120	0.059	0.005
9	10	1	12	35	0.40	0.40	5	120	0.041	0.007
10	60	1	12	25	0.40	0.40	8	72	0.067	0.035
11	10	5	12	25	0.01	0.40	8	120	0.068	0.031
12	10	1	12	25	0.01	0.01	5	72	0.024	0.011

The variables chosen for the present study were substrate concentration (jackfruit seed hydrolyzate as carbon source), incubation period, inoculum size, inoculum age, initial medium pH, incubation temperature, beef extract and $(NH_4)_2SO_4$. The effects of individual parameters on PHB production was calculated by the following equation 1.

$$E = (\sum M_+ - \sum M_-)/N \qquad \text{Eq (1)}$$

Where E was denoted as the effect of parameter under study and M+ and M – were the responses (Biomass and PHB production) of trials at which the parameter was at its higher and lower levels respectively and N was the total number of trials.

4.2.7 Response surface methodology

The levels of the significant parameters and the interaction effects between various medium constituents which influence the PHB production significantly were analyzed and optimized by Box Behnken methodology (Box and Behnken, 1960). The study was consisted an experimental plan of 15 runs and the independent variables were studied at three different levels, low (-1), medium (0) and high (+1). The experiment variables were inoculum size (X_1), $(NH_4)_2SO_4$ (X_2) and pH (X_3). The other variables in the study were maintained at a constant level which gave maximal PHB yield in the Plackett-Burman experiments. The design matrix of the variables was

shown in Table 4.2. All the experiments were performed in triplicate and the average of biomass and PHB production obtained were taken as the dependent variables or responses (*Y*).

Table 4.2 Box Behnken design for optimising the significant variables for biomass and PHB production and corresponding responses

Run Order	Inoculum size	$(NH_4)_2SO_4$	pH	Biomass (g/g PUF)	PHB (g/g PUF)
1	6.5	1.7	9.5	0.4	0.169
2	6.5	0.4	8.0	0.19	0.05
3	6.5	3.0	11.0	0.056	0.003
4	6.5	3.0	8.0	0.5	0.06
5	5.0	0.4	9.5	0.1	0.04
6	6.5	1.7	9.5	0.38	0.168
7	6.5	1.7	9.5	0.376	0.165
8	5.0	1.7	11.0	0.028	0.002
9	8.0	3.0	9.5	0.298	0.02
10	8.0	1.7	11.0	0.05	0.002
11	8.0	0.4	9.5	0.089	0.005
12	8.0	1.7	8.0	0.41	0.019
13	6.5	0.4	11.0	0.02	0.003
14	5.0	1.7	8.0	0.38	0.02
15	5.0	3.0	9.5	0.2	0.005

4.3 RESULTS AND DISCUSSION

4.3.1 Preliminary studies

4.3.1.1 Biomass recovery from fermented PUF

Since PHB is synthesized as intracellular inclusion bodies, biomass recovery from PUF after fermentation is a crucial step. Distilled water was used for the extraction of biomass from the PUF after fermentation. The biomass obtained was (0.06±0.002 g/g PUF).

4.3.1.2 Scanning electron microscopic study

PUF allows cell adsorption to a large extent because it allows a large number of cells to immobilize in a short period. The PUF after fermentation showed a continuous growth of *Bacillus sphaericus* on the surface and inside pores also. The SEM studies revealed that most of the biomass could retrieve by the fourth washing using distilled water (Fig 4.1).

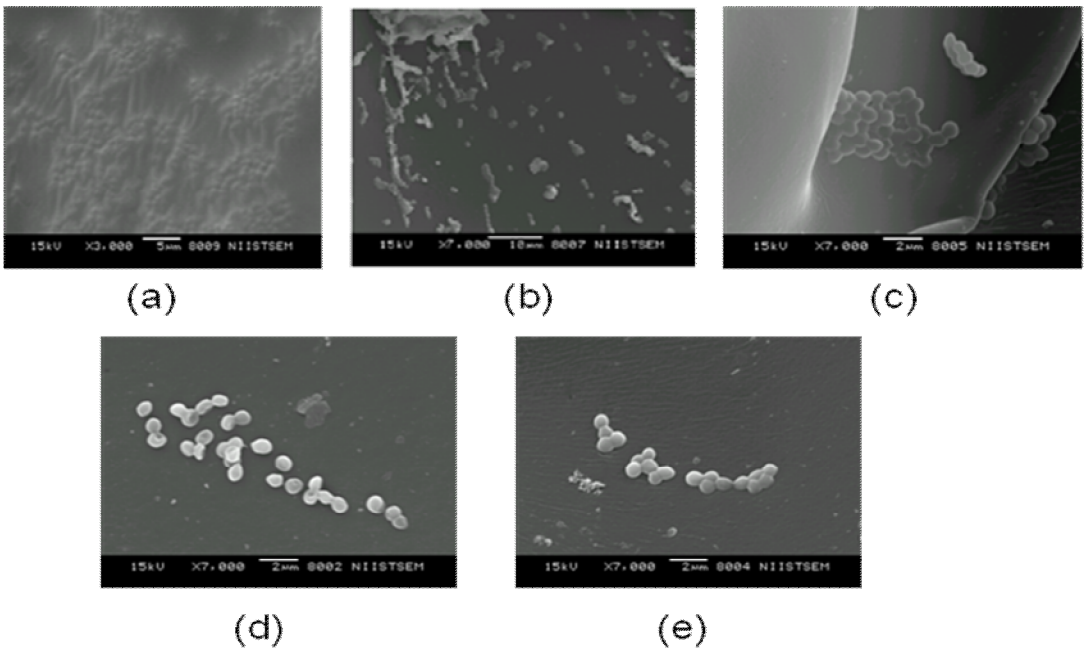


Fig 4.1 Scanning electron micrographs (a) PUF after fermentation, without washing (b) first washing (c) second washing (d) third washing (e) fourth washing

4.3.1.3 Screening of agro-industrial residues

Among the various substrates screened for SSF, jackfruit seed powder hydrolysate gave the highest PHB production (0.013±0.001 g/g PUF, 21.6%) and biomass (0.06±0.002 g/g PUF). Next position was occupied by cassava bagasse. As it is shown in Fig 4.2, none of the mixtures of substrates could give a better production. Bobbio et al. (1978) reported that jackfruit seed contained 31.9% protein, 1.3% crude lipids and 66.2% carbohydrates on dry weight basis.

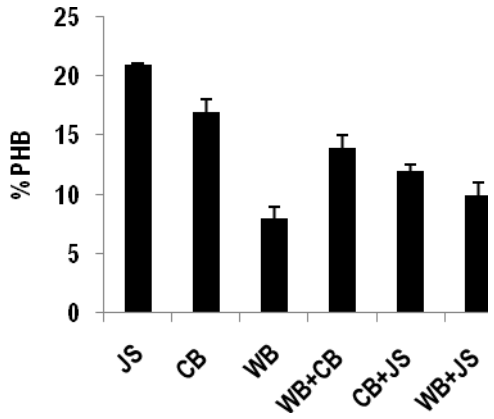


Fig 4.2 Screening of agro industrial hydrolysates on biomass and PHB production under SSF

4.3.1.4 Effect of initial moisture content of the medium on the production of PHB

Moisture is a critical factor in SSF. Generally bacteria require higher water activity for their growth. The necessary moisture in SSF exists in absorbed or complex form within the solid matrix, which is likely to be more advantageous for the growth because of the possible efficient oxygen transfer process (Raghavarao et al., 2003). If the quantity of the water becomes insufficient and does not allow a good diffusion of solutes and gas, the cell metabolism slows, or can stop, because of a lack of substrates or through too high concentration of inhibitive metabolites in or near the cell (Gervais and Molin, 2003). Similarly, a higher than optimum moisture level decreases the porosity, promotes development of stickiness, reduces gas volume and decrease diffusion, which results in lowered oxygen transfer (Ramesh and Lonsane, 1990). In this study, the maximum biomass (0.070±002 g/g PUF) and PHB (0.018±0.002 g/g PUF) production was observed when the substrate moisture was set at 75% (Fig 4.3).

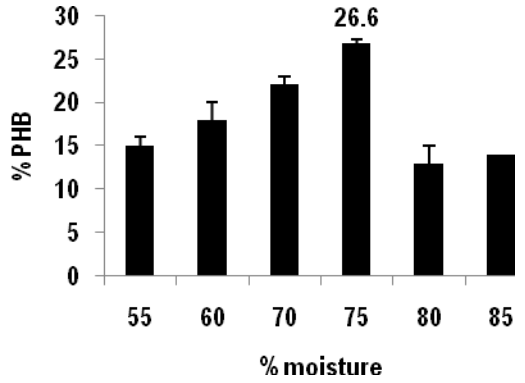


Fig 4.3 Effect of moisture content on biomass and PHB production

4.3.1.5 Effect of nitrogen source on biomass and PHB production

The result shown in Fig 4.4 and 4.5 indicated that the optimization of inorganic and organic nitrogen sources gave the same results in biomass and PHB production since the controls were the higher producers. $(NH_4)_2SO_4$ was the suitable inorganic nitrogen source for higher PHB (0.019±0.003 g/g PUF) and biomass (0.07±0.002 g/g PUF) production. PHB productions in a variety of complex nitrogen sources (peptone, beef extract, and corn steep liquor) were studied. The biomass (0.071±0.003 g/g PUF) and PHB (0.019±0.002 g/g PUF) production was higher in beef extract as compared to that obtained with peptone, corn steep liquor and yeast extract (Fig 4.5). The *Bacillus sphaericus* was not efficient for the utilization of high amounts of growth factors present in CSL.

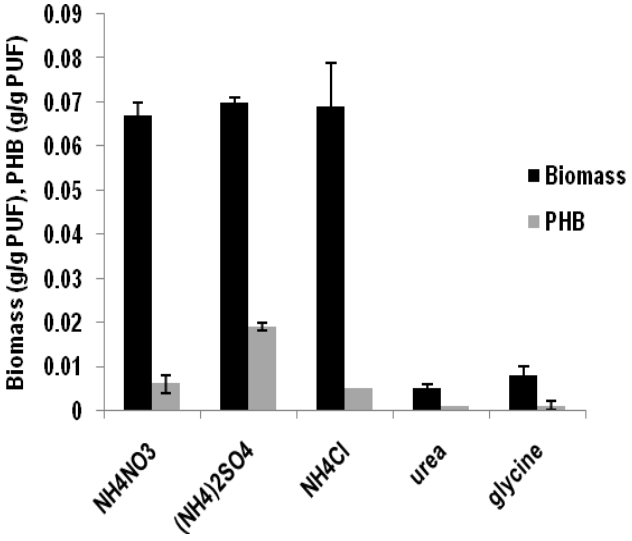


Fig 4.4 Effect of inorganic nitrogen source on biomass and PHB production

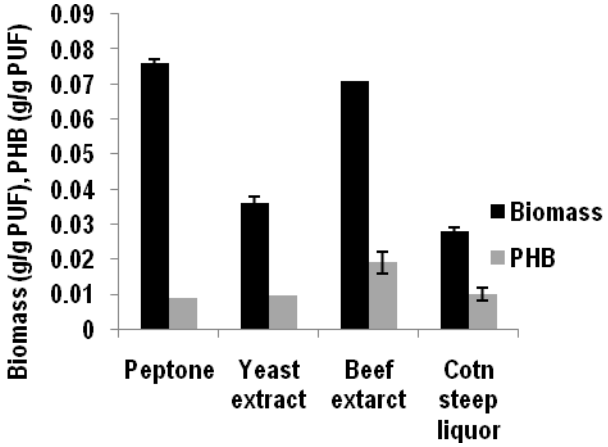


Fig 4.5 Effect of organic nitrogen source on biomass and PHB production

4.3.1.6 Screening of parameters using Plackett-Burman design

Factorial design is a statistical method helps to determine the effects of multiple variables on a response. It can overcome the disadvantages of 'one variable at a time experiment' by reduce the number of experiments one has to perform by studying multiple factors simultaneously. Additionally, it gives both main effects (from each

independent factor) and interaction effects (when both factors must be used to explain the outcome). However, factorial design can only give relative values, and to achieve actual numerical values, the math becomes difficult, as regressions (which require minimizing a sum of values) need to be performed. Regardless, factorial design is a useful method to design experiments in both laboratory and industrial settings. The Plackett-Burman experimental design is a two factorial design which identifies the critical physico-chemical parameters that have an effect on PHB production. The effect of eight factors of the fermentation for PHB production by *Bacillus sphaericus* was examined using Plackett-Burman statistical design. The experiment was conducted in 12 runs to study the effect of the selected variables. Data for both biomass and PHB production were analyzed by general linear model (GLM). ANOVA consists of classifying and cross classifying statistical results and analyzing whether the means of a specified classification differ significantly. The main effect of the medium components, regression coefficient, F values and P values of the factors investigated in the present study is illustrated in Table 4.3. The F-value identifies the influence of each controlled factor on the tested model and it is described as the ratio of the mean square due to regression to the mean square due to error. The model equation fitted by regression analysis is given for biomass and PHB were as follows (Eq 2 and 3).

Biomass = - 0.0045 - 0.000196 (A) + 0.00521(B) - 0.000722 (C) - 0.000003 (D) + 0.0762 (E) + 0.0096 (F) + 0.0102(G) - 0.000285 (H) Eq (2)

PHB = 0.0303 + 0.000023 (A) + 0.00329 (B) - 0.000986 (C) - 0.000416 (D) + 0.0252 (E) + 0.00556 (F) + 0.00417 (G) - 0.000281 (H) Eq (3)

The coded variables were A-Carbon, B- Inoculum size, C-Inoculum age, D-Temperature, E- $(NH_4)_2SO_4$, F- Beef extract, G- Incubation time and H- pH

The values of Prob < 0.05 indicated that model terms were significant. For biomass production, inoculum size, $(NH_4)_2SO_4$ and pH were significant variables, while in the case of PHB production inoculum size, inoculum age, temperature, pH and $(NH_4)_2SO_4$ were found significant. The determination coefficient (R^2) for biomass production in SSF was 95.8%, indicated that the statistical model with 95.8% variability in the response and for PHB production it was 96.30%.

Table 4.3 Analysis of variance (ANOVA) for the quadratic model for the biomass production

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Carbon	1	0.0002881	0.0002881	0.0002881	2.50	0.212
Inoculum size	1	0.0013021	0.0013021	0.0013021	11.28	0.044
Inoculum age	1	0.0002253	0.0002253	0.0002253	1.95	0.257
Temperature	1	0.0000000	0.0000000	0.0000000	0.00	0.996
$(NH_4)_2SO_4$	1	0.0026463	0.0026463	0.0026463	22.93	0.017
Beef extract	1	0.0000418	0.0000418	0.0000418	0.36	.590
pH	1	0.0028213	0.0028213	0.0028213	24.45	0.016
Incubation time	1	0.0005631	0.0005631	0.0005631	4.88	0.114
Error	3	0.0003462	0.0003462	0.0001154		

Total	11	0.0082342				

Table 4.4 Analysis of variance (ANOVA) for the quadratic model for the PHB production

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Carbon	1	0.0000041	0.0000041	0.0000041	0.14	0.735
Inoculum size	1	0.0005199	0.0005199	0.0005199	17.52	0.025
Inoculum age	1	0.0004199	0.0004199	0.0004199	14.15	0.033
Temperature	1	0.0000520	0.0000520	0.0000520	1.75	0.277
$(NH_4)_2SO_4$	1	0.0002899	0.0002899	0.0002899	9.77	0.052
Beef extract	1	0.0000141	0.0000141	0.0000141	0.48	0.540
pH	1	0.0028213	0.0028213	0.0028213	15.79	0.029
Incubation time	1	0.0005469	0.0005469	0.0005469	18.43	0.023
Error	3	0.0000890	0.0000890	0.0000297		
Total	11	0.0024046				

The maximum biomass and PHB production were 0.101±0.05 and 0.048±0.005 g/g PUF respectively; in run no 5, while the minimum PHB was 0.001±0.0 g/g PUF in run no 1. The lowest biomass production was in run no 2. The Pareto charts were created to find out the order of significance of the variables affecting the biomass and PHB production (Fig 4.6 and 4.7). The order of significance for biomass production as indicated by Pareto

chart was pH, $(NH_4)_2SO_4$, inoculum size, beef extract, temperature, inoculum age, incubation time and carbon while for PHB production inoculum size was came in first position followed by pH, $(NH_4)_2SO_4$, beef extract, temperature, carbon, inoculum age and incubation time. Since pH, inoculum size and $(NH_4)_2SO_4$ were found most significant parameters that had a positive influence on biomass and PHB production, these were considered as the variables for the next stage in the medium optimization using response surface optimization technique. The optimum values of test variables were inoculum size- 5 ml ($8x10^8$ CFU/ml), $(NH_4)_2SO_4$ -0.4% (w/v) and pH 8.0. The results suggested that SSF using inert support is an alternative method for PHB production with an effortless and cheap recovery method.

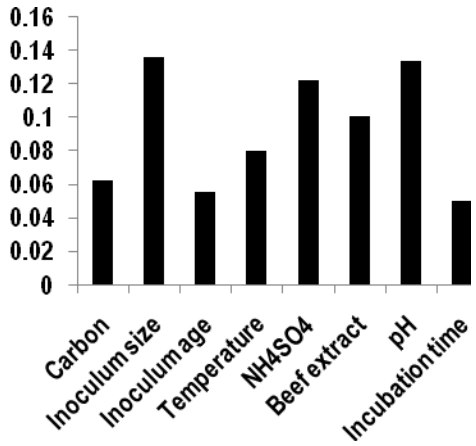


Fig 4.6 Pareto chart representing the influence of variables on PHB production in SSF using PUF as inert support

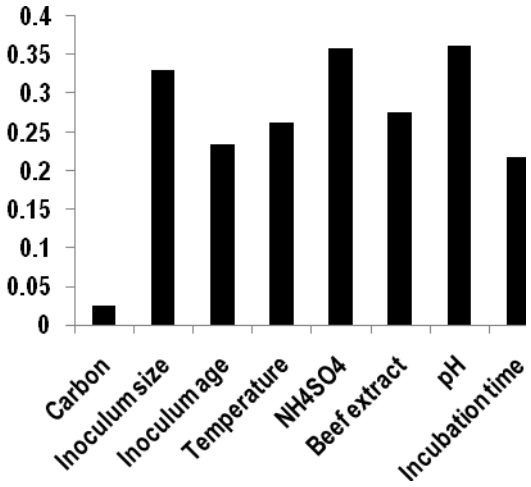


Fig 4.7 Pareto chart representing the influence of variables on biomass production in SSF using PUF as inert support

4.3.2 Box Behken design

RSM can be defined as a statistical method that uses quantitative data from appropriate experiments to determine and simultaneously solve multivarient equations. RSM determines the factor levels that will simultaneously satisfy a set of desired specifications and it helps the determination of the optimum combination of factors that yield a desired response and describes the response near the optimum. Response surface methodology (RSM) has been in use for several decades and Box Behnken design is one of the most commonly used one. It has only three levels and need fewer experiments that make it more efficient and easier to arrange and to interpret in comparison with others. Further optimization was carried out using the response surface methodology. The response surface methodology of Box Behnken design with three factors and three levels, including three replicates at the centre point, was used in order to generate 15 treatment combinations with selected variables such as inoculum size, $(NH_4)_2SO_4$ concentration

and pH with other variables were maintained at a constant level. A considerable increase was found in the biomass and PHB production depending on the three chosen variables. The maximum PHB production (0.169±0.03 g/g PUF) with 0.4±0.003 g/g PUF biomass was in run no 1(Table 4.2). Maximum PHB production was found with 6.5 ml of inoculum size, ($8x10^8$ CFU/ml), 1.7 % $(NH_4)_2SO_4$ concentration (w/v) and pH 9.5.

In order to explain the biomass and PHB production, a multiple regression analysis was applied on the experimental data and the following second order polynomial equation were obtained as shown below (Eq 4 and 5):

Y (Biomass) = -4.25298 + 0.471871(X_1) + 0.101913(X_2) + 0.602448 (X_3) – 0.0373519 (X_1^2) – 0.0389300 (X_2^2) - 0.0322407 (X_3^2) -0.00641026 (X_1X_2) +0.000111111 (X_1X_3) - 0.00128205 (X_2X_3) Eq (4)

Y (PHB) = - 4.16531+0.539623 (X_1) + 0.546363 (X_2) +0.582348(X_3) - 0.0417963 (X_1^2) - 0.0707347(X_2^2) - 0.0330185(X_3^2) + 0.0139744 (X_1X_2) - 8.88889E-04 (X_1X_3) - 0.0351282(X_2X_3) Eq (5)

Where *Y* was the responses and X_1, X_2 and X_3 were the coded values of inoculum size, $(NH_4)_2SO_4$ and pH respectively.

The goodness of the model was checked by fitting the independent variables into the second order model equation. The adequacy of the fitted model was evaluated using analysis of variance (ANOVA) and the results are shown in Table 4.5 and 4.6.

Table 4.5 Analysis of variance (ANOVA) for the quadratic polynomial model of biomass production

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	0.390097	0.390097	0.043344	116.80	0.000
Linear	3	0.275828	0.275828	0.091943	247.77	0.000
Square	3	0.092514	0.092514	0.030838	83.10	0.000
Interaction	3	0.021755	0.021755	0.007252	19.54	0.003
Residual Error	5	0.001855	0.001855	0.000371		
Lack-of-Fit	3	0.001525	0.001525	0.000508	3.07	0.255
Pure Error	2	0.000331	0.000331	0.000165		
Total	14	0.391952				

coefficient determination (R^2)=0.9953; correlation coefficient (R)=0.9867.

Table 4.6 Analysis of variance (ANOVA) for the quadratic polynomial model of PHB production

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	0.056562	0.056562	0.006285	41.27	0.000
Linear	3	0.002483	0.002483	0.000828	5.43	0.050
Square	3	0.053429	0.053429	0.017810	116.95	0.000
Interaction	3	0.000650	0.000650	0.000217	1.42	0.340
Residual Error	5	0.000761	0.000761	0.000152		
Lack-of-Fit	3	0.000753	0.000753	0.000251	57.90	0.017

Pure Error	2	0.000009	0.000009	0.000004
Total	14	0.057323		

coefficient determination (R^2)=0.9867; correlation coefficient (R)=0.9628

The value of determinant coefficient (R^2 = 0.9953) suggested that the total variation of 99.53% for biomass was attributed to the independent variables and only 0.47% of the total variation cannot be explained by the model. For PHB production the determinant coefficient was 0.9867 and 1.33% of the total variation cannot be explained by the model. R^2, or coefficient of determination, is the proportion of variation in the response attributed to the model rather than to random error (Henika, 1972). Joglekar and May (1987) suggested that R^2 should be above 80% for a good fit of a model. Here the value of R (0.9628 and 0.9867 for PHB and biomass respectively) being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. The pairs X_1X_2 and X_2X_3 showed a very good interaction and this might be contributed to the biomass production at a significant level. It was evident from the P values in Table 4.7. In the case of PHB production none of the pairs showed the interactive effect.

