Novel Polyhydroxyalkanoate blends: their characterisation and possible applications

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Declaration

This thesis is a presentation of my original research work in accordance with the guidelines and regulations of the University of Westminster. The contributions of others involved, are fully cited and referenced with appropriate acknowledgement.

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Abstract

Polyhydroxyalkanoates (PHAs) are polyesters consisting of 3-hydroxyalkanoic acids synthesised by numerous bacteria as storage compounds, in the presence of excess carbon, under nutrient limiting conditions. PHAs are biodegradable and biocompatible polymers that exhibit a variety of properties ranging from being thermoplastic to elastomeric in nature. For the first part of this study, the production of PHA in *Bacillus cereus* SPV and *Pseudomonas mendocina* was investigated. Nutrient limitations play a major role in PHA production hence, studies were carried out on the effect of nitrogen, potassium and magnesium limitations on the short chain length PHA accumulation by *B. cereus* SPV. The organism was grown in the Kannan and Rehacek medium using sucrose as the carbon source and accumulated PHA with a maximum yield of 38.0% dcw was observed in the shaken flask cultures. The study was continued with batch fermentation studies in 2 litre fermenters and an enhanced PHA yield was observed with an optimum yield of 44.6% dcw. Further, an enrichment media (MEM media) for *B. cereus* SPV was modified, with three simultaneous nutrient limitations for the production of PHA. A further improved yield of the polymer (52.64% dcw) was observed in this novel media. Chemical analysis of the extracted polymer was carried out using NMR and it was found that the organism accumulated the homopolymer of P(3HB).

When *Pseudomonas mendocina* was grown in the mineral salt media (MSM) with a sodium octanoate as sole carbon source, medium chain length PHA accumulation was observed with a maximum polymer yield of 29.43% dcw in shaken flask cultures. The study continued with batch fermentation studies on the production of the PHAs, which was carried out using 2 Litre fermenters and an improved yield of the polymer (33.5 wt% dcw) was noted. Fed batch fermentation was also explored and a further increase in polymer accumulation was obtained, giving a maximum yield of 37.09% dcw. Chemical analysis of the

polymer using NMR proved that the organism accumulated a homopolymer of Poly(3-hydroxyoctanoate) P(3HO), a rare occurrence. *P. mendocina* was also grown in MSM media with sucrose as carbon source and PHA accumulation was observed with a yield of 27.19% dcw. The polymer was structurally analysed by NMRand identified as the homopolymer of P(3HB).This is the first time that an absolute homopolymer of P(3HB) has been produced by *P.mendocina* using sucrose as the carbon source.

A detailed study on the effects of different extraction methods on the yield of the PHAs was carried out. Among different extraction methods used for PHA extraction the dispersion method gave the highest PHA yield of 30% dcw. The chloroformextraction showed the polymer yield of 28% dcw.The soxhlet extraction, gave the lowest yield of 12% dcw.A novel PHA recovery and purification method based on the osmotic and detergent based lysis and purification was also successfully developed. Higher purity (25%)of the extracted PHA compared to dispersion method, was confirmed by GC analysis.

The blending of the flexible and soft P(3HO) extracted from *P.medochina* with the brittle and stiff P(3HB) from *Bacills cereus*SPV was carried out in two ratios, 5:1 and 1:5. The thermal analysis of P(3HB) showed that the polymer sample had a high melting temperature T_m of 167.39°C, a glass transition temperature, T_g of 2.43°C and a crystallisation temperature T_c value of 54.33°C. The thermal analysis of P(3HO) showed that the polymer exhibited low melting temperature of 50.36°C, a T_g of -32.86°C), and no T_c value. The P(3HB)/P(3HO) 5:1 blend showed two melting temperature 164.91°C and 157.22 °C, single lower glass transition temperature of 5.84°C and raised T_c of 69.58°C as compared to neat P(3HB).The P(3HO)/P(3HB) 5:1 blend on the other hand higher melting temperature of (164.85°C), lower glass transition temperature of -36.99^{0}C as compared to P(3HO) and no T_c was observed.

The P(3HO)/P(3HB) (5:1) blend showed an Young modulus value of 37 MPa with a tensile strength of 1.5 MPa and elongation to break of 160%. Increasing the amount of P(3HB) as in the case of P(3HB)/P(3HO) (5:1) increased the Young modulus value to 4.99 MPa indicating an increase in the stiffness. The percentage of elongation of the film was reduced to just 35.81%. The incorporation of P(3HB) into the biopolymer matrix of P(3HO) or P(3HO) into a predominantly P(3HB) matrix thus resulted in a change in the mechanical properties of the neat PHAs. P(3HB) served the purpose of increasing the tensile strength of the blend, whereas P(3HO) served the purpose of increasing the elasticity of the material. The flexible and strong nature of the P(3HO)/P(3HB) (5:1) blend would make it suitable for a variety of application including the preparation of nerve conduits.

The water contact angle value for neat P(3HB) film was 70.37^{o} and for P(3HO) was 99.94^{o}. In the case of blend films P(3HO)/P(3HB) (5:1) and P(3HB)/P(3HO)(5:1), the θ_{H2O} was 90.39^{o} and 80.0^{o} respectively. The water contact angle studies showed that both the neat and blend PHAs are hydrophobic in nature.

Both the neat and blend films were able to support the attachment, growth and proliferation of the HaCaT cells. However, biocompatibility was better for the P(3HO)/P(3HB) 5:1 film and the topological features of this film led to improved cell attachment and proliferation. SEM analysis confirmed that the HaCaT cells had been able to grow and mature faster on the P(3HO)/P(3HB) (5:1) blendfilm. In conclusion, this work has led to the development of novel PHA blends with new properties, which can be exploited in a variety of applications including nerve tissue engineering.