Table 4.7 Estimated Regression Coefficients for biomass production from the results of Box Behnken experimental design

Term	Coef	S.E Coefficient	T	P
Constant	0.385333	0.011122	34.647	0.000
X_1	0.017375	0.006811	2.551	0.051
X_2	0.081875	0.006811	12.022	0.000
X_3	-0.165750	0.006811	-24.337	0.000
X_1^2	-0.094042	0.010025	-9.381	0.000
X_2^2	-0.119542	0.010025	-11.924	0.000
X_3^2	-0.074292	0.010025	-7.411	0.001
X_1X_2	0.027250	0.009632	2.829	0.037
X_1X_3	-0.002000	0.009632	-0.208	0.844
X_2X_3	-0.068500	0.009632	-7.112	0.001

Table 4.8 Estimated Regression Coefficients for PHB production from the results of Box Behnken experimental design

Term	Coef	S.E Coefficient	T	P
Constant	0.167333	0.007125	23.486	0.000
X_1	-0.002625	0.004363	-0.602	0.574
X_2	-0.001250	0.004363	-0.287	0.786
X_3	-0.017375	0.004363	-3.982	0.011
X_1^2	-0.084042	0.006422	-13.086	0.000
X_2^2	-0.065792	0.006422	-10.245	0.000

X_3^2	-0.072542	0.006422	-11.296	0.000
X_1X_2	0.012500	0.006170	2.026	0.099
X_1X_3	0.000250	0.006170	0.041	0.969
X_2X_3	-0.002500 -	0.006170	-0.405	0.702

The Table 4.7 and 4.8 represented the Student *T*-distribution and the corresponding *P*-value, along with the parameter estimate. The *P*-values are used as a tool to check the significance of each of the coefficients which, in turn, identifies the pattern of the mutual interactions between the selected variables. The parameter estimates and the corresponding *P*-values revealed that the PHB production had significantly influenced by the independent variables, X_1 (Inoculum size), X_2 [$(NH_4)_2SO_4$]. Positive coefficients for X_1 and X_2 indicated a linear effect to increase biomass production, while negative coefficient of X_3 (pH) revealed the opposite effect. The largest t-value of X_2 confirmed that it was the key factor that influenced the biomass production.

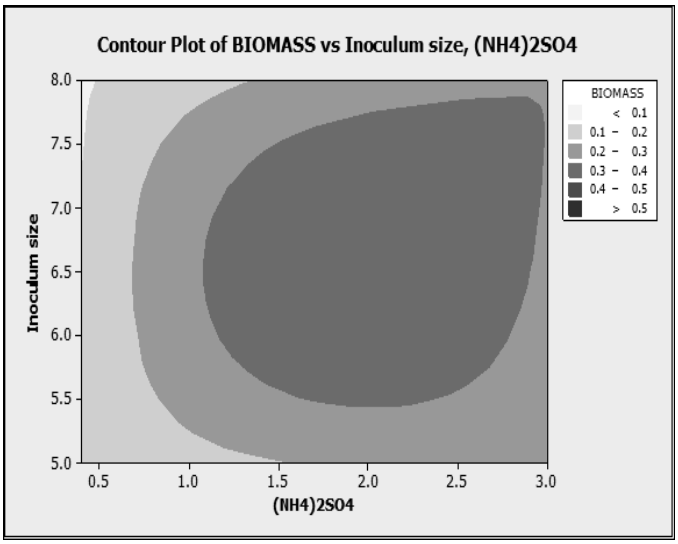


Fig 4.8 Contour plot of biomass between inoculum size and $(NH_4)_2SO_4$

Contour plots are the graphical representation of the relationship among the three variables in two dimensions and also provides information about the optimum conditions and the type of interactions between test variable. Fig 4.8 depicted the contour plot showing the interaction of inoculum size (X_1) and $(NH_4)_2SO_4$ (X_2) on biomass production, while pH (X_3) was fixed at its middle level. The nature of the contour plots confirmed the interaction between inoculum size and $(NH_4)_2SO_4$were significant and it was also evident from the p value (0.037< 0.05) from Table 4.7. The biomass production was found to increase with simultaneous increase in both the factors. As the inoculum size increases the bacteria was able to consume the available $(NH_4)_2SO_4$ and subsequently increased the biomass.

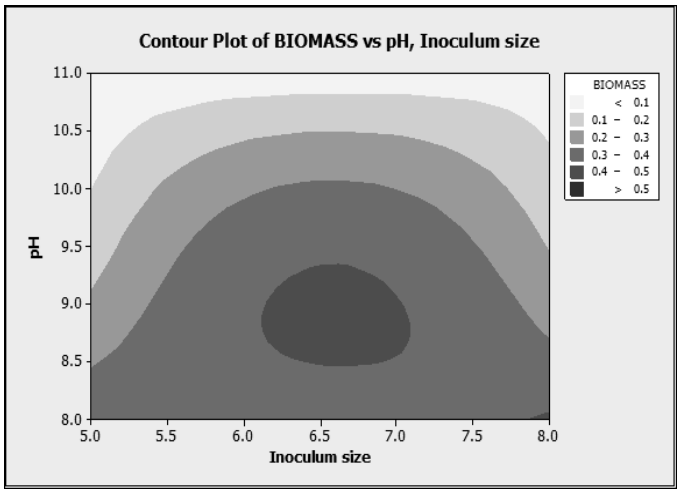


Fig 4.9 Contour plot of biomass between inoculum size and pH

The Fig 4.9 demonstrated the interactive effect between inoculum size (X_1) and pH (X_3) on biomass production. The variable $(NH4)_2SO_4$ (X_2) was fixed at its middle

level. The contour curves did not show considerable curvature, and it was concluded that the X_1X_3 term was not significant. The P value (0.844> 0.05) also supported the data (Table 4.7).The increase in pH did not support the growth of bacteria. The change in pH causes certain metals to take on different ionization states and therefore will or will not be able to be utilized. The pH also affects the solubility of many substances that bacteria need (Palleroni and Palleroni, 1978).

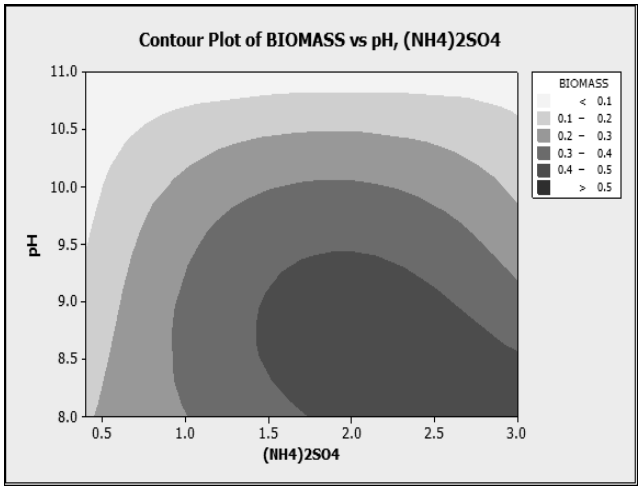


Fig 4.10 Contour plot of biomass between pH and $(NH_4)_2SO_4$

The contour plot (Fig 4.10) showed the interaction between $(NH_4)_2SO_4$ (X_2) and pH (X_3). The shape of contour plots confirmed the interaction between these two variables were significant. It was noticed that, biomass production tended to increase with gradually increasing concentration of $(NH_4)_2SO_4$. But biomass production decreased slowly with the increasing pH. The p value was 0.001(Table 4.7).

Fig 4.11 represented the interaction effect between inoculums size (X_1) and $(NH_4)_2SO_4$ (X_2). The shape of contour plots indicated that the interaction between these two variables were not significant.

The Fig 4.12 also did not show any interaction between inoculum size (X_1) and pH (X_3). The increasing pH and inoculum size tend to decrease the PHB production.

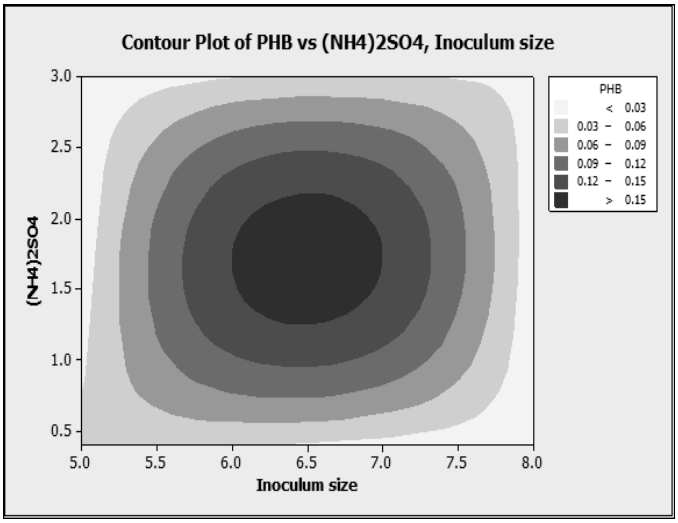


Fig 4.11 Contour plot of PHB between inoculum size and $(NH_4)_2SO_4$

This result indicted the importance in lowering of pH and inoculum size for better PHB production. Inoculum size of 6.5 ml ($8x10^8$ CFU/ml) and was found to be optimum for PHB production.

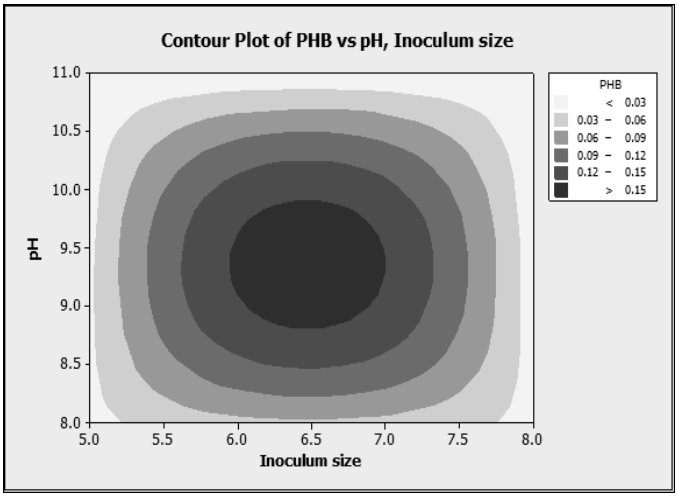


Fig 4.12 Contour plot of PHB between inoculum size and pH

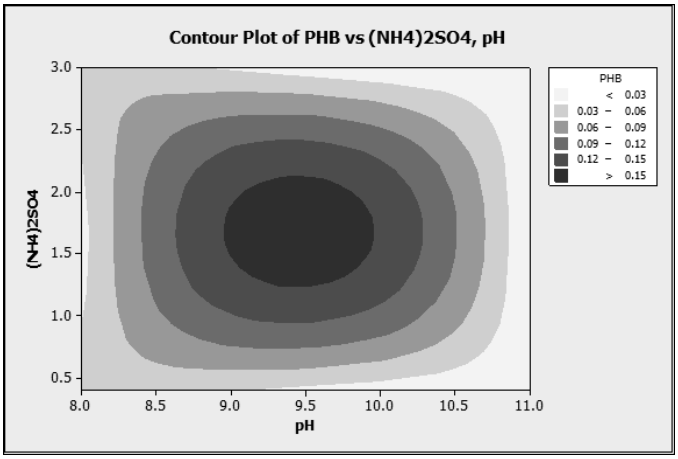


Fig 4.13 Contour plot of PHB between $(NH_4)_2SO_4$ and pH

The Fig 4.13 depicted the interaction between $(NH_4)_2SO_4$ and pH. Here also the interactive effect was very low. It is evident from the plot there was a tendency to decrease in PHB production with increasing the value of both $(NH_4)_2SO_4$ and pH.

4. 4 CONCLUSION

The *Bacillus sphaericus* was investigated and optimized for the production of the PHB under SSF using PUF as inert support. The use of inert support was a successful alternative for conventional SSF method. The biomass recovery from PUF was appreciable with less impurity.

CHAPTER 5

StudieS on polyhydroxybutyrate production in bioreactors under submerged fermentation

CHAPTER 5

Studies on polyhydroxybutyrate production in bioreactors under submerged fermentation

5.1 INTRODUCTION

The lab scale fermentation for the production of PHB had brought about promising results. However, there are still issues and obstacles to overcome before make it available for mankind. The transfer of Smf studies from laboratory scale unit to bioreactors help to identify the additional parameters that could not be provided by the flask level. But this is a challenging task due to the difficulty in assessing the factors affecting the scale-up process during the cultivation. Bacteria are more vulnerable to environmental issues such as temperature, pH, nutrient composition and dissolved oxygen during fermentation. These factors exert an ample effect on cell growth and product formation (Hsu and Wu, 2002). The bioreactor provides a suitable environment for the controlled growth of a pure or mixed culture. An ideal bioreactor provides the following facilities such as contamination free environment, close control of specific temperature and pH, maintenance of agitation and aeration, monitoring dissolved oxygen (DO), ports for nutrient and reagent feeding, ports for inoculation and sampling. Aerobic fermentation demands proper aeration to facilitate the availability of the nutrients to the microorganism rendering better performance. It is very important to provide the precise balanced dissolved oxygen in the cultivation medium to avoid the damage to the cells or death of the organisms, with a subsequent lower yield of the desired product. Also a

continuous supply of aeration is needed due to the low solubility of oxygen in broth (Hromatka et al., 1951). For the liquid fermentation, the bioreactors are equipped with internal mechanical agitation, bubble columns with gas sparging for agitation, loop reactors where mixing and liquid circulation are induced by the motion of an injected gas, by a mechanical pump or by the two. The influence of hydrodynamic conditions on the gas-liquid mass transfer in a bioprocess largely depends on the operational conditions, the physicochemical properties of the culture, the geometrical parameters of the bioreactor and also on the presence of oxygen consuming cells (Ochoa, 2009). In addition to oxygen transfer rate (OTR) and heat transfer rate, other operational factors can make changes in scale-up, such as the quality of mixing, shear stress, selection of cheaper media, foam control, physiological state of the inoculum, and sterilization of culture medium (Humphrey, 1998).

Agitation performs double purposes such as, to increase the oxygen and heat transfer, and to mix the reactor content. The insufficient agitation limits the transfer operations and thus the overall reaction performance will decline because of the appearance of zones of the fluid with insufficient nutrients or inadequate temperature or pH (Namdev et al., 1994). Agitation not only assists mass transfer between the different phases present in the culture, but also maintains homogeneous chemical and physical conditions in the culture by continuous mixing.

Aeration is beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate and product/by-product (Kongiattikajorn, 2007). Multiple impellers on a single shaft with appropriate combination and spacing are being suggested as optimum. In such cases mixing and mass

transfer are dependent on the flow rate of gas, type of agitator and its speed and properties of liquids. Power consumption per impeller decreases with an increase in the number of impellers and this increases the uniformity of energy dissipation (Nienow and Lilly, 1979).

The dissolved oxygen (DO) concentration becomes a limiting nutrient in processes of high oxygen demand (Lo et al., 2001). The supply of oxygen can be the controlling step in industrial bioprocesses, scale-up of aerobic biosynthesis systems (Al-Masry, 1999; Elibol and Ozer, 2000; Weuster-Botz et al., 1998). Efficiency of aeration depends on oxygen solubilization, diffusion rate into broths, and bioreactor capacity to satisfy the oxygen demand of microbial population. However, the DO in the broths is limited by its consumption rate on cells or the oxygen uptake rate, as well as by its oxygen transfer rate. The oxygen transfer rate could be affected by several factors, such as geometry and characteristics of the vessels, liquid properties (viscosity, superficial tension, etc.), the dissipated energy in the fluid, biocatalyst properties, concentration, and morphology of microorganisms and also depends on the air flow rate, the stirrer speed, mixing, etc. Mechanically agitated aerated vessels are widely used rather than vessels with aeration only which can be inadequate to promote the liquid turbulence necessary for small air bubble generation. Although the agitation could maintain available dissolved oxygen in the bioreactor, but the inappropriate speed of agitation results in poor oxygen transfer especially in high viscous broths.

The bioprocesses are usually conducted under previously optimized variables (temperature, pH and concentrations of nutrients) with a previously chosen mode of operation (batch fermentation). Attempts were made to study the effect of aeration and

agitation on PHB production in two stirred tank bioreactors (750 ml parallel and 5-L Biostat B bioreactor) in submerged condition.

5. 2 MATERIALS AND METHODS

Microorganism and its maintenance and inoculum preparation has been described in chapter 2.

5.2.1 Submerged fermentation

5.2.1.1 Polyhydroxybutyrate production in parallel bioreactor system

Submerged fermentation was carried out in Infors HT, Switzerland parallel bioreactor system (750 ml) with a working volume of 500 ml for the study of PHB production (Fig 5.1). The bioreactor system (WxDxH-350x500x850 mm) was equipped with DO and pH electrode. The system was also equipped with two impellers of rushton type fitted with six blades of dimensions 28x8x8 mm (outer diameter x height x Blade length). Four peristaltic pumps were used for the addition of acid, base, antifoam and feed. The pH electrode was calibrated using buffers of pH 9.0 and 4.0 and DO was calibrated by a two-point calibration method between 0 and 100% oxygen saturation. The production medium was composed of as follows (g/l): $(NH_4)_2SO_4$, 2; KH_2PO_4, 2; Na_2HPO_4, 0.6; $MgSO_4.7H_2O$, 0.2;$CaCl_2$, 0.02; beef extract, 0.2; containing 2.5% reducing sugar and 1ml trace element solution. The trace element solution contained (g/l) H_3BO_3, 0.01; $MnSO_4.H_2O$, 0.02; $CuSO_4$, 01; $ZnSO_4.7H_2O$, 0.1; and $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.02. The cultivation medium jackfruit seed hydrolyzate and trace element solution were sterilized separately at 121.5^0C for 20 min. The bioreactor was

sterilized at 121.5^{0}C for 30 min. After sterilization, the bioreactor was cooled to 30^{0}C and inoculated with 2% inoculum (8x10^8 CFU/ml of 18 h old culture). Fermentations were carried out under constant volumetric airflow rate per unit volume (Q/V=0.1 to 0.5 vvm). Agitation was controlled by the coupling of stirrer and control base, both of which were driven by magnetic stirrer. The aeration system involved an air inlet through a ring sparger with air-flow meter and filter. The temperature of the fermentation broth was monitored by temperature probe and controlled at 30^{0}C by circulating the chilled water through the jacket using a circulation pump. The foaming in the fermentation broth was monitored by a ceramic-coated antifoam probe and controlled by adding coconut oil. The DO and pH were continuously monitored by a sterilizable polarographic electrode (Mettler Toledo, Newark, Del.) and a pH sensor (Mettler Toledo), respectively. The pH was controlled at 6 using either 0.8N NaOH or HCl. The agitation was set at 200 rpm and dissolved oxygen (DO) was maintained at 50%. The effect of aeration on biomass and PHB production was studied at 0.2 vvm (Bioreactor A), 0.4 vvm (Bioreactor B), 0.6 vvm (Bioreactor C) and 0.8 vvm (Bioreactor D). The effect of dissolved oxygen on biomass and PHB production was studied at 20 and 60% DO level, where the aeration rate was set at 0.2 vvm. The effect of agitation speed on biomass and PHB production was also studied at 150 and 300 rpm with 0.2 vvm aeration rate. The batch fermentation run was performed for 48 h and the samples were withdrawn periodically at an interval of 3 h and checked the biomass, PHB, sugar utilization and total soluble protein as per described in chapter 2.

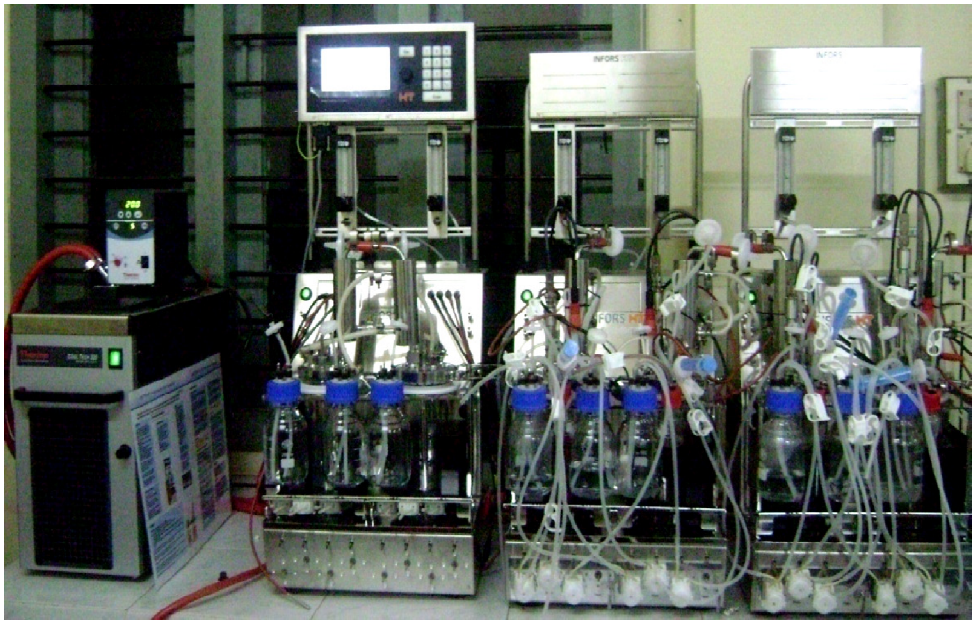


Fig 5.1 Set up of the Infors HT parallel bioreactor

5.2.1.2 Polyhydroxybutyrate production in 5- L bioreactor system

The experiments were carried out in a bench scale 5-L stirred tank bioreactor-STB (Biostat B-5; B. Braun Biotech-Sartorius) was a baffled cylindrical acrylic vessel with 3-L of final working volume. The bioreactor has an internal diameter of 160 mm and height of 250 mm with dual impellers mounted on the shaft. The baffles with a width of 12 mm were placed perpendicular to the vessel. The system was equipped with six-bladed rushton turbine impeller for agitation having a diameter of 64 mm with blade height of 13 mm and width of 19 mm. The spacing between the impellers were maintained at 110 mm and the lower impeller was located at a distance of 80 mm from the bottom of the vessel. The sparger was located at a distance of 5 mm from the bottom of the vessel through which sterile air/oxygen was sparged to the tank during the fermentation (Fig 5.2). The flow rate of sparged air was fixed at 0.5 vvm. Foaming in the fermentation broth was monitored by a ceramic-coated antifoam probe and coconut oil

was used as antifoam. The impeller rotation speed was set at 400 rpm. No pH control was exerted during the fermentation run. The DO electrode was calibrated by a two-point calibration method between 0 and 100% oxygen saturation. The production media was inoculated as in the above experiment and fermentation was carried out for 48 h, the samples were withdrawn at 3 h intervals and the biomass production, PHB, sugar utilization total soluble protein were determined in each case as described in chapter 2.