PHA		Polyhydroxyalkanoates
P(3HB)		Poly(3-hydroxybutyrate)
SCL-PHAs		Short chain length polyhydroxyalkanoates
MCL-PHAs		Medium chain length polyhydroxyalkanoate
P(3HB-co-3HHx)		Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
P(3HHx)		Poly(3-hydroxyhexanoate)
P(3HB-co-3HV)		Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HB-co-4HB)		Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)
P(3HB-co-3HO)		Poly (3-hydroxybutyrate-co-3-hydroxyoctanoate)
P(4HB)		Poly(4-hydroxybutyrate)
P(3HO)		Poly (3-hydroxyoctanoate)
P(3HHX-co-3HO-co-3HD)	1	Poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate-3hydroxydeconate
MPa		Mega paschal
P(3HB-co-HHx)		Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
PVA		Poly(vinyl alcohol)
PBA		Poly(butylene adipate)
P(3HB)/PVA-co-VA)		Polyvinyl acetate-co-vinyl alcohol
EVA		Ethylene vinylacetate,
P(3HB)/(PEC)-		Poly(epichlorohydrin)
LDPE		Low density polyethylene
(SEM)		Scanning electron microscopy
(DTA)		Differential thermal analyzer
PLA		Polylactic acid
Mv		Molecular weight
EDTA		Ethylenediaminetetraacetic acid
PEG		Polyethylene glycol
DMEM		Dulbecco's Modified Eagle media
PLA		Poly(lactic acid
PLGA		Poly (lactic-co-glycolic acid)
(NMR)		Nuclear magnetic resonance
DSC		Differential Scanning Calorimetry
(DMA)		Mechanical analysis: Dynamic Mechanical
NCIMB		National Collection of Industrial Marine Bacteria
MEM		Modified essential media
MSM		Minimal salt media
OD		Optical density
DO		Dissolved oxygen
C: N		Carbon Nitrogen ration
L		Liter
mL		Mililiter
ppm		Parts per million
rpm		Revolutions per minute
Pa		Pascal
NaOCl		Sodium hypochlorite
$CHCl_3$		Chloroform
Cm		Centimeter
mm		Millimeter
cm		Centimeter
NaCl		Sodium chloride
SEM		Scanning electron microscope
NR assay		Neutral Red Assay
PBS		Phosphate buffer saline
LPS		Lipopolysacchrides
T_g		Glass transition temperature
T_g		Glass transition temperature
T_m		Melting temperature

T_c	Crystallization temperature
Td	Degradation temperature
TS	Tensile strength
E	Young's modulus
Hf	Heat of fusion
dcw	dry cell weight
RMS	Root mean square

Table of content

Chapter 1: Introduction

Chapter 2: Materials and methods

Chapter 3: Production and charaterisation of PHAs

Chapter 4: Introduction and charaterisation and application of blend PHAs

Chapter 5: Conclusion and future work

List of Figures

List of Tables

Chapter-1: Introduction

1.1 Polyhydroxyalkanoates

Polyhydroxyalkanoates are polyesters consisting of 3-, 4-, 5- and 6-hydroxyalkanoic acids synthesised by bacteria as storage compounds for energy and carbon, in the presence of excess carbon and at least one limiting nutrient essential for growth such as, nitrogen, phosphorus, sulphur or oxygen (Anderson and Dawes, 1990). Limiting the nutrient condition means that the nutrients present in the growth media need to be able to support the organism's growth, but at much lower concentrations when compared to the high percentage of carbon in the medium. These growth conditions cause a decrease in the rate of cell division, and thereby redirect the cellular metabolism to the biosynthesis of the PHAs. Nitrogen and phosphorous have been the most commonly used limiting nutrient for PHA production. Limitation of oxygen has also been known to stimulate PHA production. For example, oxygen limitation led to increased accumulation of the homopolymer P(3HB) in *Alcaligenes eutrophus* (Gilles *et al*., 1997). The increase in P(3HB) yield by this organism was also observed in both nitrogen limited and non nitrogen limited culture conditions.

Several bacteria can accumulate PHAs as cytoplasmic inclusions that vary in number per cell and size. The observation of PHA containing organisms by transmission electron microscopy reveals the presence of inclusion bodies of 0.2-0.3 nm diameter inside the cell (Figure 1.1). In nature PHA producing organisms can accumulate polymer at a concentration of 3-20% of the cellular dry weight when grown without any nutrient limitation. However, under nutrient limited conditions, they can accumulate polymer up to a concentration of 80% of it's of its dry cell weight at the stationary phase of growth during the bacterial fermentation of sugars or lipids (Anderson and Dawes, 1990;Brandl *et al*., 1994; Misra *et al.,* 2007).

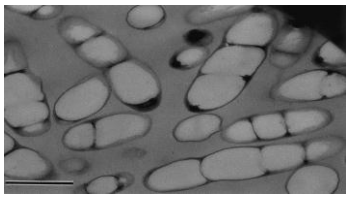


Figure1.1: Discrete intracellular granules of P(3HB) *Cuprivadus nector*

Lemoigne discovered PHAs for the first time in 1926. After this detection, intensive research has been carried out on the physiology, biochemistry and molecular genetics of PHA production. Studies on this polymer revealed that, they are thermoplastic, insoluble in water, non toxic, biodegradable, biocompatible, piezoelectric and exhibit high degree for polymerization and have molecular weight up to several million Daltons (Misra *et al*., 2007) Further, studies on the physiological and mechanical properties showed that the melting point, crystallinity, and glass transition temperature of PHAs is similar to polypropylene. Due to these similarities of physical and material properties with conventional plastics, they have immense potential for various applications. Hence, they are attracting increasing attention leading to extensive research, both in academia and in the industry (Byrom.,1987). According to the culture conditions required for PHA biosynthesis, PHAs producing bacteria can be divided into two groups. The first group of bacteria including *Cupriavidus necator, Pseudomonas oleovorans* and *Bacillus cereus* requires the limitation of essential nutrients for the synthesis of polymer, while the second group of bacteria like *Alcaligenes latus*, mutant strain of *Azotobacter vinelandii* and recombinant *E. coli*, produce PHAs alongside growth in the cultivation medium. The second group of bacteria has been widely studied because of its potential in producing significant amounts of P(3HB) from sugars and organic acids (Anderson and Dawes, 1990;(Byrom.,1987).

The general structure of PHAs is shown in Figure1.2 and it is a linear molecule of carbon and hydrogen with a carboxyl group at the end. There is an ester linkage between the hydroxyl group of each monomer and carboxyl group

of another monomer. The pendant group (R_1/R_2in Figure-1.2) varies from methyl (C_1) to tridecyl (C_{13}). The physical properties of the polymer vary mainly due to the length of side chain and additional functional groups. The structure of a particular PHA produced by bacteria can be altered by genetic or physiological manipulations. So, the type of carbon source and fermentation conditions also affects the type of polymer produced. For example, addition of specific fatty acids can lead to a variation in the monomeric composition of PHA co-polymers (Anderson and Dawes, 1990).