Fig 5.2 Biostat B - 5L stirred tank bioreactor

5.3 RESULTS AND DISCUSSION

5.3.1 Polyhydroxybutyrate production in parallel bioreactor system

5.3.1.1 Cascading effects of agitation rates and dissolved oxygen on biomass production at varying levels of aeration

The growth rate and product formation at different levels of aeration were examined by determining the biomass and PHB produced at 0.2, 0.4, 0.6 and 0.8 vvm and at 50% dissolved oxygen. Though the initial agitation speed was set at 200 rpm, the

impeller speed was cascaded to a range of 200-280 rpm. The pattern of biomass accumulation in all the four bioreactors showed an increasing trend with increase in aeration rate with maximum biomass yield. The bioreactor A with an aeration rate of 0.2 vvm resulted in the lowest biomass production of 5.12±0.4 g/l (Fig 5.3) and an increased biomass of 6.20±0.01 g/l was seen at 0.8 vvm (Fig 5.9). Interestingly, the fermentation time for maximum PHB production was shifted to 33 h in all the bioreactors, which was 48h in flask level and might be due to the aeration and controlled pH. This controlled parameters might have facilitated the bacterial cells to consume the available nutrients in the culture vessels. However, the PHB production was not growth related and the maximum PHB production (2.58±0.02 g/l) was in bioreactor A, here the aeration rate was only 0.2 vvm and the lowest production was in 0.8 vvm (0.39±0.005 g/l). The viscous nature of the medium might have decreased the DO levels at the end of fermentation process, which could be due to the ability of *Bacillus sphaericus* to synthesize exopolysaccharides.

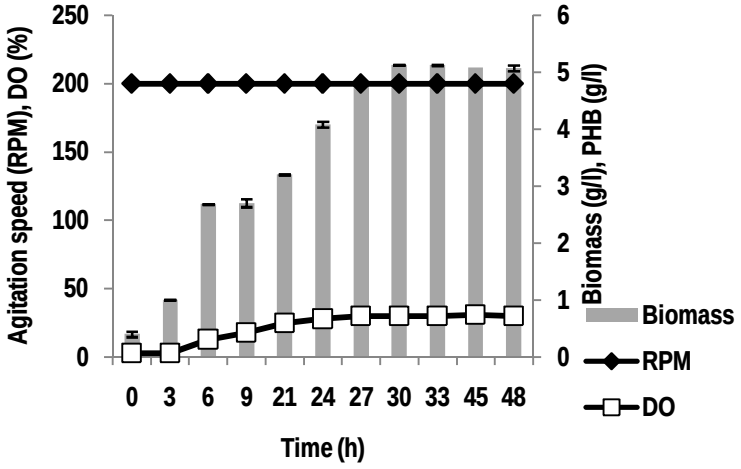


Fig 5.3 Biomass production at 0.2 vvm with 50% DO and cascading effects on impeller speed in bioreactor A

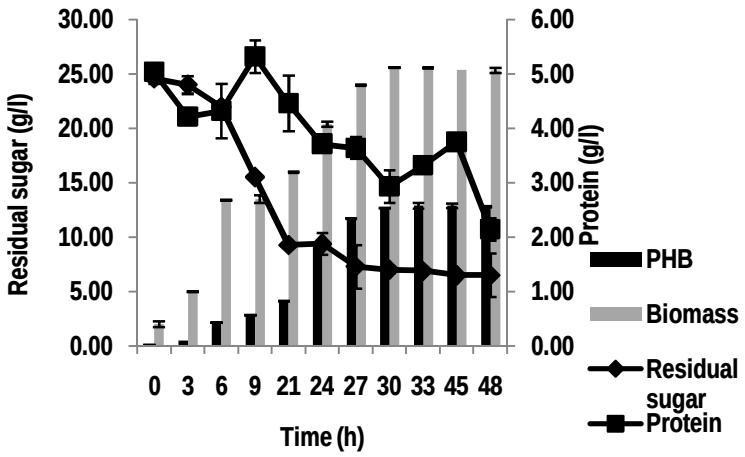


Fig 5.4 Sugar utilization and protein production in relation to biomass and PHB accumulation in bioreactor A with 0.2 vvm and 50% DO level

In bioreactor A, the agitation speed was maintained at 200 rpm till the end of fermentation run. There was an increase in DO level could be seen at 24h and after that it was constant and this might be due to the low biomass production (Fig 5.3).

In bioreactor B, a slight increase in biomass accumulation was found with 0.4 vvm aeration rate, 50% DO and agitation speed of 200 rpm. Here the initial lag phase was started with set values such as, 200 rpm and 50% DO. The DO values showed variations throughout the fermentation process and the agitation speed of 200 rpm was also adjusted according to that to maintain the aeration in the vessel. The agitation level of 200 rpm and about 50% DO was maintained in the system at initial lag phase and later the agitation speed was shifted to increased value of 250 rpm (Fig 5.5), which also resulted in decreased DO levels of 13%. As the biomass levels increased the DO levels were also found to reduce to a lower level of 13% at 45 h which indicated the biomass accumulation. The maximum biomass and PHB production were 5.42±0.03 g/l (Fig 5.5) and 1.76±0.05 g/l (Fig 5.6) respectively.

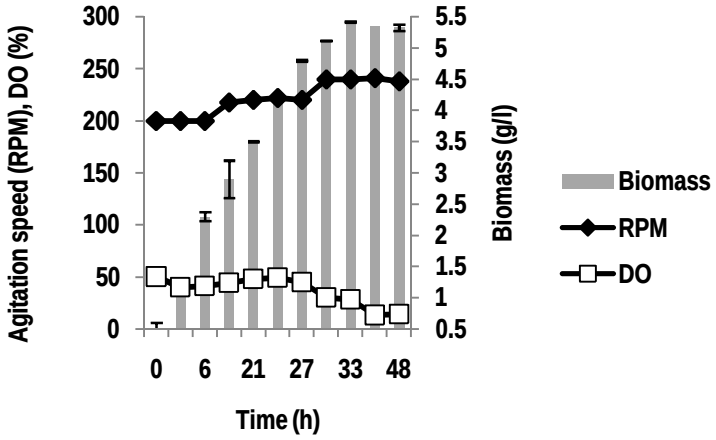


Fig 5.5 Biomass production at 0.4 vvm with 50% DO and cascading effects on impeller speed in bioreactor B

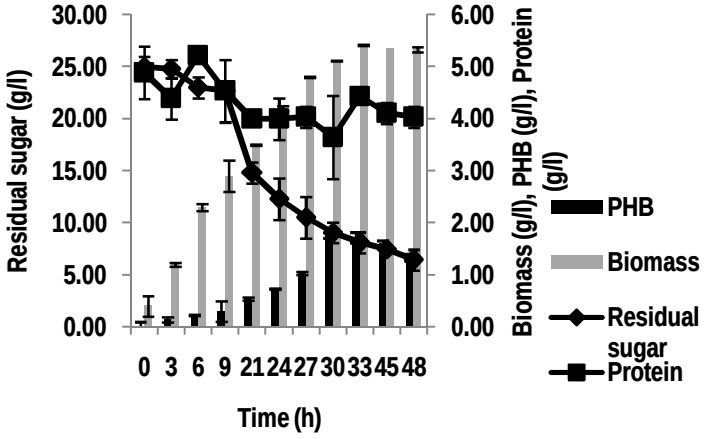


Fig 5.6 Sugar utilization and protein production in relation to biomass and PHB accumulation in bioreactor B with 0.4 vvm and 50% DO level

In bioreactor C, where the set aeration rate was 0.6 vvm, 50% DO and 200 rpm, resulted in maximum biomass of 5.62±0.3 g/l (Fig 5.7) and PHB of 1.33±0.01 g/l (Fig 5.8). The DO saturation levels in bioreactor C was found to decrease at 3 h to 13% but DO levels were restored to 46% saturation from 21h and again increased to 49% at 27 h

of fermentation which in turn resulted in the cascading effect of agitation speed (Fig 5.7). Further increase in growth had resulted in a rapid decrease in DO to 20% at 33h and correlated to higher biomass production at 0.6 vvm aeration rate.

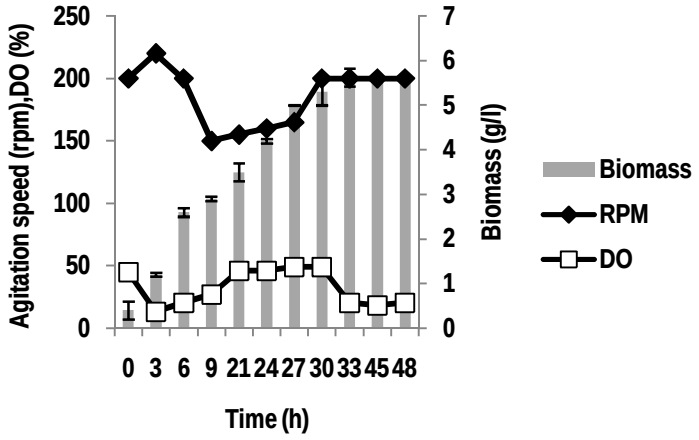


Fig 5.7 Biomass production at 0.6 vvm with 50% DO and cascading effects on impeller speed in bioreactor C

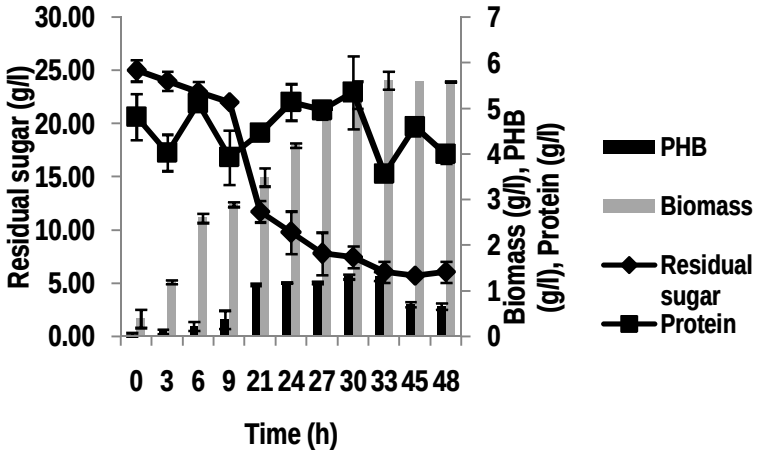


Fig 5.8 Sugar utilization and protein production in relation to biomass and PHB accumulation in bioreactor C with 0.6vvm and 50% DO level

In bioreactor D, higher variations were observed in the agitation speed and DO level and this might be due to the increased biomass production. The initial lag phase was

started with an agitation speed of 150 rpm, instead of set 200 rpm and then it was increased to 177 rpm and again decreased to 150 rpm with the increase in DO level. As the biomass levels increased, the DO levels were also reached to a lower level of 21% at 24 h, indicated high biomass accumulation (Fig 5.9 and Fig 5.10).

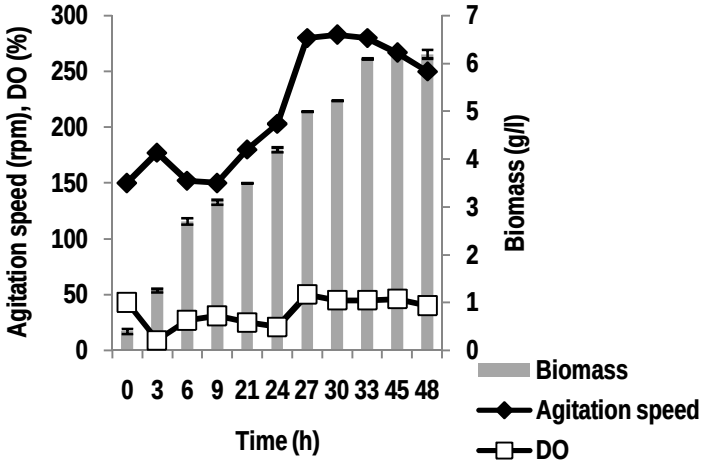


Fig 5.9 Biomass production at 0.8 vvm with 50% DO and cascading effects on impeller speed in bioreactor D

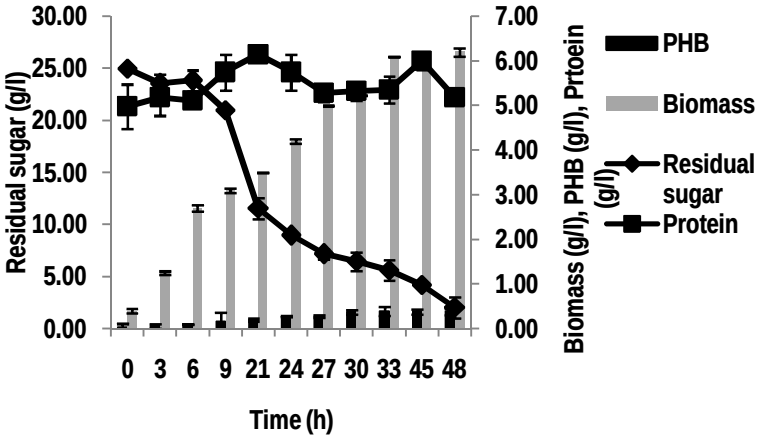


Fig 5.10 Sugar utilization and protein production in relation to biomass and PHB accumulation in bioreactor D with 0.8 vvm and 50% DO level

Further increase in biomass resulted in decrease in DO level and subsequent increase in the agitation speed up to 283 rpm at 30h. When the bacterial growth reached its stationary phase, the agitation speed found to be decreased.

5.3.1.1.1 Effect of aeration rates on substrate utilization and biomass and PHB production

The rate of substrate utilization and biomass and PHB production was studied at varying levels of aeration. The rate of substrate utilization was found to be related to the growth of biomass thus minimum residual sugar (2.03±0.04 g/l) was observed in bioreactor D (Fig 5.10) and higher residual sugar was observed at the end of fermentation in bioreactor A (6.50±0.01 g/l) (Fig 5.4). In bioreactor B the residual sugar was 6.44±0.08 g/l (Fig 5.6) and in C it was 6.08±0.12 g/l (Fig 5.8). This indicated comparatively the lower utilization of sugars by the microorganisms at lower rates of aeration due to the lower growth. It was evident from the Fig 5.4, 5.6, 5.8 and 5.10 that the protein in the production medium did not follow a regular profile. However, the maximum protein yield (5.78±0.1 g/l) was in bioreactor D, and lower protein yield was in bioreactor A (2.14±0.3 g/l), where the minimum accumulation of biomass was found and it could be correlated to the lower substrate utilization at lower levels of aeration (Fig 5.5). In all the cases the presence of protein in the initial lag phase might be contributed by the jackfruit seed hydrolyzate and beef extract (Bobbio et al., 1978). Differences in substrate utilization might be due to incomplete mixing and/or oxygen transfer resistance at the lower agitation rate and may cause disruption of free cells in the reactor by the shear forces at high stirrer speed. PHB synthesis was suppressed at higher oxygen

transfer rate. Barron (1955) proposed that the harmful effects of oxygen on biochemical materials were mainly due to non-specific oxidation of enzyme. Aeration is beneficial to the growth and performance of microbial cells as it improves the mass transfer characteristics with respect to substrate, product and oxygen (Kim et al., 2003). Oxygen transfer limitations occur commonly in bioreactor vessels due to the poor solubility of oxygen in aqueous solutions, leading to decreased performance.

5.3.1.2 Effect of dissolved oxygen (DO) concentrations on biomass and PHB production

Two levels (20 and 60%) of saturation of dissolved oxygen were studied at 0.2 vvm aeration rate. The maximum biomass (4.98±0.02 g/l) (Fig 5.11) and PHB (2.76±0.05 g/l) production resulted at 200 rpm and 20% DO (Fig 5.12), whereas a minimum yield of 0.98±0.003 g/l of biomass (Fig 5.13) and 0.25±0.001 g/l PHB was observed at 60% DO (Fig 5.14). The high DO value (60%) resulted in the reduction of the biomass and PHB production. The higher aeration also resulted in foam production. Earlier reports have indicated that during fermentation, although increases in agitation and aeration could provide better mixing and mass transfer effects, a higher agitation and aeration might have resulted in high shear stress leading to the rupture of the cells, vacuolation and autolysis (Kao et al., 2007).The control of DO created problems due to an increase in overall oxygen demand with increase of biomass.

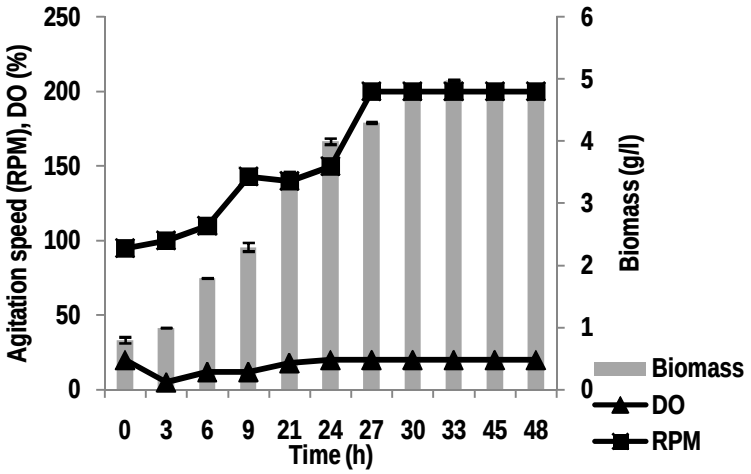


Fig 5.11 Biomass production at 0.2 vvm with 20% DO and cascading effects on impeller speed

This was accompanied by the changes in the mass transfer coefficients due to the changes in the fluid characteristics resulting from metabolic products, foaming at high aeration rates and high stirrer speeds, and the presence of anti-foaming agents. Hence, DO values influences all of these model parameters.

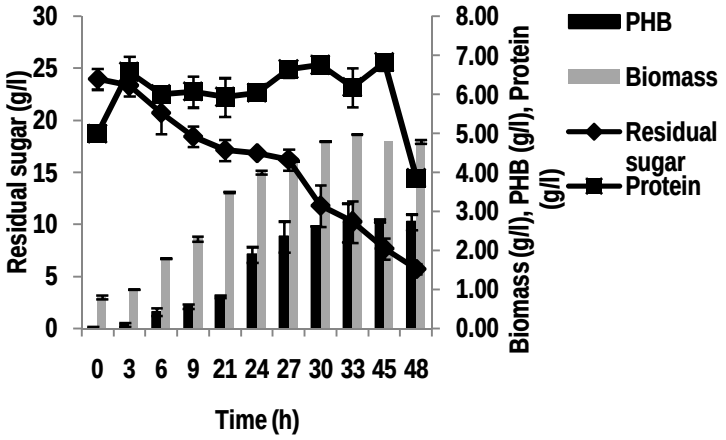


Fig 5.12 Sugar utilization and protein production in relation to biomass and PHB accumulation at 0.2 vvm and 20% DO level

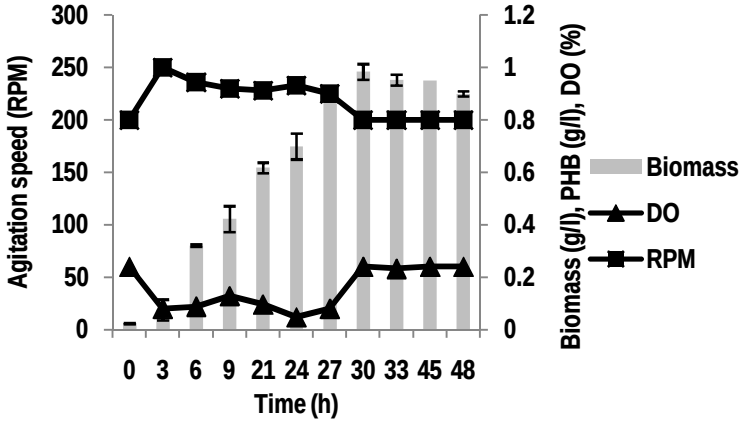


Fig 5.13 Biomass production at 0.2 vvm with 60% DO and cascading effects on impeller speed

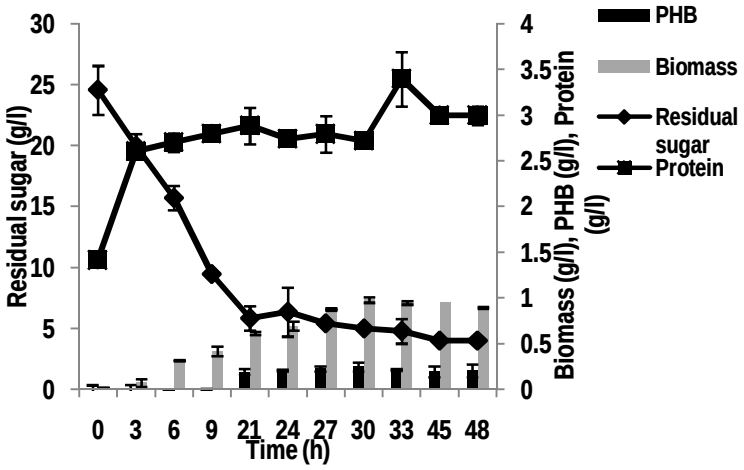


Fig 5.14 Sugar utilization and protein production in relation to biomass and PHB accumulation at 0.2 vvm and 60% DO level

The rate of substrate utilization was related to the growth of biomass and 7.12±0.06 g/l residual sugar was observed in bioreactor with 20% DO, 0.2 vvm and 200 rpm agitation (Fig 5.12). The residual sugar of 4±0.12 g/l was observed at the end of

fermentation in bioreactor with 60% DO, 0.2 vvm and 200 rpm. From 21 h, the sugar utilization was almost steady till the end of fermentation (Fig 5.14). This indicated the harmful effect of higher agitation speed on the bacterial growth. The interesting observation of sensitivity to high dissolved oxygen suggested that very high oxygen concentration also interferes with the PHB accumulation in cells resulting in lower yield. However, the requirement of moderate levels of dissolved oxygen is a positive attribute of the microbe as any process development with present bacteria may lead to less energy consumption for providing compressed air. It was reported that the oxygen limiting condition reduces the rate of re-oxidation of NADH and NADPH, via NADH oxidase and NADPH-NAD+ trans-hydrogenase, and thus trigger the PHB biosynthesis. The re-oxidation of NAD(P)H is achieved through this biosynthesis, rather than by electron transport to oxygen as the ultimate electron acceptor (Ritchie et al., 1969; Senior and Dawes, 1971).