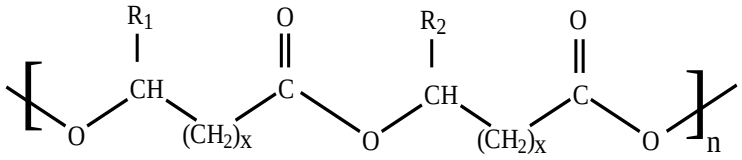


Figure1.2: The general structure of polyhydroxyalkanoates (R_1/R_2 = H, alkyl groups C_1-C_{13}, x = 1-4 and n = 100-30000)

1.2 Types of Polyhydroxyalkanoates

PHAs are found in both Gram negative and Gram positive species like *Alcaligenes*, *Azotobacter*, *Bacillus*, *Nocardia*, *Pseudomonas*, *and Rhizobium*. To date at least 150 different PHAs have been characterised from bacteria because depending on factors like growth conditions, carbon sources and the bacterial strain used, the structural composition of the polymer varies (Steinbuchel *et al.,* 1998). Further, there are more than 91 different monomer units that have been identified with a wide range of properties. Depending on the number of constituent carbon atoms present in their monomer units PHAs are classified mainly into two groups, short chain length PHAs (SCL-PHAs) which consist of monomers with 3-5 carbon atoms e.g., poly(3-hydroxybutyrate), poly (4-hydroxybutyrate),poly(3-hyroxypropionate),poly(3-hydroxyvalerate)and medium chain length PHAs (MCL-PHAs) which consist of monomers with 6-13 carbon atoms example poly(3-hydroxyhexanoate) P(3HHx), poly(3-hydroxydodecanoate), poly(3-hydroxynonanoate), poly(3-hydroxydecanoate) and

poly(3-hydroxyoctanoate). Also, depending on the kind of monomer present, PHAs can be a homopolymers containing only one type of hydroxyalkanoate as the monomer unit, e.g., P(3HB), P(3HHx) or a hetero polymer containing more than one kind of hydroxyalkanoate as monomer units, e.g., poly(3-hydroxybutyrate-co-3-hydroxyvalerate), poly(3hydroxyhexanoate-co-3-hydroxyo-ctanoate), poly-(3-hydroxybutyrate-co-3-hydroxyhexanoate) (Byrom *et al.,* 1987).The SCL-PHAs are crystalline polymers, which are quite brittle and stiff, with a high melting point, and a low glass transition temperature. They have higher tensile strength compared to polypropylene. On the other hand, MCL-PHAs are thermoplastic elastomers with low crystallinity and tensile strength, but high elongation to break. They have lower melting points and glass transition temperatures when compared to SCL-PHAs (Misra *et al.,* 2007; Lee *et al.,* 1995; Lara *et al.,* 1999).

1.2.1 Small Chain Length Polyhydroxyalkanoates (SCL-PHAs)

Poly(3-hydroxybutyricacid), P(3HB), one of the most well studied and characterised SCL PHAs, was first discovered in the bacterium *Bacillus megaterium*. There are many Gram positive and Gram negative aerobic, photosynthetic, organotrophic, lithotrophic bacterial species that have been known to produce this homopolymer (Brandl *et al*., 1994; Lee *et al.,* 1995; Vallapil *et al.,* 2006) (Table-1.1) The biosynthetic pathway of P(3HB) production consists of mainly three enzymatic reactions catalysed by three different enzymes. The pathway involves the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA catalysed by a ß-ketoacyl-CoA thiolase, encoded by the gene *phaA,* followed by the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA

dehydrogenase encoded by the gene *phaB*. Finally, the (R)-3-hydroxybutyryl-CoA monomers are polymerised into P(3HB) by the PHA synthase, encoded by the gene *pha*C (Valappil *et al.,* 2006). The PHA synthases can be divided into four classes based on their subunit composition and substrate specificity. Class I and II synthases have one subunit (*PhaC*). The major difference between Class I and II enzymes is their substrate specificity. The Class I synthase produces SCL-PHA, and the Class II mainly synthesize MCL-PHAs. Class III synthases comprised of two subunits called *PhaC* and *PhaE* and PHA IV composed of *PhaC* and *PhaR* subunits. Both these PHA synthases preferably produce SCL-PHAs (Byrom *et al*., 1994; Lee *et al.,* 1995).

Acenetobacter	*Gamphosphaeris*	*Photobacterium*
Actinomycetes	*Haemophilus*	*Pseudomonas*
Alcaligenes	*Halobacterium*	*Rhizobium*
Aphanothece	*Hypomicrobium*	*Rhodobacter*
Aquaspirillus	*Lamprocystis*	*Rhodospirillum*
Azospirillum	*Lampropedia*	*Sphaerotilus*
Azatobacter	*Leptothrix*	*Spirillum*
Bacillus	*Methylobacterium*	*Spirulina*
Beggiatoa	*Methylosinus*	*Streptomyces*
Beijerinkia	*Methylosinus*	*Syntrophomonas*
Caulobacter	*Micrococcus*	*Sntrophomonas*
Chlorofrexeus	*Microcoleus*	*Thiobacillus*
Chlorogloea	*Microcystis*	*Thiocapsa*
Chromobacterium	*Mycoplana*	*Thiocystis*
Chromobacterium	*Moraxella*	*Thiodictyon*
Clostridium	*Nitrobacter*	*Thiopedia*
Derxia	*Nirococcus*	*Thiosphaera*
Ectothiorhodospira	*Nocardia*	*Vibrio*
Ferrobacillus	*Oceanospirillum*	*Xanthobacter*

Table1.1 Small chain length polyhydroxyalkanoates accumulating microorganisms

There are several organisms capable of synthesizing P(3HB) using different substrates like *Alcaligenes latus*, mutant strain of *Azotobacter vinelandii* and *Cupriavidus necator* that have been widely studied because of their potential in producing significant amounts of P(3HB). For example, *Cupriavidus necator* is a

Gram negative soil bacterium widely studied because of it's potential in producing significant amount of P(3HB) from simple carbon substrates such as glucose, lactic acid and acetic acid. Kim *et al.,* (1994) studied the production of P(3HB) under nitrogen limiting conditions, in fed batch culture of *Cupriavidus necator* using glucose as the carbon source and the organism was able to accumulate polymer at an yield of 76% of dry cell weight at a cell concentration of 164g/L. Also, another strain of this organism is able to accumulate P(3HB) from edible oil like palm kernel oil and palm oil. For example Kang*et al.,* (2008) studied palm oil as a substrate for P(3HB) production, employing *Cupriavidus necotor* H16. The organism was able to accumulate 3.1grams of P(3HB) per litre of palm oil, under fed batch conditions. *A. latus* could be grown in a nutritionally rich media, as the organism doesn't require any nutrient limitation for PHA production. For example, *A. latus* ATCC 29713 was able to accumulate P(3HB) within 93 hours and the yield was 63% dry cell weight under fermentation conditions. In an another study, some species of *Methylobacterium* was found to be able to utilize methanol to produce P(3HB). However, further studies revealed that this organism was able to accumulate more P(3HB) from glucose (53 wt %) and fructose (25 wt %) than methanol(11wt %). PHA production from recombinant organisms have been studied to increase PHA production yield. Recombinant *E.coli* harbouring *Cupriavidus necator* PHA biosynthetic genes were able to accumulate P(3HB) with a yield of 80-90% of the dry cell weight in fed-batch fermentation. Nevertheless, in terms of the thermal and physical properties, P(3HB) was found to be a very brittle, crystalline and stiff polymer with a high melting point, and low glass transition temperature. So, it is relatively difficult to process and be commercially utilised on a large scale. This has been reported as a major drawback in the use *E. coli* for polymer production (Brandl *et al.,* 1990). To avert this problem, the production of copolymer containing 3-hydroxybutyrate and 3-hydroxyvalerate has been produced from *Cupriavidus necator*. Glucose and propionic acid were used to feed *Cupriavidus necator*as major carbon source and it was

observed that the HV content in the co polymer was controlled by glucose to propionic acid ratio in the feed. Fatty acids were used to feed *Alcaligenes latus* AK201 to synthesise P(3HB-co-3HV). In this study the molecular weight of the polymer was found to vary with the percentage of carbon used, and optimum yield of 59% dry cell weight was obtained when the C_{7-9} fatty acids were used. Non-sulphur photosynthetic bacterium like *Rhodospirilium, Rhodobacter* and *Rhodopseudomonas* were able to produce copolymers of P(3HB) under phototrophic and microaerophilic conditions. For example, *Rhodopeudomonas palustris,* a non-sulphur photosynthetic bacterium, has been extensively studied for it's potential in producing P(3HB-co-3HV) from using malate and acetate as carbon sources (Lee *et al.,*1995).Further, a homopolymer of P(4HB) is produced by organisms such as *Commomonas acidivorans,* when fed with structurally related carbon sources such as 1,4-butanediol and ß-butyrolactone. This is known to be a strong pliable thermoplastic material with a tensile strength of 104 MPa, similar to polyethylene. Poly(3-hydroxybutyrate-co-4hydroxybutyrate P(3HB-co-4HB) is another co-polymer which can be produced by *Comamonas acidovorans,* using different substrates such as carbohydrates, alkanes, and alkenes (Valappil, *et al.,* 2006; Colin *et* a*l.,* 2008; Mallic *et al.,* 2007).

The genus *Bacillus* has been widely studied for the production of P(3HB). This genus is an important candidate for PHA production due to various properties like short generation time, absence of endotoxins and the presence of both amylase and proteinase which enables it to use even food waste as a substrate (Law and Slepecky, 1961).*Bacillus sp* was found to be able to synthesize various PHAs with 3-hydroxybutyrate, 3-hydroxyvalerate and 4-hydroxybutyrate (4HB) like monomer units from structurally unrelated carbon sources such as fructose, sucrose and gluconate.(Valappil *et al.,* 2006; He *et al.,* 1998).*Bacillus* UW85has been found to produce terpolymers containing 3-hydroxybutyrate, 3-hydroxyhexanoate, and 6-hydroxyoctonoate units when, grown in a media containingß-caprolactone. Valappil and co-workers achieved P(3HB) production

with Kannan and Rehacek medium using *Bacillus cereus* SPV.Under fed batch fermentation conditions the organism gave a highest yield of 38% dcw (Valappil *et al.,* 2006).

1.2.2 Medium chain length polyhydroxyalkanoates (MCL-PHAs)

Medium chain length (MCL) PHAs consists of 6-14 carbon atom containing monomers. MCL-PHAs are produced mainly by Gram negative bacteria belonging to the rRNA homology group I, they are also synthesised by other Gram negative bacterial species, like *Comomonas* and *Aeromonas*(Table-1.2).MCL-PHAs were discovered in 1983 when *Pseudomonas oleovorans* was grown in octane (De Smet *et al.,* 1983). Since the first discovery of MCL-PHA, many fluorescent *Pseudomonas sp* have been studied for their production. But, depending up on the organism used, and culture conditions, there will be a difference in the molecular structure of the PHA produced. *Pseudomonas* has been considered to be the desirable species for MCL polymer production as they can be easily grown on different carbon sources; both structurally related and structurally unrelated carbon source i.e. precursor substrates that exhibit structures related to the desired constituents of the MCL-PHAs and structurally unrelated precursors that form the monomeric structures via specific metabolic pathway. For example, *Pseudomonas oleovorans* is able to produce MCL-PHAs utilising structurally related carbon sources i.e, the fatty acids. When this organism is grown with hexane as a sole carbon source, it was able to produce polymer containing P(3HHx), P(3HO) and P(3HD). *Pseudomonas putida* KT2442 on the other hand accumulates MCL-PHAs when grown on the unrelated carbon source, such as glucose. This organism was able to accumulate P(3HD) as a major constituent and P(3HHx), P(3HO) and P(3HDD) as minor constituent (Christelle *et al.,* 2008; Duner *et al.,* 2001; Kang *et al.,* 2001).

Pseudomonas mendocina	*Pseudomonas marginalis*	*Burkholderia caryophylii*
Pseudomonas nitroreducens	*Pseudomonas veronii*	*Comamonas testosteroni*
Pseudomonas pseudoalcaligenes	*Pseudomonas chlororaphis*	*Aeromonas caviae*
Pseudomonas stutzeri	*Pseudomonas fluorescens*	*Aeromonas punctata*
Pseudomonas resinovorans	*Pseudomonas aeruginosa*	*Pseudomonas sp. NZ096*

Table1.2 Medium chain length polyhydroxyalkanoates producing microorganisms

The MCL-PHA biosynthetic operon encodes for two PHA synthases (*PhaC1* and *PhaC2*), a depolymerase (*PhaZ*) and the PhaD protein (Figure1.3). The main enzyme in the polymerisation process is the MCL PHA synthase. *Pseudomonas* strains consist of two PHA polymerases, which are slightly different in substrate specificity. The PHA depolymerase functions under nutrient limiting conditions and degrades stored PHA. The *phaD* gene encodes a protein, which plays an important role in the stabilization of MCLPHA granules. The PHA biosynthetic gene of *P. mendocina* consists of two PHA synthase genes flanking a PHA depolymerase gene. The investigation of *pha*C1 and *pha*C2 mutants revealed the different roles played by each PHA synthase. It was found that *PhaC2* could not replace *PhaC1* very effectively because the *pha*C1 mutant exhibited a reduced ability to form PHA from gluconate and was no longer able to accumulate PHAs from fatty acids. However, disruption of *pha*C2 had no effect on the accumulation of PHAs in *Pseudomonas mendocina*. This led to the conclusion that *phaC1* is the main PHA synthase for PHA formation in *P. mendocina* (Lee *et al*., 1995).