5.3.1.3 Effect of agitation speed on biomass and PHB production

Fermentation was carried out with different initial agitation speed of 150 and 300 rpm with aeration rate set at 0.2 vvm and 20% DO level to study the effect of agitation on PHB production. As the agitation speed increased from 150 to 300 rpm, a decrease in the biomass and PHB production was noted. The highest biomass (2.82±0.05 g/l) was observed at 150 rpm (Fig 5.15) and corresponding highest PHB was 0.19±0.005 g/l (Fig 5.16). As the agitation speed increased to 300 rpm, the maximum biomass accumulation was only 2.6±0.06 g/l (Fig 5.17) and PHB was 0.11±0.003g/l (Fig 5.18).

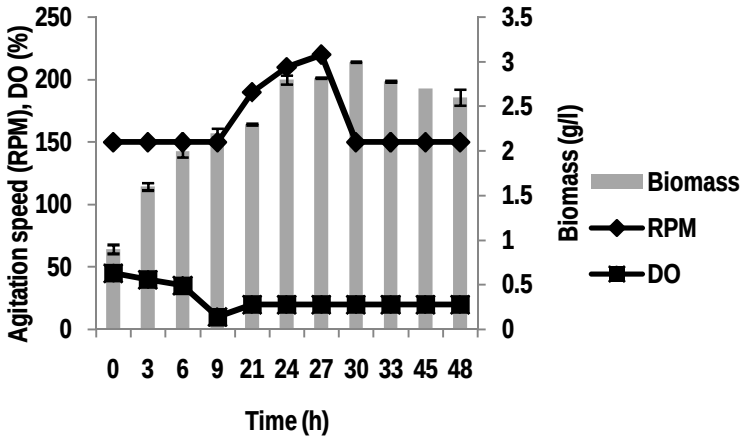


Fig 5.15 Biomass production at initial 150 rpm, 0.2 vvm and 20% DO

The reduction in biomass might be due to the shearing effects of agitation. The findings from the present study suggested that the sufficient agitation resulting in better distribution of nutrients; improved the availability of oxygen to cells by increased mass transfer, making it a significant contributor to the biomass accumulation. The agitation speed also influenced the substrate utilization by the organisms and showed a significant difference in the utilization of substrate.

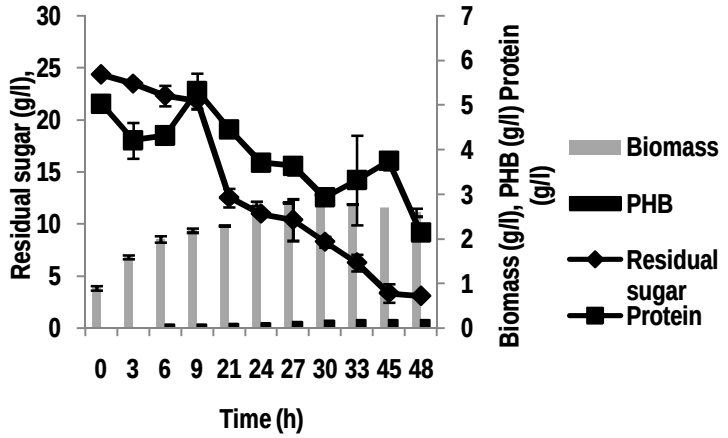


Fig 5.16 Sugar utilization and protein production in relation to biomass and PHB accumulation at initial 150 rpm, 0.2 vvm and 20% DO

The sugar utilization was low in bioreactor with initial 300 rpm, where the residual sugar was 5.98±0.09 g/l (Fig 5.18). The residual sugar of 3.1±0.02 g/l was found in bioreactor with 150 rpm (Fig 5.16).

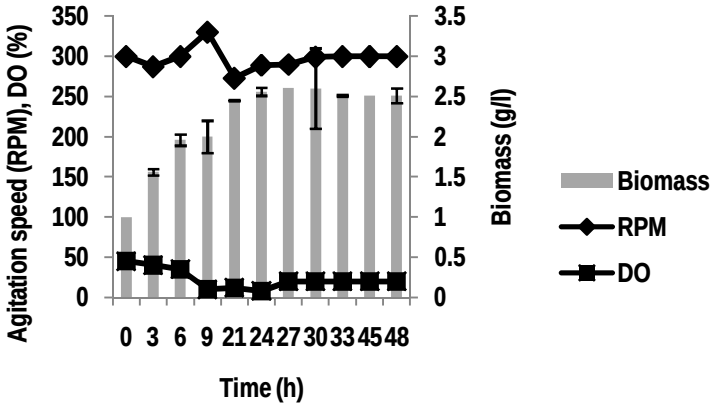


Fig 5.17 Biomass production at initial 300 rpm, 0.2 vvm and 20% DO

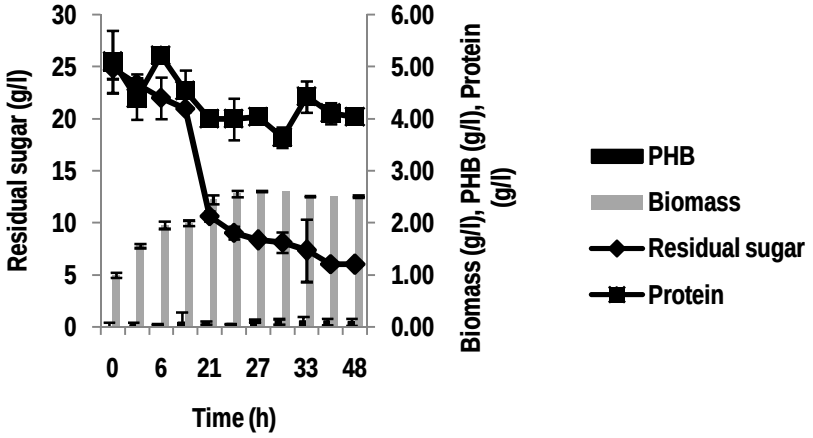


Fig 5.18 Sugar utilization and protein production in relation to biomass and PHB accumulation at initial 300 rpm, 0.2 vvm and 20% DO

5.3.1.4 PHB production in 5-L bioreactor

The specific flow rate of the air or oxygen was fixed at 0.5 vvm and impeller speed of 400 rpm. The fermentation was run at a temperature of 30^0C. The dissolved oxygen (DO) was maintained by supplying the purified oxygen automatically by operating the DO controller in cascade. Hence, under the low dissolved oxygen conditions, purified oxygen was automatically supplied to maintain the desired DO level. The production studies revealed biomass production of 7.29±0.09 g/l and corresponding PHB production was 3.21±0.006 g/l (Fig 5.19) at 0.5 vvm, and 400 rpm.

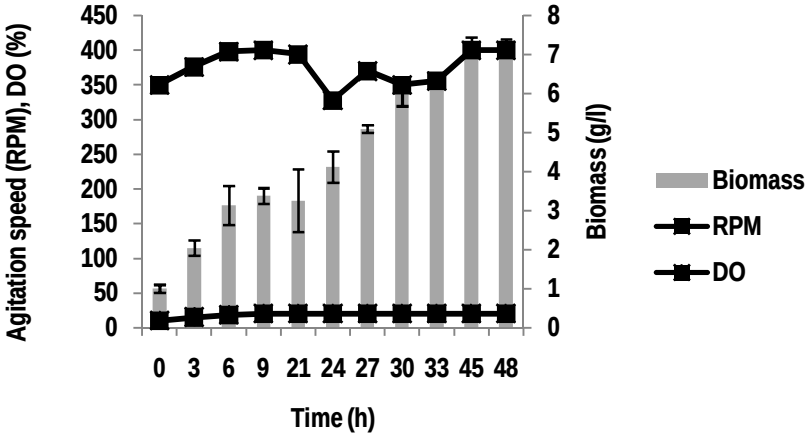


Fig 5.19 Biomass production in 5-L bioreactor at 0.5 vvm and 400 rpm

The polymer production has been scaled up to 20 L with a 14 L working volume in batch fermentations using a newly characterized *Bacillus* strain, *Bacillus cereus* SPV and the study demonstrated the production of 29% dry cell weight of PHB within 48 h (Valappil et al., 2007).

The residual sugar and the yield of soluble protein obtained were 2±0.04 g/l and 8.23±0.01 g/l (Fig 5.20), which indicated the efficient utilization of the substrate for the growth and PHB production.

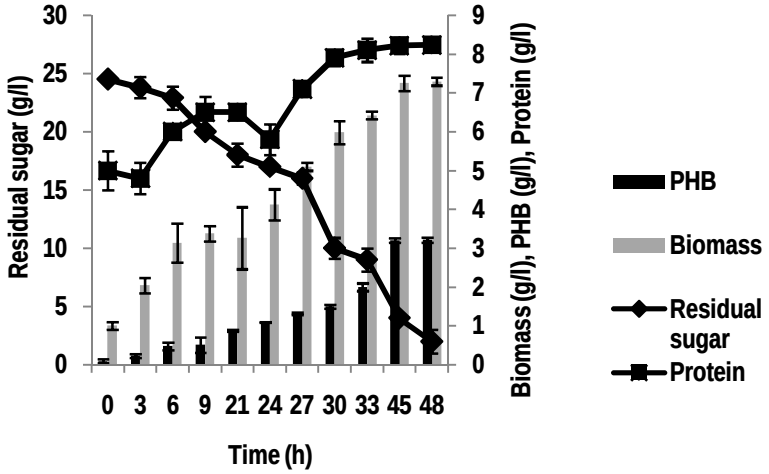


Fig 5.20 Sugar utilization and protein production in relation to biomass and PHB accumulation in 5-L bioreactor at 0.5 vvm and 400 rpm

5.4 CONCLUSION

This study concluded that the condition of aeration and agitation play significant roles in PHB production which is again related to the sugar utilization by *Bacillus sphaericus* in the bioreactors**.** A suitable mixing level should be a compromise between good mass transport and low shear. Hence, studies were conducted at different aeration rates, DO level and agitation speed. The finding from present study suggested that linear increase in agitation resulted in better distribution of nutrients; also, it improved the availability of oxygen to cells by increased mass transfer, making it a significant contributor to response. The agitation and aeration influenced the substrate utilization and

product formation. Differences in substrate utilization may be due to the incomplete mixing and/or oxygen transfer resistance at the lower agitation rate and may cause disruption of free cells in the reactor by the shear forces at high stirrer speed.

CHAPTER 6

Purification and characterization of the biopolymer and blend preparation

CHAPTER 6

Purification and characterization of the biopolymer and blend preparation

6.1 INTRODUCTION

In today's scientific community, biodegradable and eco-friendly materials produced by renewable sources are of particular interests to many researchers. PHAs are usually extracting from the producing cells using physical methods with solvents or their mixtures (Holmes and Jones, 1981). The non- polymer cell materials (NPCMs) include nucleic acids, lipids and phospholipids, peptidoglycan and proteinaceous materials. The polymer extraction using mild polar compounds like acetone and alcohols (Holmes et al., 1980) weaken or break down non-polymer cell material (NPCM), leaving PHB granules intact. But acetone may harm some longer-side-chain PHAs by solubilizing them in acetone (Brandl et al., 1988). However, chloroform and other chlorinated hydrocarbons (Stageman, 1984) dissolve all the PHB. For the effective extraction of polymer, a method has been developed by applying both types of solvents are therefore usually applied. The next step in extraction procedure is to separate out the dissolved polymer from the solvent by evaporation with acetone or an alcohol, or precipitation using methanol or ethanol. It was also noticed that drying the cells prior to the extraction steps (Holmes et al., 1980) can facilitate the subsequent polymer recovery, as can changing the pH or temperature (Walker et al., 1981; Barham and Selwood, 1982) of the polymer–solvent mixture. Another approach, in which differential digestion of NPCM can be facilitated with

alkaline solutions of sodium hypochlorite followed by the separation of undigested polymer granules from the aqueous phase, can be achieved by centrifugation. But the important drawback of this method was the loss of molecular mass of the polymer through severe damage to the granules (Nuti et al., 1972; Senior and Dawes, 1973). However, treatment of the cells with a surfactant prior to washing with hypochlorite led to further improvements in the degree of purity and molecular mass of the final product (Ramsay et al., 1990).

The high cost of large-scale solvent extraction has been answered by the development of non-solvent processes. In Zeneca, the recovery of PHB and P(3HB-*co*-3HV) has been achieved by an enzymatic method. Page and Cornish (1993) were succeed in obtaining a high molecular-mass PHB by treating the cells with 1N aqueous NH_3 in a process substantially simpler than enzymatic recovery. The addition of fish peptone to the culture to enhance polymer production rendered the cells osmotically sensitive and fragile, thus susceptible to disruption by NH_3.

After the recovery process, next chore is the determination of their monomeric compositions. Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared (FTIR) spectroscopy are the fascinating and effective methods for observe the structure and dynamics of polymer chains. Fourier transform infrared (FTIR) spectroscopy is a routine chemical technique used to explore the molecular structure; it detects the vibration characteristics of chemical and functional group in the same sample by allowing the IR radiation to pass through it. Some of the infrared radiation is absorbed by the sample and some part is transmitted. The resulting spectrum represents the molecular

absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum.

Despite its excellent properties, PHB is inherited by some drawbacks that limit its processability and effectiveness as a plastic. PHB has strong crystallizability and when crystallizes from melting state, it may form large spherulites, this ultimately gives rise to a very brittle property. Furthermore, PHB displays exceptional thermal instability at temperatures beyond its melting point, which poses great difficulty in processing using conventional methods. One possible solution for these problems is to blend PHB with another polymer to improve the properties of the system. Reactive blending is an interesting technology for preparing new polymer materials starting from different polymers. Thermal Gravimetric Analysis (TGA) is a simple analytical technique that measures the weight loss (or weight gain) of a material as a function of temperature. As materials are heated, they can lose weight from a simple process such as drying, or from chemical reactions that liberate gases. Some materials can gain weight by reacting with the atmosphere in the testing environment. Since weight loss and gain are disruptive processes to the sample material, knowledge of the magnitude and temperature range of those reactions are necessary in order to design adequate thermal ramps and holds during those critical reaction periods. Polymer crystallinity can be determined with DSC by quantifying the heat associated with melting (fusion) of the polymer. This heat is reported as percent crystallinity (Xc).

The biodegradability of plastics provides these materials with novel and additional properties which may also be beneficial during their use. For example, in agriculture biodegradable mulch films would not need to be removed to landfills or

incinerated after use, but would decompose with time and could simply be ploughed into the soil, where they would biodegrade. Hence, it is not surprising that this concept of using biodegradable plastics has become of major interest during recent years.

Aim of the present study was to extract the polymer from *Bacillus sphaericus* NII 0838 and to improve its properties by making blends. The degradation profile of these films was also analyzed.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Extracted pure biopolymer was used in this study. PEG with molecular weight 1450 (Merck, India) and PHB (Sigma-Aldrich Chemicals, USA).

6.2.2 Microorganism and preparation of inoculum

Microorganism, its maintenance and inoculum preparation has been described in chapter 2.

6.2.3 Extraction of biopolymer

6.2.3.1 Chloroform extraction

The polymer was extracted from cells by stirring 1 g of freeze dried cell in 100 ml chloroform for 48 h at 37^0C and extracted by re-precipitation with 10 volumes of ice-cold methanol (Hahn et al., 1995).

6.2.3.2 Dispersion of sodium hypochlorite and chloroform.

A 1g portion of cell powder was treated with a dispersion containing 50 ml of chloroform and 50 ml of a diluted sodium hypochlorite solution. The hypochlorite concentrations in the aqueous solutions used were 5, 10, 20 and 30% (v/v). After the cell powder was agitated at 30^0C for 1 h, the mixture was then centrifuged at 4,000x*g* for 10 min, which resulted in three separate phases. The upper phase was a hypochlorite solution, the middle phase contained non-PHB cell material and undisrupted cells, and the bottom phase was chloroform containing PHB. The upper phase was removed first with a pipette, and the middle phase was separated by filtration from the chloroform phase. Finally, pure PHB was obtained by adding ice cold methanol (10 times volume of chloroform). The pure PHB get precipitated and which was separated by filtration.

6.2.4 Characterization of extracted PHB

6.2.4.1 Fourier Transform Infrared (FTIR)

The presence of different functional group in PHB was checked by FTIR. FTIR spectra of polymer films were recorded on a Shimadzu IR prestige 21 spectrometer in the range from 4000 to 500 cm^{-1} with a resolution of 4.0 cm^{-1}. The sample films and commercial PHB (Fluka, Sigma -Aldrich chemicals, USA) (2 mg) were cast from a $CHCl_3$ solution on a sodium chloride crystal. The films were dried at room temperature until $CHCl_3$ was completely removed and 25 scans were co added and averaged.

6.2.4.2 Nuclear Magnetic Resonance (NMR)

^{1}H NMR spectra obtained in a Bruker Avance II 500 spectrometer. The polymer was suspended in spectrochem grade deuterochloroform (CDCl3). The 500 MHz ^{1}H-NMR spectra were recorded from this $CDCl_3$ solution of the samples. The standard PHB (Fluka, Sigma Aldrich chemicals, USA) (10 mg/ml) and the extracted biopolymer sample (10 mg/ml) were used for the analysis. The ^{13}C NMR spectrum was also done using 20 mg of the standard and extracted biopolymer which were dissolved in $CDCl_3$ and subjected to the analysis. The spectra were referenced to internal Tetra methyl silane (TMS). For ^{1}H NMR spectra the sample was submitted to a delay (D1) and acquisition time (AQ) of 1.00 s and 3.17 s, respectively, whereas for ^{13}C NMR, the D1 and AQ were 2.0 and 1.1 s respectively.

6.2.5 Preparation of blends

6.2.5.1 PHB-Thermoplastic starch (TS)

PHB and thermoplastic starch were mixed in different ratios (1:1, 3:2 and 2:3). Thermoplastic starch (TS) was obtained by mixing starch powder, water, and glycerol in 50:15:35 (w/v/v) ratios (Ramsay et al. 1995). The contents were thoroughly mixed for 15–30 min to obtain a paste. It was then heated in a boiling water bath with continuous stirring for 15 min. This thermoplastic starch was mixed with PHB in different ratios and solvent cast films were obtained from chloroform. The mechanical properties were analyzed using TGA and DSC.

6.2.5.2 PHB- PEG blend

PEG is a synthetic polymer known to be highly hydrophilic, biocompatible and flexible. PEG is believed to be able to accelerate the degradation process by reducing the crystallinity of PHB. Different ratios of PHB to PEG were dissolved in chloroform (1:1, 3:2 and 2:3). These solutions were stirred for 1h to form a homogenized and clear solution. It was then cast onto a Petri dish and completely dried to a constant weight at room temperature. The mechanical properties were analyzed using TGA and DSC.

6.2.6 Analysis of mechanical properties

6.2.6.1 Thermal Gravimetric Analysis (TGA)

Thermal gravimetric analysis (TGA) of extracted polymer was performed in a Shimadzu H-50 TGA apparatus. The sample films were heated from 30^0C to 500^0C at a heating rate of 10^0C/min. The TGA curve was generated by plotting the TGA signal, converted to percent weight change on the Y-axis against the reference material temperature on the X -axis.

6.2.6.2 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry is a thermo-analytical technique, by which the melting and crystallization temperature of polymer can be determined. DSC analysis of biopolymer was performed using a Perkin-Elmer Pyris 6 DSC instrument which was calibrated with indium. The samples of cast films were sealed in aluminum pans and then heated from 20^0C to 200^0C at a scanning rate of 10^0C/min with a nitrogen atmosphere around the sample. The melting enthalpy (ΔH_f), melting temperature (Tm) were

determined from the DSC endothermal peaks. After 1 min annealing, the samples were cooled to 20^0C. The thermal properties of extracted biopolymer and its blends were compared with commercial PHB.

6.7 Biodegradation

Biodegradation study was carried out in this investigation using preweighed extracted biopolymer and its blend films by immersing in different kinds of samples kindly supplied from the Waste water treatment plant of MILMA and Solid waste treatment plant, Vilappilsala, Trivandrum (Table 6.1). The samples were collected in sterile polythene containers. Biodegradation was monitored after one month by measuring the residual mass. For this, the buried samples were recovered, washed with distilled water and dried at room temperature before being weighed. The residual mass was calculated as the ratio between the final and the initial weights.

Table 6.1 Samples used in biodegradation studies

Leachate fresh (pH 4)	
Final compost	**Vilappilsala solid waste treatment plant, Trivandrum**
Semi compost 1.5yr	
Leachate 1.5 yr	
Landfill soil	
Activated sludge	**MILMA (waste water treatment plant), Trivandrum**
Pontibacter sp. (NII 905)	

Citrobacter sp. (NII 908)	
Paracoccus sp. (NII 917)	
Peanibacillus sp. (NII 918)	
Alicycliphilus sp. (NII 922)	
Serratis sp. (NII 935)	**Bacterial strains from NII Culture Collection, NIIST, Trivandrum**
*Pontibacter*sp. (NII 905)	
Citrobacter sp. (NII 908)	
Paracoccus sp. (NII 917)	
Peanibacillus sp. (NII 918)	
Alicycliphilus sp.(NII 922)	**Fungal strains from NII Culture Collection, NIIST, Trivandrum**
Serratis sp. (NII 935)	
Pontibacter sp. (NII 905)	
Citrobacter sp. (NII 908)	
Paracoccus sp. (NII 917)	

6.7.1 Isolation of PHB degrading microbes from the above samples

6.7.1.1 Enrichment method

One gram soil sample was added to 10 ml sterile mineral media without any carbon source and incubated it for two days. To which 0.1% PHB was placed to it and further incubated for five days. The enriched samples were spread plated onto mineral salt agar plates containing PHB as the sole source of carbon and incubated for three days. The organisms were isolated by pure culturing on PDA plates. The organisms were

screened for PHB degradation as follows. Mineral medium with agar was prepared and PHB was used as the sole carbon source in the medium. The cultures were inoculated in the mineral agar plates containing PHB, kept at 30^0C for three days. The formation of clear zones around the bacterial colonies on PHB-agar plates were considered as the indication of PHB-degrading microorganisms.

6.7.2 Screening of PHB degraders among isolates from NII Culture Collection, NIIST, Trivandrum

6.7.2.1 Fungal isolates

Fungal isolates (Nine known and 14 unknown) from NII Culture Collection, NIIST, Trivandrum (Table 6.1) were screened for PHB degradation in mineral medium with agar containing PHB as sole source of carbon. The cultures were inoculated in the mineral agar plates containing PHB, kept at 30^0C. Since some fungi grow slowly and show enzyme activity late, determination of PHB degradation required an incubation time of three weeks. PHB-degrading microorganisms were indicated by the formation of halos around the colonies on PHB-agar plates.