Two main pathways are involved in the synthesis of MCL-PHAs. The first pathway involves the fattyacid degradation pathway by ß-oxidation. This pathway is mainly found in fluorescent *Pseudomonas*, such as *Pseudomonas oleovorans*, *Pseudomonas putida* and *Pseudomonas aeruginosa,* which are known to accumulate PHA. Here, the fatty acids are first converted to the

corresponding acyl CoA thioesters that are then oxidized by fattyacid ß-oxidation via trans-2-enoyl-CoA and (*S*)-3-hydroxyacyl-CoA to form 3-ketoacyl CoA. Subsequently acyl-CoA is completely converted to acetyl-CoA. The enzymes enoyl-CoA hydratase (encoded by *phaJ*) and 3-ketoacyl-CoA reductase (encoded by *fabG)* take active part in converting acetyl-CoA to (R)-3-hydroxyacyl-CoA, which act as substrates for the PHA synthase (Klinke *et al.*, 2000).The second pathway is the *de novo* fatty acid biosynthesis, which leads to the formation of PHA monomers from structurally unrelated, simple, inexpensive carbon sources. (Reddy *et al.,* 2003, Philip, *et al.,* 2006)Fatty acid *de novo* biosynthesis is involved in this pathway and is of significant interest because it helps generate PHA monomers from structurally unrelated, simple, inexpensive carbon sources such as glucose, sucrose and fructose. In this pathway, the enzyme acyl-CoA-ACP transferase (encoded by *phaG*) transfers the hydroxyacyl moiety from (R)-3-hydroxy-acyl carrier protein to coenzyme A, thus forming (R)-3-hydroxyacyl-CoA, which acts as the substrate for the PHA synthase enzyme. The expression of enzymes such as malonyl-CoA-ACP transacylase (*fabD*) is also known to generate monomers for PHA biosynthesis (Sudesh *et al.,* 2000; Philip *et al.,* 2006).

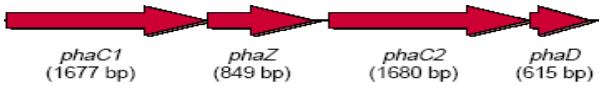


Figure1.3 Organization of the PHA synthesis genes in *Pseudomonas sp*

Pseudomonas putida IPT 046 has been shown to accumulate 60% dry cell weight (dcw) of MCL-PHA copolymer from glucose and fructose. The MCL PHA produced was composed of 3-hydroxydecanoate 60–70 % and 3-hydroxyoctanoate (20–25%). Different *Pseudomonas* strains isolated from sugarcane fields were studied for polymer production and two bacterial strains (*P. putida* and *P. aeruginosa)* were found to be able to efficiently utilize plant oils to grow to accumulate MCL-PHA.

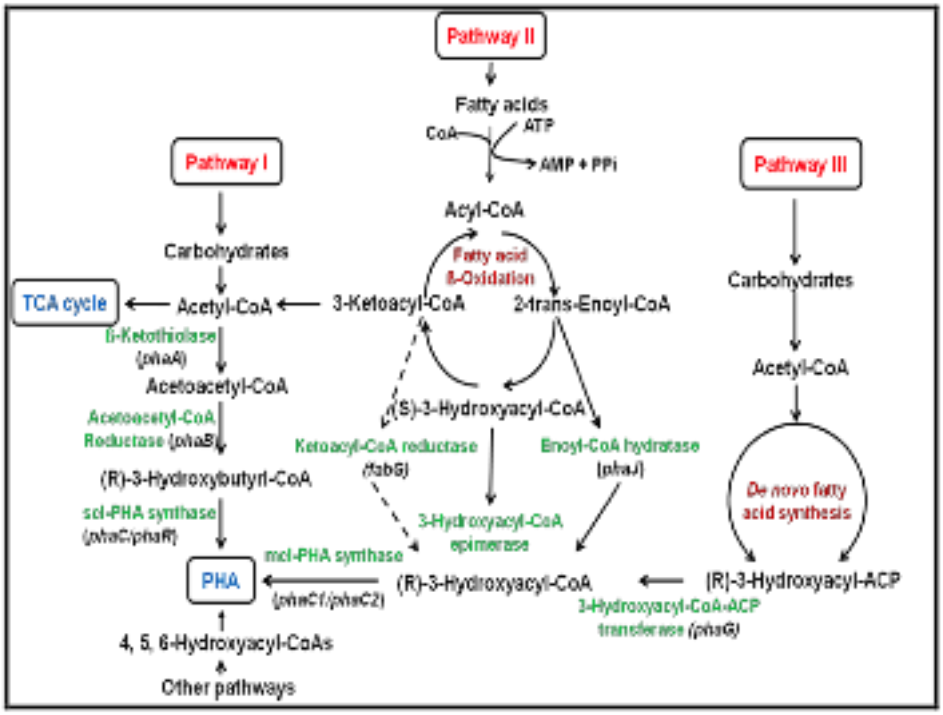


Figure 1.2 Metabolic pathways for the production of PHAs (Kim *et al* 2007)

The molar fraction of 3-hydroxydodecanoate detected in this PHA was linearly correlated to the amount of oleic acid supplied (Luengo *et al.,* 2003; Christelle *et al.,* 2008; Hong *et al.,* 2009; Silva *et al.,* 2009).

The results from Dr. Ipsita Roy's laboratory at the University of Westminster have shown that, the production of MCL-PHAs can be achieved using different *Pseudomonas* strains like *P. oleovorans*, *P. mendocina* and *P. putida*. Among these, *P. mendocina* showed versatility in using both structurally related carbon sources, like octanoate, heptanoate and the structurally unrelated carbon sources, like sucrose, glucose for polymer production (Rai *et al* 2011). Interestingly, *Pseudomonas mendocina* could produce a copolymer, P(3HHX-co-3HO-co-3HD), using glucose. This result was considered to be important, since this is a relatively unexplored polymer. Moreover, *Pseudomonas mendocina* was able to synthesis P(3HO), using sodium octanoate as the sole source of carbon and an yield of 29% dcw was obtained (Silva *et al.,* 2009; Hein *et al.,*2002; Rai *et al.,* 2011).

The polymers produced by *Aeromoanas hydrophila, Aeromonas cavis* grown in a medium containing oleic acid were also able to synthesise MCL PHAs. *Comamonas testosteroni* has been studied for its ability to produce MCL PHAs from vegetable oils. In this study it was observed that *C. testosteroni* was able to convert oil to PHAs with yield ranging from 53.1% to 58.3% for different vegetable oils like castor oil, coconut oil, mustard oil, and olive oil. In another study, by Takor *et al.,*(2003) *C. testosteroni* was able to produce P(3HB) from naphthalene. Intracellular PHA degradation caused by the endogenous PHA depolymerase encoded by *pha*Z is one of the main disadvantages in the production of MCL-PHAs. To avoid this problem, Cai *etal,* constructed a PHA depolymerase knockout mutant of *Pseudomonas putida* KTMQ01.

This mutant organism accumulated up to 86 % dcw MCL-PHA, containing 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate and3-hydroxydodecanoate, when cultured in mineral medium containing sodium octanoate as the carbon source, while the wild type*, Pseudomonas putida* KT2442, produced only 66% of yield. Recombinant *E. coli* harbouring *Aeromonas caviae* biosynthetic genes produced terpolymers of 3-hydroxybutyrate (3HB),3-hydroxyvalerate (3HV) and3-hydroxyhexanoate (3HHx) from decanoate (Hiroki *et al.,*1999; Kek *et al.,* 2008).

1.2.3 Properties of polyhydroxyalkanoates

The chemical structure of the polymer greatly affects its physical and material properties. For example the SCL-PHA P(3HB), is a crystalline, brittle and stiff polymer with a melting temperature of 180^{o}C and a glass transition temperature , of 4^{o}C. The brittleness of P(3HB) is due to the presence of crystalline domains called spherulites which form upon cooling of the melt polymer. Tm is the melting temperature of the polymer at which a solid,

given sufficient heat, becomes a liquid and the glass transition temperature (T_g) The temperature at which a rigid solid becomes pliable and can be formed, shaped, or moulded. The average molecular weight (M_w) of P(3HB) has been found to range between 530,000 to 1,100,000. The mechanical properties of P(3HB) are similar to that of polypropylene with similar Young's modulus (3.5GPa) and tensile strength (40 MPa). However, the elongation to break is about 5%, which is significantly lower than that of polypropylene (400%) However, another example of SCL PHA, P(4HB) has a Young's modulus value of 149 MPa, tensile strength of 104 MPa and a high value of elongation to break (1000 %) (Anderson and Dawes, *et al.,* 1990).

MCL-PHAs are highly elastomeric in nature because of their low degree of crystallinity and a low melting temperature, mainly due to an increase in the length of the side chain. These are biopolymers, which exhibit properties of thermoplastic elastomers and resemble natural rubbers. In fact due to the T_g values below room temperature and a low degree of crystallinity these polymers exhibit elastomeric properties. The low crystallinity is due to the presence of large and irregular pendant side groups, which inhibit close packing of the polymer chains in a regular three dimensional fashion. MCL-PHAs have melting temperatures (T_m values) ranging between 40^{o}C and 60^{o}C and glass transition temperatures (T_g values) ranging between -50^{o}C and -25^{o}C. In MCL-PHA the number average molecular weight, $M_n$is between 40,000 and 231,000 (Sánchez *et al.,* 2003; Bernard and Birgit, 1999).

1.2.4 SCL/MCL PHAs

As discussed above, the homopolymer P(3HB) is the most well studied member of the family of SCL-PHAs. P(3HB) is highly crystalline, rigid, and brittle, with poor elastic properties, making it difficult to process, thus limiting its range of applications. The degree of brittleness depends on the degree of crystallinity, glass temperature and microstructure. On the other hand MCL-PHAs are semi

crystalline elastomers with low tensile strength, low melting point and high elongation to break. A comparison of the properties of SCL and MCL PHAs are shown in Table-1.3 (Christelle *et al.,* 2008; Sodian *et al.,*2000).

Properties	SCL-PHAs	MCL-PHAs
Crystallinity (%)	40-8	20-40
Melting Points (^{o}C)	80-180	30-80
Density (gcm^{-1})	1.25	1.05
Tensile Strength (MPa)	43	20
Glass transition temperature (^{o}C)	4	-35
Extension to break (%)	6-10	300-450

Table 1.3 Comparisons between SCL- PHAs and MCL-PHAs

There are two main approaches to improve the physical properties of PHAs. The first approach is microbial synthesis of co-polymers of SCL and MCL-PHAs (Bo*et al.,* 2008; Silva *et al.,* 2009).To overcome the brittleness of P(3HB), the US company Teph introduced copolymers of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), P(3HB-co-3HHx).This known SCL-MCL copolymer exhibits a good tensile strength value which is of intermediate value between that of P(3HB) and P(3HHx) (Table-1.5). Also, this polymer shows a higher elongation at break (850%) compared to 2.56% of the brittle P(3HB).To improve physical and thermal properties of P(3HB), random copolysters of P(3HB)–co-3HV) or P(3HB-co-4HV) have been produced by *Alcaligenes eutrophus* from alkanoic acids. The varying copolymer composition can regulate the physical and thermal properties of this polymer. These co-polyesters are found to be more ductile and elastic than P(3HB) (Luengo *et al.,* 2003; Bo *et al.,* 2008).

Aeromonas species like, *A. caviae, A. hydrophila,* can produce P(3HB-co-HHx) from carbon sources like oleic acid, olive oil, and lauric acid. The highest production of polymer observed was 33 wt%, when the organism was fed with lauric acid in batch fermentation. Interestingly there was an improvement of 11-20% yield, when the concentration of lauric acid increased from 5-30 g/L.

P(3HB-co-HHx) are semi crystalline in nature and the crystallinity of the polymer is mainly due to the presence of P(3HB) in the co-polymer. These bacteria are able to produce SCL-MCL PHAs mainly due to the PHA synthase substrate specificity, which accepts precursors of a certain range of carbon length (Bo *et al.,* 2008).

The presence of copolymers in the polymer backbone also affects the mechanical and thermal properties of the polymer. For example, the copolymer of P(3HB-co-HHx) with 2.5 % 3HHx exhibits a tensile strength of 25 MPa and Young's modulus of 631.3 GPa. But when the HHx content was increased to 9.5% HHx, the tensile strength and Young's modulus reduced to 8.8 MPa and 9.5 GPa respectively. P(3HB-co-3HHx) also exhibits better biodegradability and biocompatibility as compared to P(3HB). Recent studies showed that (P3HB-co-3HHx) has better biocompatibility as compared to that of poly(L-lactic acid) (PLA), P(3HB), and P(3HB-co-3HV) (Bo *et al.,* 2008; Bian *et al.,* 2009).