6.7.2.2 Bacterial isolates

Bacterial isolates (Table 6.1) were cultured in LB broth, and 2 ml was transferred to 250 ml Erlenmeyer flask containing 50 ml sterile mineral medium and pre-weighed thin films of commercial PHB, extracted polymer and its blends. The flasks were incubated at 30^0C for four weeks. The residual films were separated by filtration and

washed thoroughly with distilled water. It was dried to a constant weight and the % weight loss was calculated.

6.3 RESULTS AND DISCUSSION

6.3.1 Extraction of biopolymer

6.3.1.1 Chloroform extraction

Chloroform extraction method relied solely on the solubility of the PHB in chloroform and did not involve any treatment with sodium hypochlorite. The PHB yield was very low in this method, might be due to the inefficient lysis of bacterial cell wall.

6.3.1.2 Dispersion of sodium hypochlorite and chloroform.

Dispersion technique could significantly reduce the degradation of PHB by hypochlorite, and thus minimize the problems associated with conventional PHB digestion methods. An inverse relationship exists between the molecular weight and hypochlorite concentrations. Sodium hypochlorite with 30% gave best result.

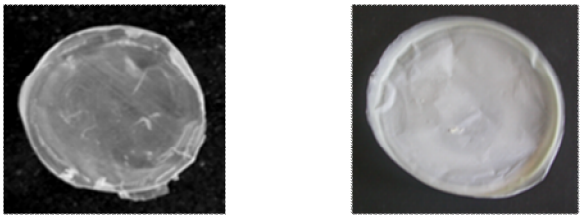


Fig 6.1 Film of commercial PHB and extracted biopolymer

6.3.2 Characterization of the extracted biopolymer

6.3.2.1 Fourier Transform Infrared spectroscopy

IR spectra of commercial PHB (Sigma- Aldrich) (a) and extracted biopolymer (b) is shown in Fig 6.2.The standard PHB represented the spectrum of mainly two intense absorption bands at 1722.43 cm $^{-1}$ and 1278.81 cm^{-1} corresponding to C=O and C–O stretching groups respectively. The existence of exactly the same transmission at 1722.43 cm $^{-1}$ and 1278.81 cm^{-1} in the IR spectra of extracted biopolymer indicated that *Bacillus sphaericus* could synthesize PHB when jackfruit seed hydrolyzate used as sole source of carbon in the production medium.

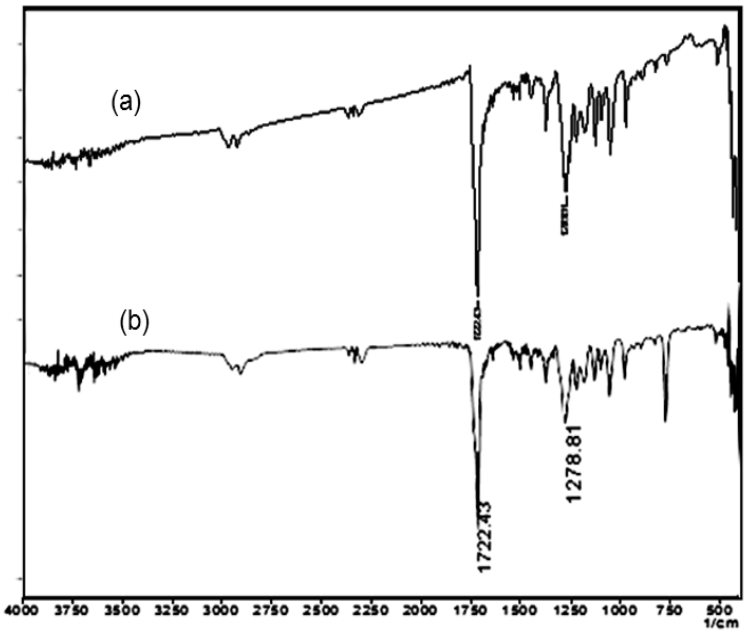
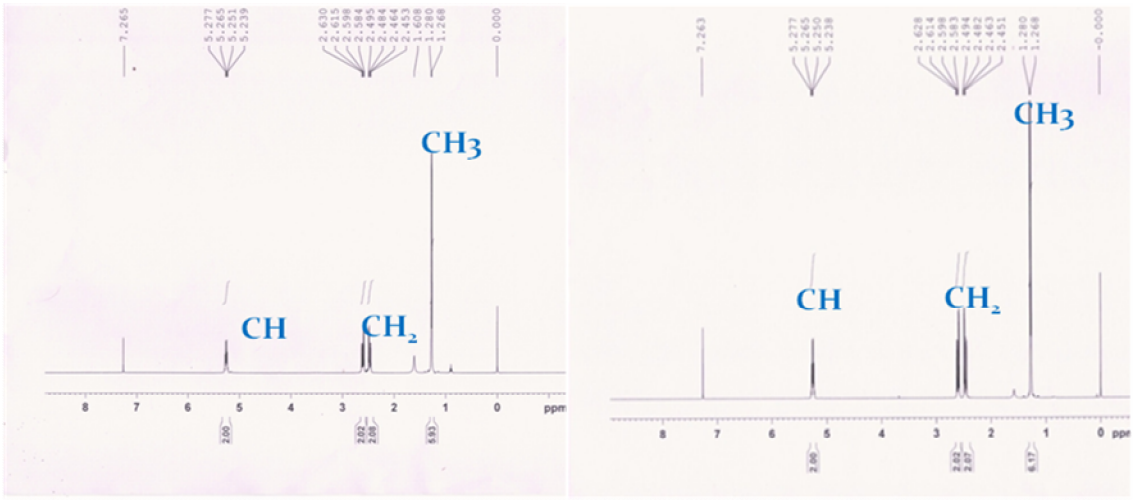


Fig 6.2 IR spectra of commercial PHB (a) and extracted biopolymer (b)

6.3.3 Nuclear Magnetic Resonance (NMR)

The quality of structural composition of PHB was easily determined by NMR analysis. The ^{1}H and ^{13}C NMR spectra obtained from PHB samples produced from jackfruit seed hydrolyzate, are shown in Fig 6.3 and 6.4, respectively compared with the commercial PHB. Both were found to match perfectly with each other. The peaks in the spectra were coincide, corresponding to the different types of carbon atoms presented in the PHB structure, [-O- CH- (CH_3)- CH_2- (C=O)-] $_n$. The chemical shift scale was in parts per million (ppm) and showed in Table 6. 2 and 6.3. The spectrum showed a doublet at 1.28 ppm which was attributed to the methyl group coupled to one proton and a doublet of quadruplet at 2.58 ppm, which was a characteristic of an asymmetric carbon atom bearing a single atom. The multiplet at 5.7 ppm was contributed by the methylene group. Another prominent peak was observed in the spectrum at 7.2 ppm which was contributed by the solvent used i.e. $CDCl_3$.



(a) (b)

Fig 6.3 ^{1}H NMR spectra of commercial PHB (a), extracted biopolymer (b)

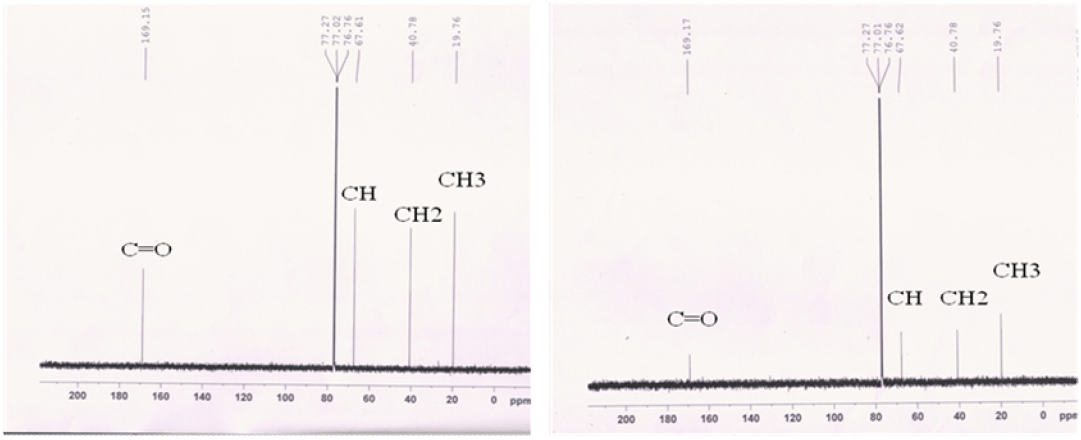


Fig 6.4 ^{13}C NMR spectra of commercial PHB (a), extracted biopolymer (b)

The chemical shift signals of ^{1}H and ^{13}C NMR spectrum obtained in the present work and the commercial PHB were agreed with those obtained by Fabiane et al. (2007) and is shown in Table 6.2 and 6.3. Thus the extracted polymer was confirmed as PHB.

Table 6.2 Comparison of chemical shift signals of ^{1}H NMR spectrum of commercial and extracted biopolymer with literature data

Hydrogen type	PHB extracted	PHB commercial	PHB (Kunioka , 1989, 1990)
CH3	1.26- 1.28	1.26- 1.28	1.24–1.30
CH2	2.45-2.62	2.45-2.63	2.48–2.59
CH	5.23-5.27	5.23-5.27	5.16–5.35

Table 6.3 Comparison of chemical shift signals of ^{13}C NMR spectrum of commercial and extracted biopolymer with literature data

C atom	PHB extracted	PHB commercial	PHB
CH3	**19.76**	**19.76**	**19.65**
CH2	40.78	40.78	40.66
CH	67.62	67.61	67.48
C=0	169.17	169.15	169.03

6.3.2 Blends

Blending is a common practice in polymer science to improve unsatisfactory physical properties of the existing polymer. The techniques most commonly adopted to blend polymers are either melt blending (direct mixing of the component polymers in the molten state), or film casting from a common solvent. While the former method tends to mimic industrial processing conditions, the latter is a simple laboratory approach. In this study the latter approach was adopted to study the effect of blending on the physical properties of extracted biopolymer.

6.3.2.2 PHB- Thermoplastic starch (TS)

Many efforts have been exerted to develop starch-based polymers for conserving the petrochemical resources, reducing environmental impact and searching more applications. PHB and starch were good candidates for polymer blends preparation because these are biodegradable and derivable from renewable resources. The importance in making thermoplastic starch was to enhance the interfacial affinity. Starch is

gelatinized to disintegrate granules and overcome the strong interaction of starch molecules in the presence of water, which lead to well dispersion (Martin and Avérous, 2001, Park et al., 2000). Out of the various proportions studied, PHB to starch ratio of 1:1 gave a very good film formation (Fig 6.5).

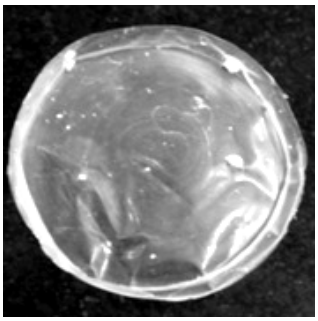


Fig 6.5 Film of extracted PHB-TS

However, the hydrophilic nature of starch did not allow interacting well with hydrophobic polyesters resulting to unfavorable qualities of the blends. Starch could improve the biodegradability and lower the cost while PHB was able to control the mechanical properties of the blend.

6.3.1.2.2 PHB-PEG

PEG is a synthetic highly hydrophilic polymer and believed to be able to accelerate the degradation process by reducing the crystallinity of PHB. On the other hand, its mechanical tensile strength and elongation at breaking are decreased. The film of PHB-PEG (1:1) was transparent, homogeneous and flexible compared to the brittle pure PHB film. The incorporation of low molecular weight PEG into a PHB matrix could improve the hydrophilicity and flexibility by increasing the availability of water with the matrix.

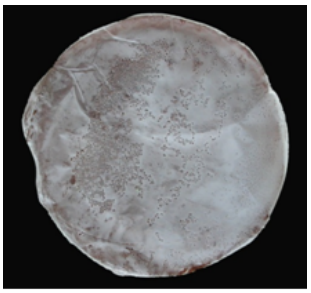


Fig 6.6 Film of extracted PHB-PEG

6.3.4 Analysis of mechanical properties

6.3.4.1 Thermal gravimetric analysis

TGA involves the measurement of change of sample mass with change of temperature. It measures the variation of heat flux in a sample with variation of temperature. It helps gain insight on the degradation behavior of both polymer constituents of the blend. The test result of the samples were represented by a graph of the TGA signal on the Y -axis plotted versus the sample temperature in ^{0}C on the X -axis which is shown in Fig 6.7 to 6.10. The Fig 6.7 and 6.8 represented the typical TGA curves of commercial PHB and extracted biopolymer respectively. The onset of degradation temperature, the temperature at which weight loss is maximum (Tmax) and the residual weight in percentages are given in Table 6.4. The increasing in temperature caused a decrease in the viscous nature of the polymer. At some points the molecules might obtained enough freedom of motion to arrange themselves spontaneously into a crystalline form. It is known as the crystallization temperature (Tc). The transition from amorphous solid state to crystalline solid was an exothermic process, and resulted in a peak in DSC signal. As the temperature increases the sample reached the melting temperature and the melting process resulted in an endothermic peak. The thermal

degradation of commercial and extracted PHB proceeded by one-step process with a maximum decomposition temperature at 279 and 290^{0}C respectively. The thermal degradation was started from 195.36^{0}C with 3.61% weight loss whereas the extracted polymer was stable at the same temperature and started degradation only at 222.05^{0}C. But at Tmax (290^{0}C), 99.156% polymer was lost due to the thermal degradation. At the end, the residual weight was 3.6 and 0.85% for commercial PHB and extracted PHB respectively. It was reported that the thermal degradation at maximum decomposition temperature of about 300^{0}C is mainly due to the ester cleavage of PHB component by β-elimination reaction (Choi et al., 2003). This reaction was favored because the hydrogen atoms in the methyl side group of the PHB were chemically active due to the proximity to the carbonyl groups that grant them an acidic character and lead to random chain scission. However, the blends (PHB-TS and PHB-PEG) exhibited a different thermal decomposition pattern, with decreased thermal stability, in which two separated degradation steps could be seen (Fig 6.9 and 6.10). The PHB- TS blend in which the first one between 150 and 220^{0}C might be attributed by the degradation of thermoplastic starch. The second event exhibited somewhat the same characteristics of the mass loss of the extracted PHB. The onset of thermal degradation for PHB-PEG was drop to 178^{0}C, because the melting temperature of PEG is 60^{0}C. Hence when PHB mixed with PEG, the two components were partially compatible, that shifted the melting temperature. At 178^{0}C the weight loss was 46.212% and the maximum degradation temperature was extended up to 342.47^{0}C.

Table 6.4 Data obtained by Thermal gravimetric analysis

Sample	Temperature of onset of degradation (^{0}C)	Tmax (^{0}C) First step	Tmax (^{0}C) Second step	Residual weight (%)
Commercial PHB	195	279	-	3.6
Extracted PHB	222	290	-	0.85
Extracted PHB - TS	179	227	259	5.5
PHB-PEG	178	269	342	4.54

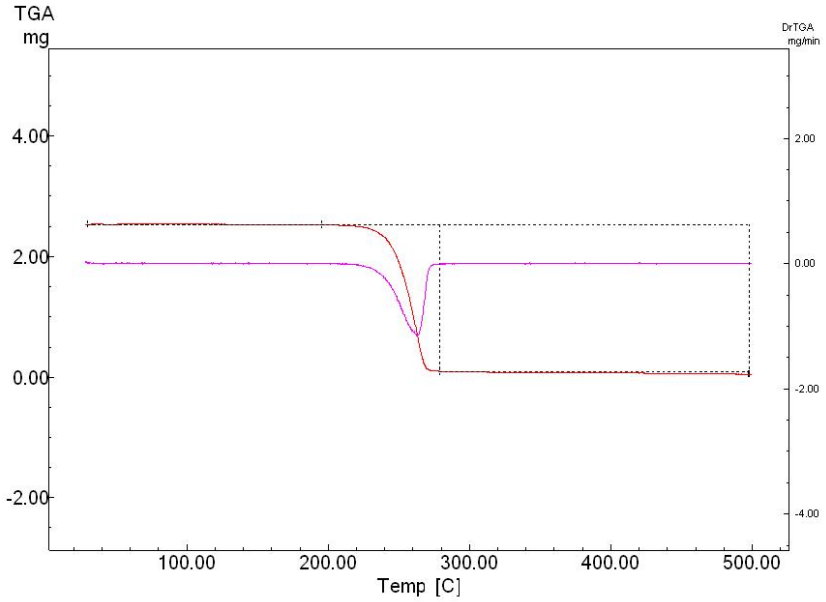


Fig 6.7 TGA curve of commercial PHB

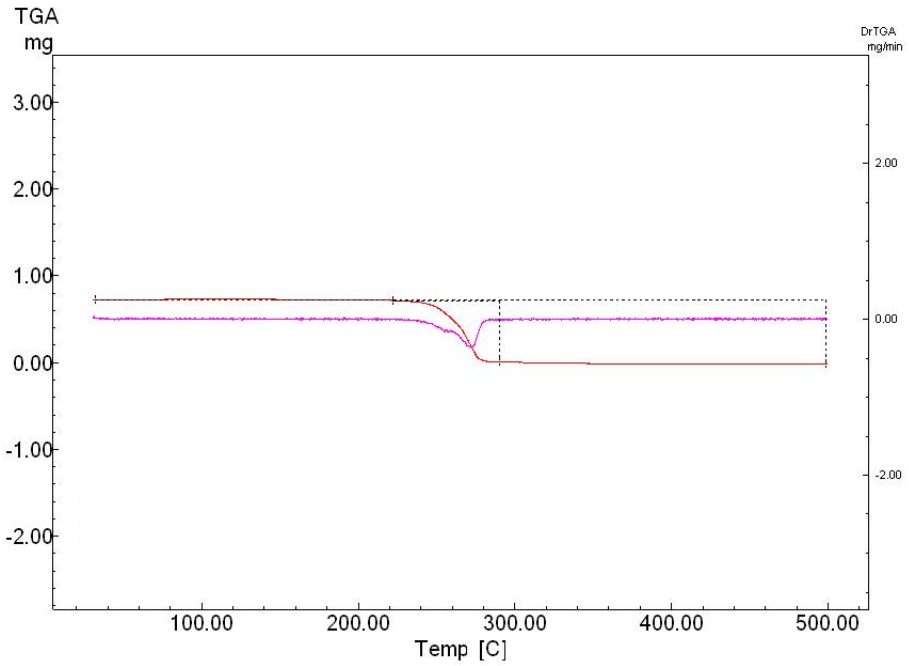


Fig 6.8 TGA curve of extracted biopolymer

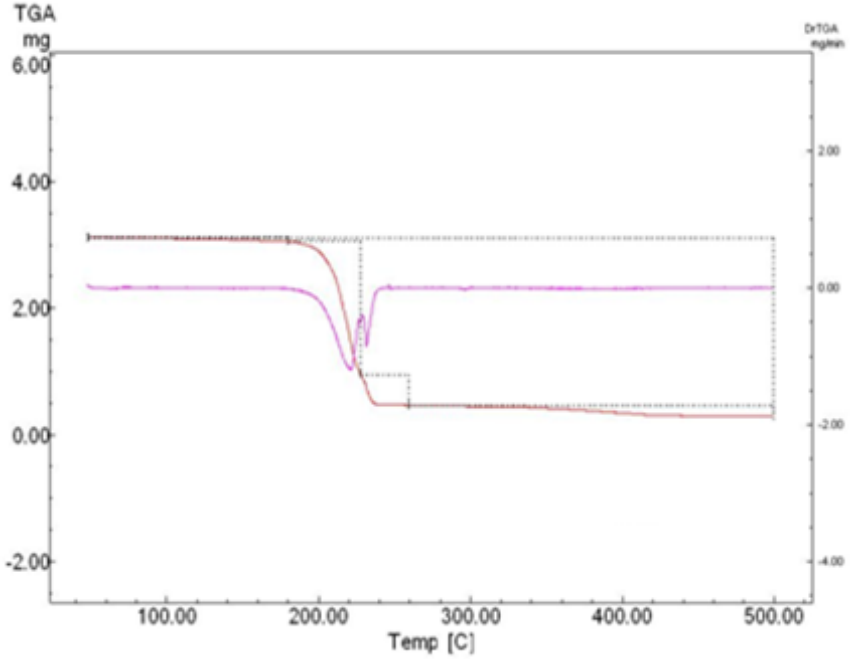


Fig 6.9 TGA curve of extracted biopolymer – Thermoplastic Starch (TS) blend

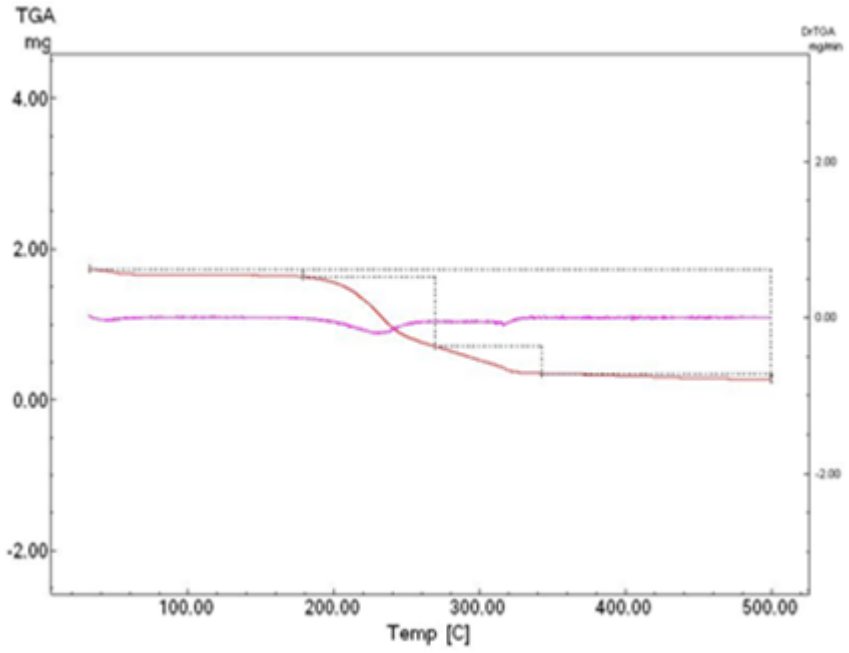


Fig 6.10 TGA curve of Extracted biopolymer-PEG blend

6.3.4.2 Differential Scanning Colorimetry (DSC)

DSC is a technique that measures the heat flow into or out of a material as a function of time or temperature. The DSC curves of extracted PHB and its blends showed two endothermal peaks in between 140 to 180. The peak at the higher temperature was attributed to the melting of the crystalline film. The DSC curves in the region above the glass transition temperature for all samples are presented in Fig 6.11 to 6.14. The DSC curves for the extracted PHB, shown in Fig 6.12, represented an exothermic peak between 70 and 90^0C and two endothermic peaks between 140 and 180^0C.