Pseudomonas sp DSY-82 is able to produce polyesters containing 3-hydroxyvalerate, as well as various MCL 3-hydroxyalkanoate monomeric units, when valerate is cofed with either nonanoate or undecenoate. Further, structural analysis proved that copolymer consists of different concentrations of 3-hydroxyvalerate, 3-hydroxyoctanoate, and 3-hydroxydecanoate.Yoshiharu *et al*, (1995) observed that *Aeromonas caviae* produced a copolymer from olive oil. Further, structural studies showed that this copolymer comprised of 83 mol% 3-hydroxybutyrate (3HB) and 17mol% 3-hydroxyhexanoate. So, SCL-MCL-PHA co-polymers have properties between the SCL-PHAs and MCL-PHAs depending on the ratio of SCL and MCL monomer unit Table 1.5. Hence, they are expected to exhibit physical, thermal and mechanical properties intermediate to that of SCL and MCL PHAs (Haywood *et al.,* 1989; Ha *et al.,*2002).

Samples	T_m (^{0}C)	T_g (^{0}C)	Tensile strength (MPa)	Elongation at break (%)
P(3HB-co-10% HV)	150	-	25	20
P(3HB-co-20% HV)	135	-	20	100
P(3HB-co-10% HHx)	127	-	21	400
P(3HB-co-17% HHx)	120	2	25	850
Polypropylene	170	-	34	400
Polystyrene	110	-	50	-
P 3(HO)	50.8	-	20	300-450
P(3HB)	177	4	40	5

Table.1.5 Comparison of mechanical properties of SCL-MCL copolymers with that of P(3HB) and P(3HO) Ha *et al.*, 2002.

The second approach to improve the properties of SCL and MCL monomer is the blending of the PHAs with other polymers or preparation of blends with between SCL and MCL PHAs. In this method the blends are made from using two polymers which are mixed together in order to get a material with properties somewhere between those of the two individual polymers. There would not be any covalent bond formation between the blend polymers. P(3HB) has been already blended with non biodegradable polymers like polyvinyl acetate, polymethacrylate, and also with biodegradable polymer such as polyethylene oxide, and poly(vinyl alcohol). Various studies have shown that polymers like poly(vinyl acetate) cellulose, poly(ethylene glycol), poly(vinylidene fluoride) are miscible with PHAs. Immiscible blends have also been made using P(3HB) mixed with polymers like poly(1,4-butyleneadipate), poly(ε-caprolactone), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-3HV). Immiscible blends have polymers not forming a homogeneous mixture when added together .In all these studies because of the blend partner the thermal, physical and biodegradability properties of the P(3HB) changed significantly (Ha *et al.,*2002).

Scandola *et al.,*(1997) studied the miscibicility of P(3HB) with cellulose acetate and cellulose acetate propionate. The investigation of the mechanical properties of the blend revealed that both the blends are miscible in amorphous state. The miscibility of P(3HB) and poly(vinyl alcohol) (PVA) was studied by Azuma *et al.,*

(2000) they found that blends are partially miscible in the amorphous phase at high PVA concentration. The degradation studies showed that the blend with more PVA degraded faster than pure P(3HB). In another study on the chitosan-P(3HB) blend revealed that both polymers are miscible and the crystallization of P(3HB) was suppressed with increased concentration of chitosan. Blending of poly(caprolactone) (PCL) and poly(butylene adipate) (PBA) with P(3HB) showed that the immiscibility affected the mechanical properties of the blend polymers (Table-1.6). The Young's modulus and tensile strength of P(3HB)-PBA blends decreased with the increase of PBA concentration in the blend. In the case of P(3HB)-PLC film, it was observed that the crystallisation and melting point of P(3HB) was decreased by the addition of PLC in the blend film (Ha*et al.,*2002). Xing *et al.,*(1998) investigated the miscibility and crystallisation behaviour of polyvinyl acetate-co-vinyl alcohol) and P(3HB). When they studied the crystallization behaviour and morphology of P(3HB)/PVA-co-VA (polyvinyl acetate-co-vinyl alcohol) blends, and found that the PVA-co-VA with a vinyl alcohol content of 9, or 15 mol% made a miscible phase with the amorphous part of P(3HB).

However, in the melt state P(3HB)/PVA-co-VA) blend with 20/80 composition formed a partially miscible blend. On the other hand, P(3HB) and PVA-co-VA with 22 mol% vinyl alcohol were found to be immiscible. In a another investigation, Yoon *et al* studied the miscibility of P(3HB) with ethylene vinyl acetate, (EVA) containing 70 or 85 wt% of vinyl acetate. Based on the result of the studies they found that the blend of P(3HB) and EVA was immiscible. This conclusion was based on the fact that individual the glass transition temperature, the melting temperature and crystallization conditions were observed for both P(3HB) and (EVA) (Ha *et al.,*2002).

Sample	Blend composition	Young's modulus MPa	Tensile strength MPa	Elongation at break %
P(3HB)	-	1560	38	5
P(3HB)/PCL	77/23 49/51 25/75 0/100	1560 730 110 220	21 4 8 15	9 11 18 24
P(3HB)/PBA	75/25 49/51 24/76 0/100	1050 860 480 -	32 19 10 -	7 4 3 -

Table-1.6 Mechanical properties of the films of P(3HB)-PLC and P(3HB)-PBA blends(Ha *et al* 2002)

Paglia *et al.,*(1993) studied the changes in the crystallization and thermal behaviour of P(3HB) in the P(3HB)/poly(epichlorohydrin) (PEC) blends. According to their studies, the T_m and T_g behaviours, the blends were found to be miscible in the amorphous phase. Further, Finelli *et al.,* (1998) also analysed P(3HB)/(PEC) blends by Differential scanning calorimetry and Dynamic mechanical analysis and concluded that both polymers are miscible with each other. Olkhov *et al* carried out studies on the properties of P(3HB)/low density polyethylene (LDPE) and the scanning electron microscopy (SEM) results concluded that both polymers formed an immiscible blend (Ha *et al.,*2002).Gassner and Owen studied the melting, crystallization and dynamic mechanical behaviour of blends of P(3HB) and P(3HV). The thermal analysis results, gave two melting regions detected by the differential thermal analyser (DTA). The different separate melting point shows that both P(3HB) and remain unaffected by the blend formation. Thus, their results showed that P(3HB)/P(3HV) blends contained phase separated domains. Polylactic acid is a semi crystalline polymer with melting point ranging between 164-180^{0}C, and the P(3HO) is an another semi crystalline polymer with melting point of 57 ^{0}C. Renard *et al,* prepared blends of P(3HO) and PLA in different proportions and found that P(3HO)/PLA blends are immiscible. Electron microscopy observations revealed that the mixture film clearly divided into two phases. So the miscibility, thermal behaviour, and mechanical properties of the PHAs they vary depending

up the type of PHA selected for blending. Hence through blending approach, it is possible to obtain polymer with desirable mechanical and thermal properties. The blending approach has several advantages in comparison to the syntheses of copolyesters, such as easy processability, balanced properties and low production costs.