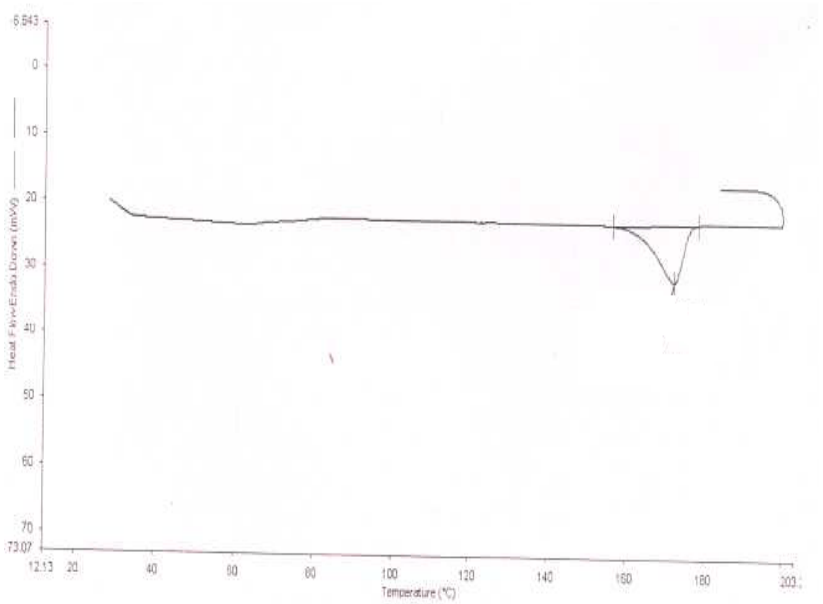


Fig 6.11 DSC of Commercial PHB

The dominant endothermic peak indicated the melting of the crystalline film while the small endothermic peak might be due to the melting of the imperfect crystals formed during the sample preparation. The PHB crystallization peak was less sharp with a maximum temperature at 172.26^0C. All the blends presented exothermic signals in the cooling step related to crystallization that occurs during this stage.

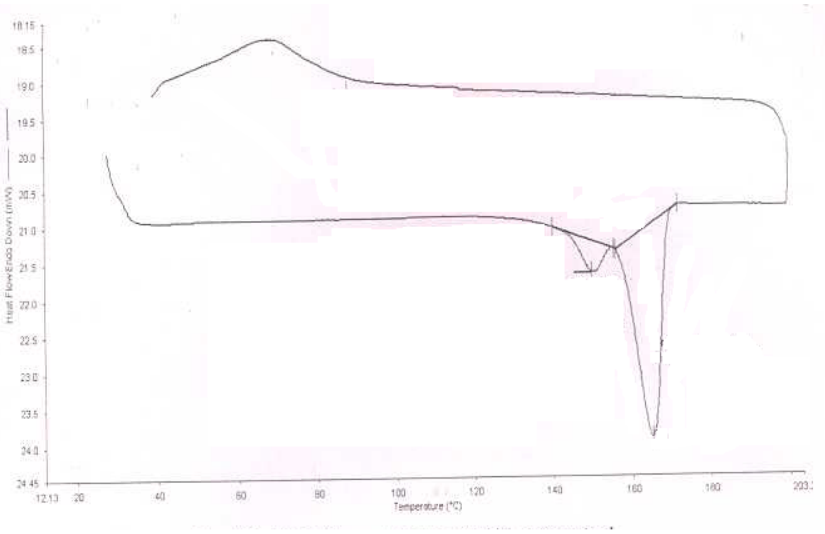


Fig 6.12 DSC of extracted PHB

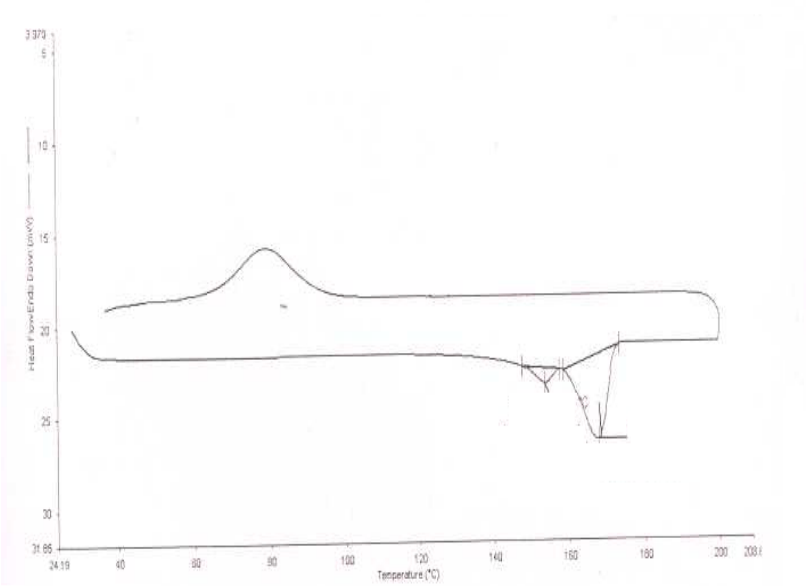


Fig 6.13 DSC of PHB-PEG blend

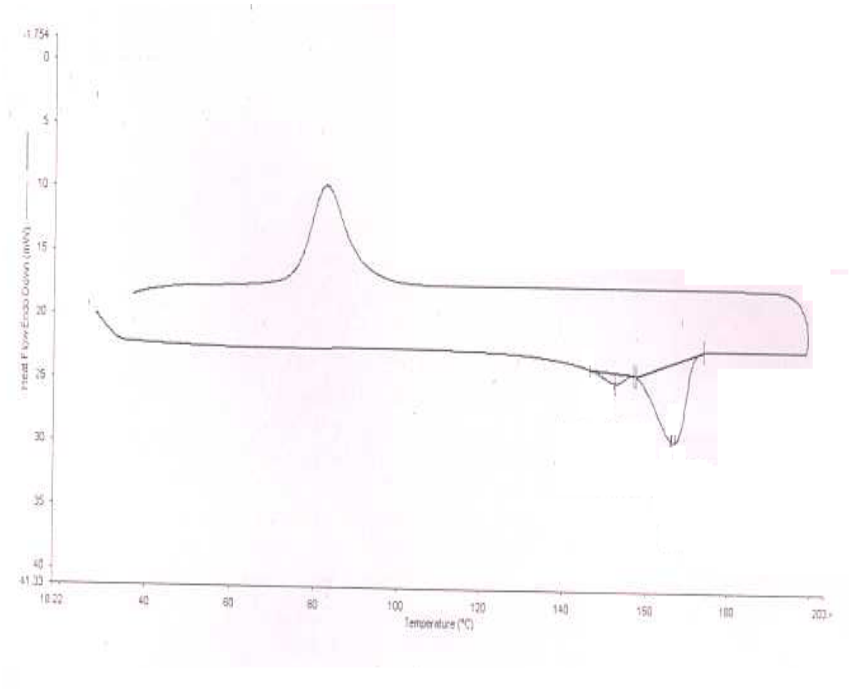


Fig 6.14 DSC of PHB-Thermoplastic starch blend

Table 6.5 Data obtained by DSC analysis

Sample	**Tm (^{0}C)**	**ΔHf (J/g)**
Extracted PHB	165.24	36.50
Commercial PHB	172.26	40.34
PHB-TS	167.31	35.11
PHB-PEG	167.55	28.09

The value obtained for the pure PHB film was low compared to the literature data. This was probably due to the favorable conditions of the casting process. From the Table 6.5 it was evident that extracted PHB exhibited a lower melting temperature (165.24^{0}C)

compared with other samples. The blending process causes only a slight increase in the melting temperature compared to the extracted PHB. The crystallization rate decreased in the presence of the plasticizer. Highly crystalline polymers are usually stiff and brittle resulting in very poor mechanical properties with low extension at break (Savenkova et al., 2000).

6.3.5 Biodegradation

Biodegradable polymers generally decompose in the various media in our environments. These environments contain soils, seawater, and activated sludge. If biodegradable materials waste is discarded, they decompose in these media. Environmental factors have a great role in the degradation process of polymer and also have an influence on the microbial population and on the activity of the different microorganisms themselves. Biodiversity and occurrence of polymer-degrading microorganisms vary depending on the environment, such as soil, sea, compost, activated sludge, etc. It is necessary to investigate the distribution and population of polymer-degrading microorganisms in various ecosystems. The plastic biodegradation is usually a heterogeneous process. Because of a lack of water-solubility and the size of the polymer molecules, microorganisms are unable to transport the polymeric material directly into the cells where most biochemical processes take place. The adherence of microorganisms on the surface of plastics followed by the colonization of the exposed surface is the major mechanisms involved in the microbial degradation of plastics followed by the excretion of extracellular enzymes which depolymerize the polymers outside the cells. The enzymatic degradation of plastics by hydrolysis is a two-step process: first, the enzyme

binds to the polymer substrate then subsequently catalyzes a hydrolytic cleavage. Polymers are degraded into low molecular weight oligomers, dimers and monomers and finally mineralized to CO_2 and H_2O. Count of the main microorganism groups in soil, post film incubation was studied. The clear zone method with agar plates is a widely used technique for screening the polymer degraders and for the assessment of degradation potential of different microorganisms towards a polymer.

Table 6.6 Biodegradation profile of the PHB (commercial and extracted) and blends in samples from waste treatment plants.

Polymer	Sample	Residual Wt of polymer	% wt loss
Commercial PHB	Leachate fresh (pH 4)	78.11	22.89
Commercial PHB	Leachate 1.5 y	88	12
Commercial PHB	Final compost	93.95	6.05
Commercial PHB	Landfill soil	87	13
Commercial PHB	Semi compost 1.5y	80	19
Extracted PHB	Leachate fresh (pH 4)	70.59	29.41
Extracted PHB	Leachate 1.5 y	93.94	6.06
Extracted PHB	Final compost	84.06	15.94

Extracted PHB	Landfill soil	66.04	33.96
Extracted PHB	Semi compost 1.5y	94.50	5.50
PHB-TS	Final compost	80.77	19.23
PHB-TS	Leachate fresh (pH 4)	77.27	22.73
PHB-TS	**Leachate 1.5 y**	**10**	**90.00**
PHB-TS	Landfill soil	14	86.00
PHB-TS	Semi compost 1.5y	28.57	71.43
PHB -PEG	Leachate fresh (pH 4)	74.68	25.32
PHB -PEG	Final compost	86.73	13.27
PHB -PEG	Leachate 1.5 y	92.54	7.46
PHB –PEG	Semi compost 1.5y	88.06	11.94
PHB -PEG	Landfill soil	95.24	4.76
Commercial PHB	Activated sludge (Milma)	80	20
Extracted PHB	“	73	27
PHB-TS	**“**	**0**	**100**

PHB-PEG	“	53	47

The PHB and its blends were exposed to activated sludge and showed the weight losses are reported in Table 6.6. All the samples showed evidence of PHB degraders. This was predictable, in view of the biodiversity of polymer-producing prokaryotes in these environments and the consequent availability of PHB as a nutrient source (Brandl et al., 1990). The biodegradation of blends were more difficult to interpret because of the different biodegradation rates of the component polymers and leaching of plasticizers and additives can impact the data (Mayer et al., 1996). Considerable weight losses were obtained for extracted polymer-TS films after exposure to the mixed bacterial population present in the wastewater treatment plants. These film showed greatest degradation of 90% and 100% in presence of leachate (1.5 y) from Vilappisala waste treatment plant and activated sludge from Milma waste treatment plant respectively. Starch is totally biodegradable in a wide variety of environments. It can be hydrolyzed into glucose by microorganism or enzymes, and then metabolized into carbon dioxide and water (Primarini and Ohta, 2000). The search for the PHB degraders revealed that both samples were rich in different kinds of microorganisms and was regarded as being capable of decomposing the biopolymers. These result suggested that the microorganisms in these samples secreted depolymerizing enzymes with less stringent requirements. In addition to that the colonization of the sample surface by bacteria might have created particularly favorable local conditions for biodegradation of blends. The biodegradation for the commercial PHB extracted PHB and extracted PHB-PEG blends were the least. The reduced biodegradation of PHB probably reflected the absence of a larger proportion of

tertiary carbons in this polymer, making it more resistant to biodegradation than PHB – TS. In PHB there were a small number of ramification involving CH_3 groups. The hydrophobicity of the PHB also contributed the resistance to degradation.

The screening of microbes present in samples from Vilappilsala waste treatment were showed the presence of a total of 11bacteria, 18 fungi and 12 actinomycetes in which five fungi and two actinomycetes gave positive results. The occurrence of clear zones on a turbid plate indicated the degradation of polymer particles to soluble molecules due to enzyme activity arising from the PHB degrader. Recently the contribution of fungi on the degradation of bioplastic has been evaluated (Gonda et al., 2000, Kim and Rhee, 2003). However, in contrast to bacterial polyester degradation, which has been extensively investigated, the microbiological and environmental aspects of fungal degradation of polyesters are unclear.

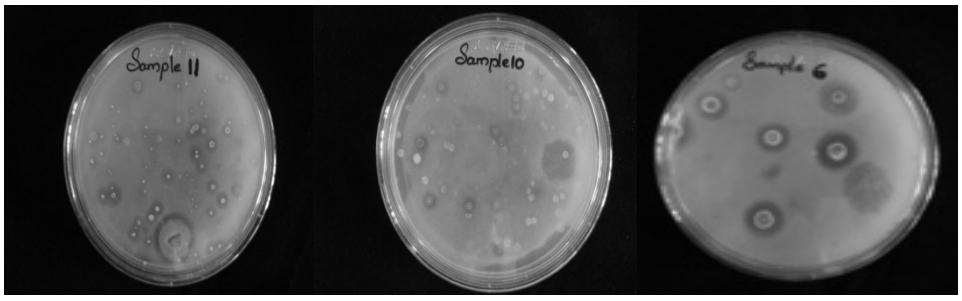


Fig 6.15 Isolation of PHB degraders from Vilappilsala waste treatment plant, Trivandrum

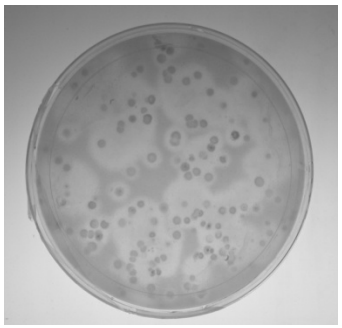


Fig 6.16 Isolation of PHB degraders from Milma waste treatment plant, Trivandrum

6.3.5.1 Screening of NIIST bacterial and fungal isolates for PHB degradation

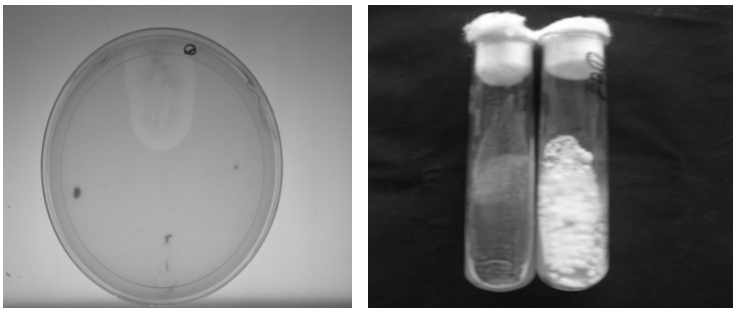
The biodegradation in presence of bacterial isolates are shown in Table 6.7. All the bacterial strains showed PHB degrading capability. Out of six bacterial strains studied *Paracoccus* sp. gave the maximum biodegradation for PHB- TS films and the degradation rate estimated was 95.10%. In which PHB degrading bacteria secreted specific PHB depolymerase which hydrolyze the polymer to water soluble monomers or oligomeric esters. The hydrolytic products were taken up by the cells and metabolized.

Table 6.7 Biodegradation by bacterial isolates, NIIST, Trivandrum

Polymer	Strain	Residual wt (mg)	% wt loss
PHB- TS	*Pontibacter* sp. (NII 905)	7.3	92.70
PHB- TS	*Citrobacter* sp. (NII 908)	10.6	89.40
PHB-TS	*Paracoccus* sp. (NII 917)	**4.9**	**95.10**

PHB-TS	*Peanibacillus* sp. (NII 918)	25.6	74.40
PHB-TS	*Alicycliphilus* sp. (NII 922)	11.9	88.10
PHB-TS	*Serratis* sp. (NII 935)	24.5	75.50
PHB-PEG	*Pontibacter*sp. (NII 905)	88.14	11.86
PHB-PEG	*Citrobacter* sp. (NII 908)	91.83	8.17
PHB-PEG	*Paracoccus* sp. (NII 917)	95.74	4.26
PHB-PEG	*Peanibacillus* sp. (NII 918)	90.95	9.05
PHB-PEG	*Alicycliphilus* sp.(NII 922)	89.80	10.20
PHB-PEG	*Serratis* sp. (NII 935)	91.5	8.50
Commercial PHB	*Pontibacter* sp. (NII 905)	98.5	1.5
Commercial PHB	*Citrobacter* sp. (NII 908)	96	4
Commercial PHB	*Paracoccus* sp. (NII 917)	90	10
Commercial PHB	*Peanibacillus* sp.(NII 918)	95 .7	4.3
Commercial PHB	*Alicycliphilus* sp. (NII 922)	97.2	2.8
Commercial PHB	*Serratis* sp. (NII 935)	98	2
Extracted PHB	*Pontibacter* sp. (NII 905)	91	9
Extracted PHB	*Citrobacter* sp. (NII 908)	89.5	10.5
Extracted PHB	*Paracoccus* sp. (NII 917)	87	13
Extracted PHB	*Peanibacillus* sp. (NII 918)	91.3	8.7
Extracted PHB	*Alicycliphilus* sp. (NII 922)	90	10
Extracted PHB	*Serratis* sp. (NII 935)	98	2

NIIST fungal isolates (9 known and 14 unknown) were screened for PHB degradation by inoculating the cultures in mineral agar plates containing PHB as the sole carbon source. Those organisms which showed growth along with clear zone formation were chosen as positive degraders. Among the 14 unknown fungal isolates one gave positive result (Fig 6.13a) Out of the 9 known fungal isolates, two isolates such as *Penicillium chrysogenum* and *Geotrichum candida*) (Fig 6.13 b and c) showed PHB degradation. Some fungal depolymerases are relatively nonspecific hydrolyase that are capable of giving rise to chain scission while acting upon various types of polymer substrates. For example the depolymerase enzyme from *Aspergillus fumigatus* effectively degrades bacterial and synthetic polyesters in the following order PEA>PES>PBA>PHV (Scherer et al., 1999).



(a) (b) (c)

Fig 6.17 Unknown fungal culture showing clear zone in PHB agar plate (a), *Geotrichum candida* (NII 08115) (b) and *Penicillium chrysogenum* (NII 08138) (c)

In general, the biodegradation of bioplastic proceeded actively under different environmental conditions according to their properties, because the microorganisms responsible for the degradation differ from each other and have their own optimal growth conditions were different in each environments. Polymers, especially bioplastics, were potential substrates for heterotrophic microorganisms (Glass and Swift, 1989). So it was

clear that the biodegradation rate was very fast in the case of subsurface burial for all films.

6.4 CONCLUSION

The treatment of the bacterial pellet with dispersion of sodium hypochlorite and chloroform showed more efficiency in NPCM solubilization and purity increase of the biopolymer. The NMR and FTIR characterization studies of the extracted biopolymer confirmed it as PHB. Attempts were made to improve the properties of PHB by blending with PEG and thermoplastic starch but the TGA and DSC results indicated that the blending did not improve the melting temperature but the crystallization properties. As an alternative to field tests, various simulation tests in the laboratory had used to measure the biodegradation of plastics. Biodegradation were tested in sludge samples and also in presence of isolated microbes. Very good results were obtained in the case of PHB-TS blend in presence of sludge sample from Milma, waste water treatment plant, Trivandrum.

CHAPTER 7

StudieS on mutation of *Bacillus sphaericus* NII 0838 by classIcal mutageNesIs

CHAPTER 7

Studies on mutation of *Bacillus sphaericus* NII 0838 by classical mutagenesis

7.1 INTRODUCTION

After the identification of the ability of an organism to produce a valuable product, it is essential to increase the product yield from fermentation to minimize the cost of the production. The main strategies in this regard are developing a suitable medium for the fermentation, refining the fermentation process and improving the productivity of the strain. The techniques and approaches used to genetically modify the strains to increase the production of the desired product are referred as strain improvement or strain development. In recent years, new approaches such as rational screening and genetic manipulations have begun to make a significant contribution to this activity. However, mutagenesis and selection the so called 'random screening' - is still an effective procedure, and for reliable short-term strain development is frequently the method of choice. Mutagenic procedures can be optimized in terms of type of mutagen and dose and mutagen specificity effects can be taken into account and mutagenesis itself can be enhanced or directed in order to obtain the maximum frequency of desirable mutant types among the isolates to be screened. Screens can be designed to allow the maximum expression of the desirable mutant types. The strain improvement requires painstaking lengthy and tedious procedures to identify superior isolates among a mutagen-treated population. Normally, this problem can be solved by recombination technologies. The mechanisms for protection against low pH and high solute

concentration are controlled by large number of loci and multiple genes which are the major difficulties in insertional mutagenesis. Without an understanding of the actual mechanism of pH tolerance in bacteria, a rational approach to engineering pH tolerance is impractical. Environmental tolerance, a particularly complex phenotype, is not an apparent target for sensible engineering. In such a case the only method of strain improvement is classical mutagenesis (Patnaik et al., 2002). The first report of mutagenic action of a chemical was in 1942 by Charlotte Auerbach, who showed that nitrogen mustard (component of poisonous mustard gas used in World Wars I and II) caused mutations in the cells. Since that time, many other mutagenic chemicals have been identified.

Point mutations are thought to be induced in many cases by modification of a base in the DNA by a mutagen and subsequent mis-incorporation of an 'incorrect' deoxynucleoside triphosphate at the site opposite to the modified base in a reaction catalyzed by DNA polymerase (Sidorkina et al. 1997). Nitrous acid, a potent chemical mutagen, exerts its effect in the DNA by deamination of adenine, cytosine and guanine residues present in them (Kotaka and Baldwin 1964; Sidorkina et al., 1997). It acts on both replicating and non-replicating DNA. It leads to the oxidative deamination of cytosine (removal of 6-NH_2), adenine (removal of 6-NH_2) and guanine (removal of 2-NH_2) in which amino groups are replaced by keto groups to yield uracil, hypoxanthine and xanthine, respectively. Uracil pairs with adenine resulting in G–C to A–T transition and hypoxanthine pairs with adenine resulting in A–T to G–C transition. Since, xanthine pairs with cytosine (similar to guanine); deamination of guanine does not yield mutation. Ethyl methanesulfonate (EMS) is another well known mutagen, which produces random

mutations in genetic material by nucleotide substitution, particularly by guanine alkylation. The ethyl group of EMS reacts with guanine in DNA, forming the abnormal base O-6-ethylguanine. During DNA replication, DNA polymerase that catalyzes the process, frequently place thymine, instead of cytosine, opposite to O-6-ethylguanine. Following subsequent rounds of replication, the original G:C base pair become an A:T pair. The exposure to UV radiation leads to the major lethal lesions such as the formation of pyrimidine dimers in DNA. These are the result of a covalent attachment between the adjacent pyrimidines in one strand. These dimers are able to block transcription and DNA replication and are lethal if unrepaired. They can stimulate mutation and chromosome rearrangement as well. UV has been suggested as a mutagen of first choice since it can induce both base pair substitution and frame shift mutation. Moreover lethality is high and it is relatively safe mutagen for the experimenter (Bridges, 1976).

The present study was aimed in improving the PHB synthesis ability of *Bacillus sphaericus* through classical mutagenesis.

7.2 MATERIALS AND METHODS

Microorganism, its maintenance and inoculum preparation were carried out as described in chapter 2.

7.2.1 Materials

The chemical mutagens used were ethyl methanesulfonate (Hi-media) and nitrous acid (freshly prepared).

7.2.2 UV Mutation

The *Bacillus sphaericus* culture was prepared by transferring a loopful of culture to the LB broth and keeping it for 24 h at 30^0C under shaking conditions (200 rpm). From this, 1 ml ($45x10^7$ CFU/ml) inoculum was serially diluted and spread on LB agar plates. The plates were exposed to UV radiation for different periods of time (5 sec to 60 min) at the distance of 10 cm from UV irradiation (200–300 nm, $1.7Wm^{-2}$). UV exposure was followed by five days incubation at 30^0C under static condition in dark to avoid the photo reactivation. The colonies were sub-cultured in LB till a consistent growth appeared at 30^0C at 200 rpm. From this, 2 ml ($8x10^8$ CFU/ml) inoculum was transferred to the PHB production medium for the screening of mutation. Submerged fermentation was done as described in chapter 3.

7.2.3 Chemical mutagenesis

Two chemicals used as mutagens were ethyl methanesulfonate (EMS) and freshly prepared nitrous acid (HNO_2). Fresh HNO_2 was generated by mixing 0.1N chilled HCl and 0.1N $NaNO_2$ (2.5 ml each). For mutation studies, 5.0 ml of EMS (0.05, 0.1and 0.25%) and 0.1N HNO_2 were transferred separately to individual sterilized centrifuged tubes containing 5.0 ml ($45x10^7$ CFU/ml) of bacterial suspension and placed at 30^0C for 10-60 min. After this, the tubes were centrifuged at 8,000x*g* for 15 min. The supernatant was discarded to remove the EMS. The pellet was washed three times with saline water to remove the traces of EMS from the bacterial cells. The pellet was suspended in 10 ml sterilized phosphate buffer (pH 7.0). One milliliter of the suspension was serially diluted

in sterile phosphate buffer and 0.1 ml was spread on the Petri plates containing LB agar. The plates were sealed with paraffin film and the cultivable counts were determined after incubation at 30^0C under static condition for five days. The Petri plates with minimum number of colonies were selected for further studies. These colonies were sub- cultured in LB till a consistent growth appeared at 30^0C at 200 rpm. From this, 2 ml ($8x10^8$ CFU/ml) inoculum was transferred to the PHB production medium and SmF was carried out as described in chapter 3.

7.3 Assay of PHB

Assay of PHB was performed as described in chapter 2.

7.4 RESULTS AND DISCUSSION

Genetic improvements have proved to be the promising approach for increased production of desired product by industrially important microorganisms. The present investigation on effect of mutation on *Bacillus sphaericus* was carried out by UV irradiation treatment and by treating the culture with EMS and nitrous acid.

7.4.1 UV Irradiation

In this study, attempts were made to generate the mutants by subjecting the *Bacillus sphaericus* to UV irradiation at different periods of time. It was evident from the Fig 7.1 that the morphology of the mutants was different from the wild type and the description of the colony morphology is shown in Table 7.1. The survival rate decreased with increasing in time of exposure to UV radiation and results are shown in Table 7.2.

The UV treated samples showing 99% kill was selected best suited for the strain improvement because the fewer survivors in the sample would have undergone repeated or multiple mutations which may lead to the enhancement in the productivity of the culture. The 1% survival rate was obtained with 15 min UV exposure at 10 cm distance which resulted in the survival of five colonies (Table 7.2). These colonies were analyzed quantitatively under SmF in shake flasks for their potential for biomass and PHB production. Even though the time course study did not reveal significant differences in the growth pattern of the mutant derivatives, when compared with the wild type, one mutant (UV4) showed a slight increase in the biomass and PHB production (Table 7.3). The UV4 mutant produced 5.03±0.04g/l biomass and 2.45±0.03g/l PHB, while the biomass and PHB production of wild type was 4.5±0.02g/l and 2.21±0.01g/l respectively (Table 7.3). This showed that UV irradiation was not efficient in the improvement of *Bacillus sphaericus* for increased PHB production.

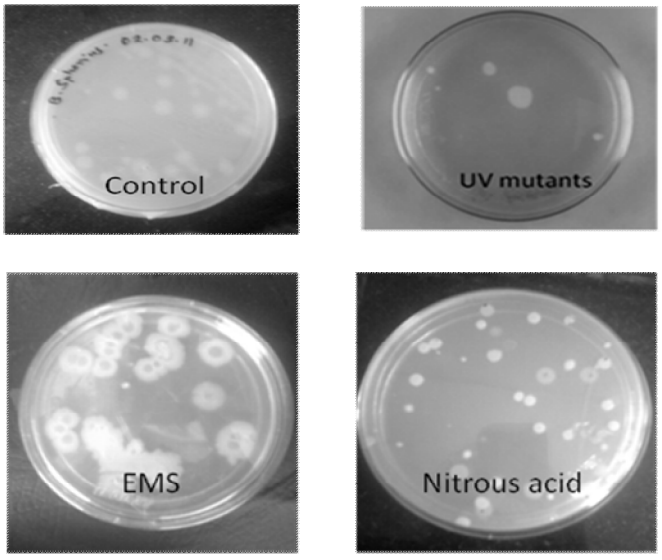


Fig 7.1 Morphology of mutants

The UV4 mutant was sub -cultured and checked the biomass and PHB production in two successive generations to confirm the stability for PHB production and results are shown in Table 7.8.The mutant (UV4) did not show stability in two successive generations.

Table 7.1 Effect of classical mutagenesis on the phenotype of *Bacillus sphaericus*

Strain	Phenotype
Wild type	Circular, smooth, pale yellow, entire margin and medium size
UV mutant	Irregular, rough, white and medium size
EMS mutant	Irregular, spreading, rough, white and large size
Nitrous acid mutant	Circular, smooth, pale yellow, and medium size

Table 7.2 Effect of UV irradiation on *Bacillus sphaericus*

Time for exposure to UV irradiation	No. of survivors
5 sec	46
1 min	40
5 min	31
10 min	5

15 min	-
30 min	-
45 min	-
60 min	-

Table 7.3 Comparison of biomass and PHB production of UV mutants with wild type

Strains	24 h		48 h		72 h	
	Biomass (g/l)	**PHB (g/l)**	**Biomass (g/l)**	**PHB (g/l)**	**Biomass (g/l)**	**PHB (g/l)**
Wild type	3.08±0.02	1.5±0.005	**4.5±0.02**	**2.21±0.01**	4.5±0.02	2.0±0.02
UV1	3.14±0.02	1.0±0.05	4.08±0.06	1.8±0.02	4.09±0.03	1.5±0.02
UV2	3.09±0.01	0.55±0.02	3.33±0.02	0.9±0.01	3.0±0.0.02	0.4±0.01
UV3	3.02±0.05	1.33±0.03	4.02±0.04	1.5±0.01	4.02±0.02	1.5±0.01
UV4	**3.66±0.03**	**1.5±0.05**	**5.03±0.04**	**2.45±0.03**	**5.03±0.0.04**	**2.43±0.03**
UV5	3.0±0.04	1.9±0.01	3.59±0.02	2.2±0.005	3.6±0.01	2.01±0.05

7.4.2 Chemical mutagenesis

7.4.2.1 Ethyl methanesulfonate (EMS)

The results obtained after the treatment of *Bacillus sphaericus* with different doses of EMS for different exposure time are shown in Table 7.4. The results showed that exposure of the bacterial strain to EMS also produced a dose-dependent killing of it, which increased gradually with the increase of EMS concentration. The variants were characterized with respect to changes in colony morphology, such as size and color and changes were observed from the parental strain (Table 7.1 and Fig 7.1). The mutants

obtained were rough colonies with irregular margin and larger in size compared with the wild type colonies. Cells treated with 0.25% EMS for 30 min resulted in the survival of four colonies (Table 7.4) and these were selected for the screening of biomass and PHB production. The screening studies resulted in two mutants, namely EMS1and EMS2 and EMS1 was a better biomass producer compared with the wild type strain (Table 7.5). The mutant EMS1 showed an increased growth and at 48 h, the biomass accumulation was 6.7±0.05 g/l. But the strain failed to synthesize PHB (2.12±0.02 g/l) according to this increased biomass production. The biomass and PHB production by the second mutant, EMS2, was 4.74±0.04 g/l and 2.77±0.03g/l respectively (Table 7.5). To confirm the stability, the mutants (EMS1 and EMS2) were sub -cultured and analyzed the biomass and PHB production in two successive generations and the results indicated that these mutants were unable to continue the stability in two successive generations and the results are shown in Table 7.8.

Table 7.4 Effect of Ethyl methanesulfonate treatment on *Bacillus sphaericus*

EMS Concentration (%)	**Time for exposure to EMS (min)**				
	10	**15**	**30**	**45**	**60**
0.05	40	36	20	12	-
0.1	35	30	11	3	-
0.25	20	12	4	-	-

Table 7.5 Comparison of biomass and PHB production of EMS mutants with wild type

Strains	24 h		48 h		72 h	
	Biomass (g/l)	**PHB (g/l)**	**Biomass (g/l)**	**PHB (g/l)**	**Biomass (g/l)**	**PHB (g/l)**
Wild type	3.08±0.02	1.5±0.005	4.5±0.02	2.21±0.01	4.5±0.02	2.0±0.02
EMS1	**4.14±0.02**	**1.0±0.03**	**6.7±0.05**	**2.12±0.02**	**6.71±0.01**	**2.0±0.02**
EMS2	**3.52±0.03**	**1.85±0.05**	**4.74±0.04**	**2.77±0.03**	**4.72±0.01**	**2.48±0.04**
EMS3	2.01±0.02	0.55±0.04	1.3±0.02	0.9±0.01	1.3±0.02	0.9±0.01
EMS4	3.22±0.04	1.33±0.02	3.88.±0.01	1.5±0.03	3.80.±0.02	1.5±0.06

7.4.2.2 Nitrous acid

Freshly prepared nitrous acid was also used as chemical mutagen. In contrast to UV and EMS treatments, nitrous acid proved to be an effective mutagen, as it enhanced the biomass and PHB production to a greater extent. The treatment of *Bacillus sphaericus* using 0.1N nitrous acid for 45 min resulted in the survival of seven mutants (Table 7.6). The analysis of biomass and PHB production under SmF confirmed that out of these seven mutants, two mutants (NA2 and NA3) were able to synthesize increased biomass and PHB. After 48 h of incubation, the NA2 produced a biomass of 9.92±0.01 g/l and 4.9±0.01 g/l PHB, while the second mutant, NA3 accumulated 6.09±0.02g/l biomass and 2.5±0.04g/l PHB (Table 7.7). The hyper producing mutants, NA2 and NA3 were sub - cultured and checked the biomass and PHB production in two successive generations to confirm the stability of mutants for biomass and PHB production. These two mutants

exhibited stability in biomass and PHB production in two successive generations compared with other mutants (Table 7.8).

Table 7.6 Effect of 0.1N nitrous acid treatment on *Bacillus sphaericus*

Time for exposure to 0.1N nitrous acid (min)	No. of survivors
10	30
15	23
30	16
45	7
60	-

Table 7.7 Comparison of biomass and PHB production of nitrous acid mutants with wild type

Strains	24 h		48 h		72 h	
	Biomass g/l)	**PHB (g/l)**	**Biomass (g/l)**	**PHB (g/l)**	**Biomass (g/l)**	**PHB (g/l)**
Wild type	3.08±0.02	1.5±0.005	4.5±0.02	2.21±0.01	4.5±0.02	2.0±0.02
NA1	3.17±0.05	1.0±0.05	6.08±0.02	1.8±0.06	6.09±0.01	1.5±0.04
NA2	**4.81±0.02**	**2.55±0.02**	**9.92±0.01**	**4.9±0.01**	**9.60±0.05**	**4.1±0.05**
NA3	**4.88±05**	**2.32±0.05**	**6.09±0.02**	**2.5±0.04**	**6.02±0.02**	**2.4±0.02**
NA4	3.8±0.008	1.5±0.06	4.3±0.005	2.05±0.05	5.03±0.0.04	2.03±0.03
NA5	2.8±0.03	0.9±0.01	3.22±0.02	1.92±0.05	3.6±0.01	1.01±0.05

NA6	3.5±0.04	0.2±0.005	4.02±0.003	0.8±0.01	3.9±0.02	0.78±0.001
NA7	3.72±0.006	1.5±0.04	4.33±0.02	2.1±0.07	4.3±0.001	1.98±0.007

Table 7.8 Biomass and PHB production at 48 h by the mutants in two successive generations

Strains	Biomass (g/l)		PHB (g/l)	
	2nd Generation	3rd Generation	2nd Generation	3rd Generation
Wild type	4.5±0.02	4.48±0.04	2.19±0.01	2.21±0.01
UV4	5.03±0.04	4±0.005	2.15±0.007	2.45±0.03
EMS1	6.7±0.05	5.8±0.05	1.98±0.04	2.12±0.02
EMS2	4.74±0.04	4.51±0.06	2.0±0.03	2.77±0.03
NA2	9.92±0.01	9.97±0.02	4.78±0.01	4.9±0.01
NA3	6.09±0.02	6.02±0.05	2.42±0.01	2.5±0.04

7.5 CONCLUSION

From the results, it was concluded that UV irradiation was not suitable for generating the mutant for enhanced PHB production. Chemical mutagenesis using nitrous acid was effective in producing the mutants which showed increased biomass and PHB production than the wild type strain.

CHAPTER 8

StudieS on application of purified biopolymer from *Bacillus sphaericus* on drug delivery system

CHAPTER 8

Studies on application of purified biopolymer from *Bacillus sphaericus* on drug delivery system

8.1 INTRODUCTION

The non toxicity, biodegradability, biocompatibility make the PHAs suitable for several applications such as packaging, disposal items, cosmetic containers, making of osteosynthetic material etc. Also the low *in vivo* toxicity made the biopolymers useful in the usage of long-circulating drug carriers for the protection of the encapsulated drug, controlled release and drug targeting of pharmacologically active substances. Also the flexibility of polymers aid to engineer multiple functionalities required for efficient drug delivery, simultaneously maintaining biocompatibility, facile manufacturing and stable formulation (Langer and Tirrell, 2004; Langer, 1998; Dan and Saltzman, 2000; Lavigne and Górecki, 2006; Eliyahu et al., 2005). The higher molecular weight and their instability in the gastrointestinal environment make most of the drugs limiting in their oral administration. Other options like frequent injections are also painful and expensive. The treatment of many diseases using nanoparticles or colloidal carriers had won the excellent possibility of drug targeting to specific organs or tissues. Although the research concerning formulation of nanoparticles into drug delivery devices has been extensive, only a few polymeric nanoparticulate products have reached the market. One known product, Abraxane™, consist of intravenously administered 130-nm nanoparticles prepared from the protein albumin bound with paclitaxel, a drug used in cancer therapy (Rios, 2005). Designing of a drug delivery system is based on the fact that the drug concentration can be maintained at a target level for an extended time in a patient's body

(Kost and Langer, 2001). The drugs are more stable with nanoparticles and possess useful controlled release properties and subsequent clearance of the drug from the body so as to achieve increase in drug therapeutic efficacy and reduction in side effects. Particle size and surface characteristics of nanoparticles can be easily manipulated to achieve both passive and active drug targeting after parenteral administration. The drug loading efficiency of nanoparticle is relatively high and drugs can be incorporated into the systems without any chemical reaction; this is an important factor for preserving the drug activity. Site-specific targeting can be achieved by attaching targeting ligands to surface of particles or use of magnetic guidance. In spite of these advantages, nanoparticles do have limitations. Their small size and large surface area can lead to particle-particle aggregation, making physical handling of nanoparticles difficult in liquid and dry forms. In addition, small particles size and large surface area readily result in limited drug loading and burst release. These practical problems have to be overcome before nanoparticles can be used clinically or made commercially available.

The size of the nanoparticles is one of the major physical properties because it has a significant effect in its *in vivo* absorbance and mode of action (Yoshioka et al., 1981). The definition for nanoparticle is suitable for the particulate dispersions or solid particles with a size in the range of 10-1000 nm. Depending upon the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained.

Chloramphenicol is an effective bactericidal antimicrobial agent against a wide variety of Gram- positive and Gram- negative bacteria including anaerobic organisms because it acts by binding to the 50S subunit of the ribosome and thus inhibiting the protein synthesis (Mandal et al.,2004). It is a small molecule with good lipid solubility

and shows the ability to penetrate into all tissues of the body, including the brain. It is used in the treatment of bacterial meningitis, typhoid and brain abscesses. WHO recommends chloramphenicol as the first line treatment for meningitis in low income countries (Wali et al., 1979).

The prime aim of this study was to prepare stable PHB nanoparticles with a suitable size for the effective delivery of chloramphenicol. Three methods were attempted to get the nanoparticles with desired properties.

8.2 MATERIALS AND METHODS

8.2.1 Microorganism and inoculum preparation

Microorganism and its maintenance and inoculum preparation has been described in chapter 2.

8.2.2 Extraction of the biopolymer

Extraction of the biopolymer was carried out using dispersion of sodium hypochlorite and chloroform solution as described in chapter 6.

8.2.3 Preparation of Nanoparticles

8.2.3.1 Nanoprecipitation technique

The PHB nanoparticle containing chloramphenicol was prepared by nanoprecipitation method. PHB (90 mg) was dissolved in DMSO, to which 40 mg of chloramphenicol was added and dissolved. The solution was then sonicated for 2 min. The resultant solution was added by means of a syringe by inserting the needle directly

into the aqueous dispersing phase containing 2% polysorbate 80, which was magnetically stirred. The nanoparticles were separated by centrifuging at 5,000xg for 30 min and the washing steps were performed using distilled water to remove the unbounded drug. A 2% (w/w) sucrose solution was added to the PHB nanoparticles as a cryo-protective agent and lyophilized. Hence the stability of polymer nanoparticles in liquid is a controversy; freeze drying has been selected as a most suitable method for storage and the protective ability of carbohydrates to improve the freeze-drying process of the nanoparticles was evaluated (Abdelwahed et al., 2006).

8.2.3.2 Emulsification- solvent evaporation technique

In this study, the biopolymer was dissolved in 10 ml chloroform and subjected for ultrasonication for 15 min (59 on/off cycle). It was then added to a dispersing phase containing 0.01% polysorbate 80 in water (40 ml) which again sonicated for 15 min (59 on/off cycle). From the emulsion, the solvent was removed by using rotavapour.

8.2.3.3 Double emulsion-solvent evaporation technique.

The drug and the polymer were dissolved separately in dichloromethane in different ratios (1:2, 1:2.5 and 1: 3). It is then mixed well and 5 ml aqueous phase containing 3% PVA was added as an emulsifier and subjected for ultrasonication for 10 min with 59 seconds on/off cycles. The emulsion was added to 30 ml of PVA solution and sonicated for further 5 min. The emulsion was stirred continuously using a magnetic stirrer for 8 h at room temperature, until complete evaporation of dichloromethane. The suspension was centrifuged and the resultant pellet was resuspended in phosphate

buffered saline (PBS) to form a nanoparticle suspension. The nanoparticles were collected by centrifugation at 5,000xg for 30 min, followed by washing using phosphate buffer to remove the unloaded drug. Sucrose was used as a cryo- protective agent for storage**.**

8.2.4 Characterization of the Nanoparticles

8.2.4.1 Particle size analysis

The characterization of the nanoparticle was determined through size analysis and zeta potential measurements by dynamic light scattering which is the fastest and most routine method and it determines the diameter of the particle by Brownian motion and light scattering properties. The significance of zeta potential is that its value can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. Size of nanoparticles affects the release rate of the drug. Increase in size, decreases the effective surface area which ultimately decreases the release rate.

8.2.4.2 Morphology of nanoparticles

The nanoparticles were uniformly dispersed in distilled water and a diluted drop suspension was placed on a piece of cover glass and air dried at room temperature. The nanoparticles, spread on the cover glass were coated with a thin layer of gold and examined using a scanning electron microscopy (JEOL JSM 5600LV, 115 Japan). Scanning electron microscopy was used to determine the size, surface morphology and shape of the nanoparticles obtained using various techniques.

8.2.4.3 Encapsulation efficiency

A known amount of dried drug-loaded PHB nanoparticles was added to 2 ml dichloromethane to dissolve the biopolymer. Phosphate buffer pH 7.4 (2 ml) was added into the resultant solution and agitated for 30 min to extract the drug. The sample was filtered and amount of encapsulated chloramphenicol was assayed by a spectrophotometer at 278 nm. The encapsulation efficiency was calculated as follows:

Encapsulation efficiency (%) = (actual drug loading /theoretical drug loading) x 100

8.2.4.4 *In vitro* drug release profile

An exact quantity of nanoparticles was added to 50 ml of phosphate buffer pH 7.4. The assays were performed using light protected flasks at 37^0C and 70 rpm. Samples (2 ml) were removed at predetermined time intervals over the period of 20 days. All samples were filtered and analyzed by a spectrophotometer at 278 nm. The analyses were carried out using three different batches.

8.2.4.5 *In vitro* cytotoxicity of nanoparticles

After sterilization, nanoparticles were tested for the cytotoxicity towards the viability of HePG2 cells. HePG2 cells were seeded ($1x10^6$ cells / well) into a 96 well plate and pre-incubated for 24 h at 37^0C in 5% CO_2 incubator. The samples with different concentrations were added to wells, incubated at 37^0C for 48 h in 5% CO_2. After incubation, the cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyl tetrazolium bromide (MTT) assay. The number of living cells in the culture was determined from the MTT absorbance standard curve.

8. 3 RESULTS AND DISCUSSION

8.3.1 Nanoprecipitation technique

Fessi and co-workers introduced the nanoprecipitation technique, in which the particle formation is based on precipitation and subsequent solidification of the polymer at the interface of a solvent and a non-solvent (Fessi et al., 1989). The polymer which is soluble in a water miscible organic solvent (or solvent mixture) was added to an aqueous solution, in which the organic solvent diffuses (Fig 8.1). Although the nanoprecipitation process was a simple procedure, this method led to the formation of a heterogeneous population of nanoparticles with size ranged from ~ 83-356 nm, probably due to the surfactant adsorption on the particle surface. The drop wise addition also created problem by not spreading the organic solvent better into the outer phase. The DMSO is believed to be a harmless chemical with less acute and chronic toxicity for animal, plant and aquatic life. But the biopolymer did not completely dissolve in it. The spontaneous particle formation in the nanoprecipitation process involved complex interfacial as outer phase/solvent, outer phase/polymer and solvent/polymer interactions (Quintanar-Guerrero et al., 1998; Galindo-Rodriguez et al., 2004; Barichello, 1999). The organic (polymer) phase viscosity, stabilizer, drug, solvent properties and polymer properties such as, molecular weight and ability to precipitate, among others, gave their contribution to the final particle properties.

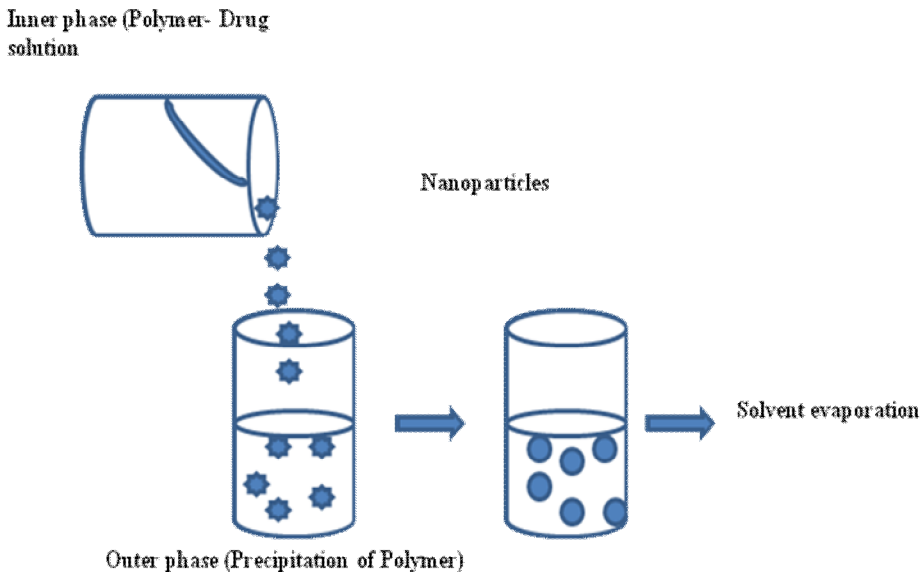


Fig 8.1 Schematic representation of the nanoprecipitation technique

8.3.2 Emulsion solvent evaporation

In this emulsion solvent evaporation method, the process efficiency was very low. PHB formed surface film during solvent removal instead of forming nanoparticles.

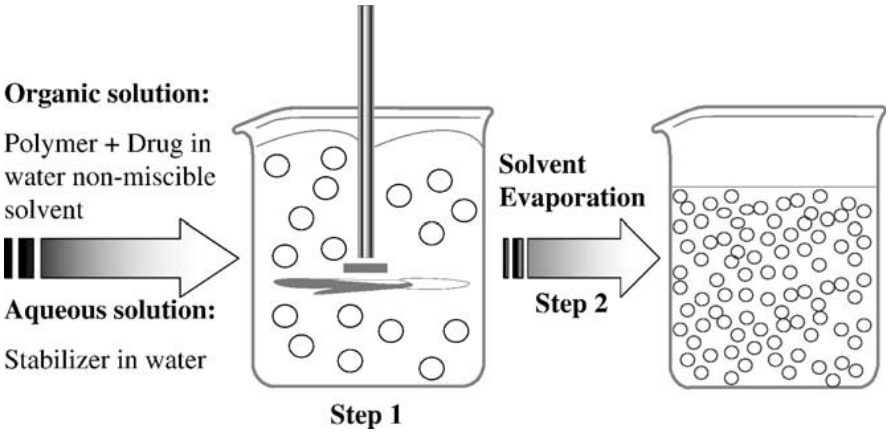


Fig 8.2 Schematic representation of the emulsification-evaporation technique

Emulsification-solvent evaporation involves two steps. The first step requires emulsification of the polymer solution into an aqueous phase (Fig 8.2). During the second step polymer solvent is evaporated, inducing polymer precipitation as nanoparticle.

8.3.3 Double emulsion solvent-evaporation

Drug-PHB (1:2 ratio) nanoparticles formulations with ~ 166.1 nm size were successfully fabricated by a double emulsion solvent evaporation technique using extracted PHB as the polymer (Fig 8.3). Particle size and size distribution are the most critical features of nanoparticle systems and it was determined by DLS. The DLS data suggested that particle size increased with increasing the polymer concentration. This might be due to the increase in viscosity of the solution and the decrease in stirring efficiency with increased polymer concentration as reported by Choi et al., (2007) (Table 8.1). The formation of nanoparticles was preferred over microparticles for drug delivery, since nanoparticles have relatively higher intracellular uptake compared to microparticles and available to a wider range of biological targets due to their small size and relative mobility. Desai et al (1997) found that 100 nm nanoparticles had a 2.5 fold greater uptake than 1 μm microparticles, and six fold greater uptake than 10 μm microparticles in a Caco-2 cell line. They determine the *in vivo* distribution, biological fate, toxicity and the targeting ability of nanoparticle systems. In addition, they can also influence the drug loading, drug release and stability of nanoparticles. The transition from the microparticles to nanoparticles can lead to the changes in physical properties such as, increase in the area of surface area to volume and the size of the particle moving into the realm where quantum effects predominate. The hydrophobicity of the drug and biopolymer facilitated the nanoparticle formation.

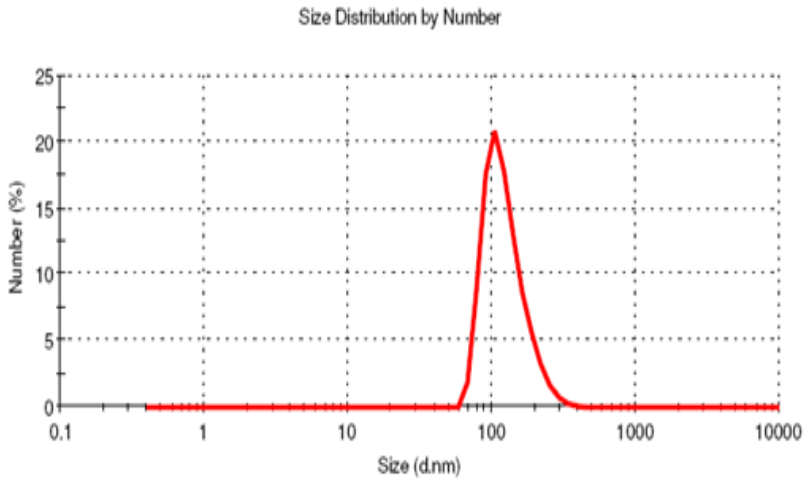


Fig 8.3 Size analysis of nanoparticle by DLS

8.3.4 Zeta potential

Surface charge of nanoparticles determines the performance of the nanoparticle system in the body and zeta potential measurements provides information about the particle surface charge. The results of zeta-potential measurements (Fig 8.4) revealed that the drug-loaded PHB nanoparticle containing PVA as emulsifier exhibited negative zeta potential of -21.9 mV. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate.

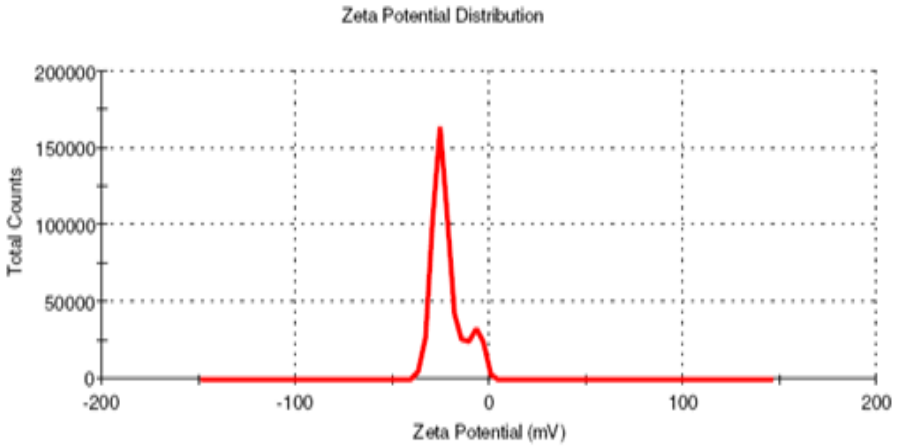


Fig 8.4 Zeta potential analysis of nanoparticle by DLS

8.3.5 SEM

The result obtained by dynamic light scattering was verified by scanning electron microscopy. SEM indicated that the prepared particles were in nano size and were homogeneous suspension of spherical nanoparticles with smooth surface (Fig 8.5). The solvent elimination from the emulsion droplets had an influence on the particle morphology, encapsulation and release behavior. The spherical nanoparticles were formed by accelerated solvent elimination due to the combined effects of continuous stirring, high solvent volatility and polymer precipitation.

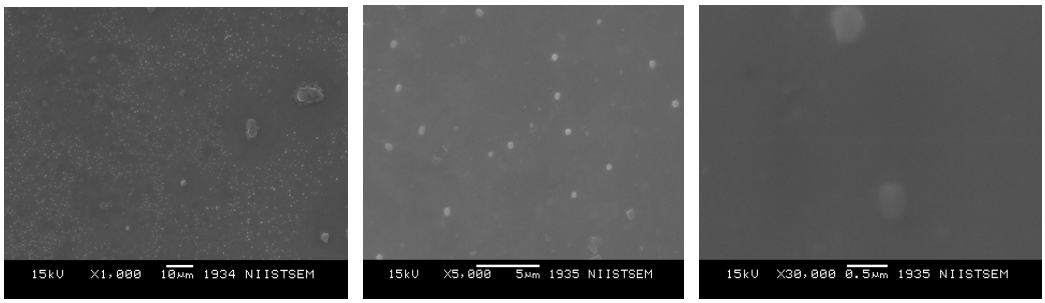


Fig 8.5 Scanning electron microphotograph of nanoparticle

Table 8.1 Encapsulation efficiency of nanoparticles prepared by Double emulsion solvent-evaporation method.

Drug-polymer ratio	Surfactant	Particle size (nm)	Encapsulation efficiency (%)
1:2.5	PVA	186	65.12±1.54
1:2	PVA	166.1	73.05±2.16
1:3	PVA	715.15	53.08±2.65

8.3.6 Encapsulation efficiency

The drug entrapment efficiency in the prepared nanoparticles ranged from 53 to 73% of the theoretical content. The drug-PHB in the ratio of 1:2 exhibited the highest entrapment of 73.05±2.16, designated the formation of the most stable emulsion (Table 8.1). However, during the intense solvent elimination, the encapsulated substance was drained and it affected the loading efficiency. Furthermore, it will concentrate towards the nanoparticle surface contributing to the initial burst release.

8.3.7 *In vitro* drug release

In vitro drug release data, shown in Fig 8.6, indicated that all the three combinations sustained drug release for over a period of 20 days and also revealed that all the formulations exhibited burst release on the first day. During the shrinkage due to the incompressibility of the inner nanodroplets, the precipitating polymer wall around them may break forming holes through which the encapsulated substance was partly expulsed. Through these holes, the encapsulated substance was further partitioning with the external aqueous phase during solvent evaporation and contributed to the initial burst

release during the application. Drug release from 1:2 ratios was higher than other two combinations (1:3 and 1:2.5). The stronger binding of the drug to the biopolymer might have contributed for the reduced drug release from these 1:3 and 1:2.5 formulations.

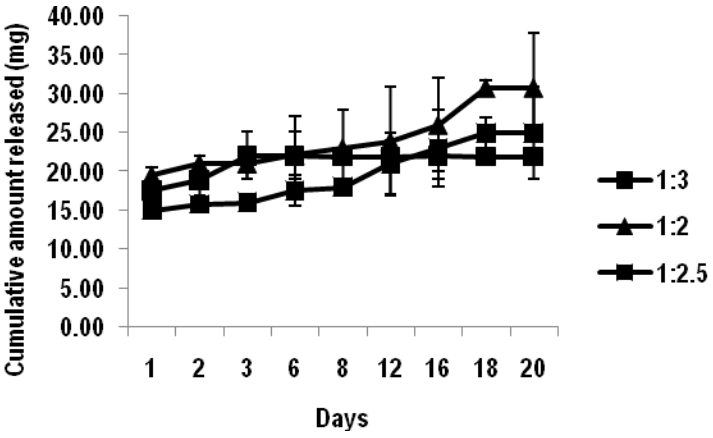


Fig 8.6 *In vitro* drug release profile

8.3.8 *In vitro* cytotoxicity of nanoparticles

The cell viability was analyzed by MTT assay and the PHB nanoparticles showed slight toxicity of 26.6%. This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilized with an organic solvent (eg. isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically. Since

reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

8.4 CONCLUSION

Simple, safe, and reproducible techniques are now available to prepare drug-loaded nanoparticles. Out of these methods, double emulsion-solvent evaporation method was found to be effective in the nanoparticle preparation using extracted PHB as the polymer. Three nanoparticle formulations prepared and drug-PHB nanoparticle with 1:2 ratio was the most suitable one, and it could successfully encapsulate the drug and the *in vitro* release rate was also appreciable. Nevertheless, there are several problems that remain to be solved and could be improved on and even tailored to meet various needs.

Chapter 9

Summary and conclusion

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Polyhydroxyalkonates have gained major importance due to their structural diversity and close analogy to synthetic plastics and these are gaining more and more importance world over. However, much more effort is required in this area to increase the production of inexpensive bioplastic for the successful replacement of the non-degradable plastics. Thus the future of bioplastic depends on the efforts towards fulfilling requirements of price and performance. Research efforts have specifically addressed the issue of productivity.

The present study has successfully demonstrated the potential of various agro-industrial residues as most inexpensive and highly energy rich substrates for fermentation. Out of seven agro industrial residues screened, jackfruit seed hydrolyzate has been selected as an efficient substrate for the PHB production by *Bacillus sphaericus.*

The product formation is strongly influenced by the media components and thus the optimization of media components plays an important role in any fermentation process. A statistical approach has been preferred over the single parameter optimization as a large number of variables can be screened and their interaction effects studied. The employment of the statistical approach also enables validation of the experiments and the statistical analysis ensures precision of experiments. Response surface methodology of central composite design used in this investigation suggested the importance of various factors at different levels for PHB production in submerged

fermentation. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize the process for PHB production. The CCD results predicted that jackfruit seed hydrolysates containing 2.5% reducing sugar, inoculum age of 18 h, and initial medium pH 6 could enhance the production of PHB to reach 49% of the biomass (biomass 4.5±0.04 g/l and PHB concentration 2.22±0.001 g/l).

Even though the submerged fermentation is preferred for the PHA production, solid state fermentation using polyurethane foam also proved to be a successful alternative for conventional SSF method for production of PHB with ease. The response surface methodology of Box Behnken design aided the identification of optimum level of significant factors which influenced the PHB production. The maximum PHB production was 0.169±0.03 g/g PUF and biomass was 0.4±0.003 g/g PUF.

The behavior of *Bacillus sphaericus* in parallel (750 ml) and BIOSTAT B (5L) bioreactors was assessed. This study concluded that the condition of aeration and agitation play significant role in PHB production which was again related to the sugar utilization by *Bacillus sphaericus* in the bioreactors**.** The growth rate and product formation at different levels of aeration were examined by determining the biomass and PHB produced at 0.2, 0.4, 0.6 and 0.8 vvm and at 50% dissolved oxygen. The maximum biomass and PHB production was obtained at maximum and minimum aeration respectively. Low rate of aeration of 0.2 vvm resulted in maximum PHB production (2.58±0.02 g/l) and lowest biomass accumulation (5.12±0.4 g/l) and maximum biomass of 6.20±0.01 g/l was seen at 0.8 vvm. The rate of substrate utilization was found to be related to the growth of biomass thus minimum residual sugar

(2.03±0.04 g/l) was observed in bioreactor with 0.8 vvm and higher residual sugar was observed at the end of fermentation in bioreactor with 0.2 vvm (6.50±0.01 g/l). However, the maximum protein yield (5.78±0.1 g/l) was with 0.8vvm and minimum accumulation of biomass and lower protein yield was with 0.2 vvm. Where as in 5-L the specific flow rate of 0.5 vvm and impeller speed of 400 rpm resulted in the biomass production of 7.29±0.09 g/l and corresponding PHB production was 3.21±0.006 g/l.

The biopolymer was extracted from the bacterial pellet using dispersion of sodium hypochlorite and chloroform solution. The extracted PHB was characterized by ^{1}H NMR, ^{13}C NMR and FTIR and confirmed as PHB. In order to improve the properties of extracted biopolymer it was blended with PEG and thermoplastic starch, and their thermal properties were characterized. But the TGA and DSC results indicated that the blending did not improve the melting temperature but the crystallization properties. The biodegradation of these extracted biopolymer and its blends were tested in sludge samples and in presence of isolated microbes and compared with that of commercial PHB. A very good result was obtained in the case of PHB-thermoplastic blend in presence of sludge sample from Milma, waste water treatment plant, Trivandrum.

Attempts were made to improve the *Bacillus sphaericus* strain for the enhanced production of PHB. The improvement of microbial strains for the over-production of industrial products has been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics. Strain improvement is usually done by mutating the microorganism by classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, UV rays, etc., and/or

chemical mutagens such as EMS, nitrous acid, MNTG, etc. The UV treatment could not generate hyper producing mutants. Two chemical mutants such as EMS and freshly prepared nitrous acid were checked for their efficiency to generate hyper producing mutants and nitrous acid treatment resulted in seven mutants. Out of these seven, two showed increased biomass and PHB production than the wild type strain.

The biocompatibility, biodegradability and low toxicity, *in vivo*, made the biopolymers useful the in the usage of long-circulating drug carriers for the protection of the encapsulated drug, controlled release and drug targeting of pharmacologically active substances. Also the flexibility of polymers aid to engineer multiple functionalities required for efficient drug delivery, simultaneously maintaining biocompatibility, facile manufacturing and stable formulation. Out of three methods used, double emulsion-solvent evaporation method was found to be effective in the nanoparticle preparation using extracted PHB as the polymer. Three nanoparticle formulations prepared and drug-PHB nanoparticle with 1:2 ratio was the most suitable one, and it could successfully encapsulate the drug and the *in vitro* release rate was also appreciable. Nevertheless, there are several problems that remain to be solved and could be improved on and even tailored to meet various needs.

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ANNEXURE 1

Table A1 - List of major instruments used

Equipment	Model
1. **Autoclave**	Tomy, Japan
2. **Incubator**	Sanyo, Japan; Innova4230, New Brunswick Scientific, USA
3. **Weighing balance**	Mettler Toledo, Mumbai, India
4. **pH meter**	Systronics, India
5. **Deep freezer**	Sanyo, Japan
6. **Hot air Oven**	Kemi Instruments, India
7. **Centrifuge**	Remi instruments, India; Eppendorf, Germany; Hitachi, Japan
8. **Laminar Air Flow Chamber**	Clean Air System, India
9. **Incubating water bath**	Julabo, Germany
10. **UV-VIS Spectrophotometer**	UV-160A, Shimadzu, Japan
11. **Phase contrast microscope**	Leica DMLS, Leica Microsystems, Germany
12. **Electron Microscope**	JEOL JSM 5600LV, 115 Japan
13. **Bioreactors**	INFORS HT, Switzerland; Biostat B-5; B. Braun Biotech-Sartorius, Germany
14. **Lyophiliser**	hDB-5503,Operon, Korea
15. **Sonicator**	VCX-750, Sonics, USA
16. **Vacuum concentrator**	5301- Eppendorf, India

Table A2 Composition of Luria Bertani medium

Constituents	Concentrations (g/l)
Yeast extract	5
Tryptone	10
NaCl	10
Agar	15

ANNEXURE

ANNEXURE II

LIST OF PUBLICATIONS

1. Nisha V Ramadas and Ashok Pandey (2010), A statistical approach for optimization of polyhydroxybutyrate production by *Bacillus sphaericus* NCIM 5149 under submerged fermentation using central composite design, *Appl. Biochem. Biotechnol*., 162, 996–1007.

2. Nisha V Ramadas, Sudheer Kumar Singh, Carlos Ricardo Soccol and Ashok Pandey (2009), Polyhydroxybutyrate production using agro-industrial residue as substrate by *Bacillus sphaericus* NCIM 5149, *Braz. Arch. Biol. Technol*., 52, 17-23.

3. Lima L F, Habu S, Gern J C, Nascimento B M, Parada J L, Noseda M D, Goncalves A G, Nisha V R, Pandey A, Soccol V T, Soccol C R, (2008) Production and characterization of the exopolysaccharides produced by *Agaricus brasiliensis* in submerged fermentation, Appl. Biochem. Biotechnol., 151 283–294.

4. Nisha V Ramadas and Ashok Pandey (2011) An unconventional bioprocess method for solid-state fermentation for the production of polyhydroxybutyrate by *Bacillus sphaericus* NII 0838.Communicated.

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1. Nisha V Ramadas and Ashok Pandey, Effect of nutrient sources on enhanced production of polyhydroxybutyrate, International Conference on New Horizons in Biotechnology (NHBT), November 26-29 Trivandrum, India (2007).

2. Nisha V Ramadas and Ashok Pandey Statical optimization of polyhydroxybutyrate by *Bacillus sphaericus* NCIM 5149 under submerged fermentation using central composite design. International Conference on New Horizons in Biotechnology (NHBT-2008), November 6-8 Osmania University, Hyderabad, India (2008).

3. Nisha V Ramadas and Ashok Pandey Effect of agitation and aeration on the production for polyhydroxybutyrate in submerged fermentation. International conference on Emerging Trends in Biotechnology, December 4- 6, Banaras Hindu University, Varanasi, India (2009).