1.3 Extraction of PHAs

Isolation of PHAs from bacterial cells is a major step in polymer production. Numerous separation processes have been proposed for the recovery of PHA from microbial mass. For example, *Alcaligenes eutrophus* can accumulate large quantities of P(3HB). Extraction of PHA from this organism can be done by solvent extraction. This extraction method initially involves a pre-treatment step to disrupt the cells then the polymer is extracted using an organic solvent like chloroform. The efficiency of the extraction method relies on the solubility of the polymer in the solvent. Finally, the polymer is precipitated using ice cold methanol. Other organic solvents such as methylene chloride, 1,2-dichloroethane, and tetrahydrofuran, also can be used for the extraction of this polymer. Vanlautem *et al.,*(1982) investigated extraction of P(3HB), from *Ralstonia eutropha* using halogenated solvents like chloroethanes, and chloropropanes and found that P(3HB) can be extracted using these solvents(Jiang *et al.*, 2006; Hahn *et al.*, 1994;Jacquel*et al.,* 2007). P(3HO) is soluble in chloroform, n-hexane, dimethyl carobonate, acetone and the solvent based extraction of the polymer is usually done either by dispersion method and also by the soxhlet extraction method. The dispersions method is a quick and easy method in which cells are put together in chloroform and sodium hyphochlorite solution, and the lysis of the cells carried out by sodium hypochlorite solution. But the main disadvantage of this method is the degradation of PHA by the sodium hypochlorite solution, which mainly affected the quality of the polymer and the average molecular weight of recovered P(3HB) is only half that of the original. Moreover, all these extraction methods generally use large quantities of toxic

and explosive solvents. In soxhlet extraction method the bacterial biomass is placed inside a thimble which is then loaded into the main chamber. The solvent is heated to reflux on the bacterial biomass and the polymer will then dissolve in the warm solvent. After the extraction the solvent is removed by means of rotary evaporator for yielding the extracted compound. Imperial Chemical Industries proposed an alternative method to the solvent extraction. This was enzymatic digestion method with thermal treatment of P(3HB) containing biomass, followed by washing with an anionic surfactant to dissolve non PHA cell materials. No molecular weight reduction and polymer degradation is observed in the enzymatic extraction method, which is the major advantage.

However, the main disadvantage of this enzymatic extraction is the complication of the method and the cost. So, less expensive chemicals were tried to reduce the cost of recovery of PHAs (Cochi and Lee., 1999). Among these chemicals NaOH and KOH were found to be good for P(3HB) recovery. The advantage of using NaOH treatment was the reduced endotoxin level in the polymer. Furthermore, in some other studies EDTA/Tris buffer was considered to be ideal choice for cell lysis and P(3HB) recovery (Jacquel*et al.,* 2008; Brandl *et al.,* 1990; Byrom *et al.,* 1994; Reddy *et al.,* 2003).

At the same time, development of automatic PHA liberating cells by genetically modified lysis of recombinant cells has attracted attention. In this method phage lytic genes were used in *E-coli* for release of PHA. For this, expression *phaCAB* genes, along with lytic gene of bacteriophage PhiX174 was successfully carried out in *E. coli*. In order to control the expression of the lysis gene a thermo sensitive expression system was used. Another study showed that, the lytic genes of phage ë and the P(3HB) biosynthetic genes (*phbCAB*) were employed for PHA production and automatic release of polymer. In addition, a few mechanical methods also have also been developed for extraction of PHA.

For example, Tamer *et al.,* (1998) have proposed the use of bed mill as the method for the large scale disruption of microbial cells in the recovery of P(3HB) (Valappil *et al.,* 2006; Reddy *et al.,* 2003).

1.4 Biocompatibility and biodegradability of PHAs

One of the important properties of PHAs is biocompatibility Shishatskaya *et al.,* (2005) conducted *invivo* biocompatibility studies on P(3HB) and P(3HB-3HV) and showed that PHAs have no toxic effects in living organisms. In mammals, the hydrolysis of P(3HB) gradually occur during degradation to form 3-hydroxybutyric acid, a known constituent of blood plasma. Moreover, PHAs are present in all organisms in the form of short chain units and they are widely distributed in the all cellular compartments and intracellular fluids. These types of PHAs are low molecular weight in nature closely associated with other cellular macromolecules such as proteins, phospholipids, and polyphosphates and hence they are referred as complex PHAs. Human blood plasma also consists of some complied PHAs. So, all these results give evidence of biocompatibility of PHAs in the human body (Brandl*et al.,*1990; Byrom *et al.,* 1994).Biodegradation of PHAs in the natural environment is affected by enzymatic activities of microorganisms, which are capable of depolymerising P(3HB)using the PHA depolymerase enzyme. PHAs are water insoluble substrates, while PHA depolymerases are soluble in water, the enzymatic degradation is therefore a heterogeneous reaction involving two steps; adsorption of enzyme onto the surface of the P(3HB) material followed by hydrolysis of polymer chains by the active site of the enzyme. The intracellular degradation occurs to release of stored carbon within the microorganism, when there is a lack of nutrients in the media. (Sujatha *et al.,* 2007; Valappil, *et al.,* 2006).The enzymatic degradation of PHAs is affected by the chemical structure and the proportion of individual monomer unit and their solid state structure. It was found that the degradation rate of the polymer decreased when the crystallinity increases.

This is mainly due to the decrease in the chain mobility rendering the material more resistant to degradation. However the degradation rate of P(3HB) can be increased by the addition of plasticizers. The addition of a hydrophilic polymer increases the water absorption rate on the increases hydrolysis of the composite. The water absorption rate can determine the degradation rate in blend polymer For example, incorporation of hydrophilic polymers like polyethylene glycol(PEG) in to the P(3HB-3HV) matrix, resulted in increased the water absorption rate, there by accelerating the rate of biodegradation of the P(3HB-3HV). When hydrophobic polymers like polycaprolactone blend with P(3HB) the water absorption rate composite is reduced. So, the addition of polycaprolactone to the homopolymer of P(3HB) resulted in decreased the rate of biodegradation of the P(3HB-3HV). This mainly because of the presence of polycaprolactone in this copolymer, which causes the rearrangement of polymer chains which leads to the increased water penetration into the polymer. However, the studies conducted by the Renard *et al.,* (2004)revealed that, the blend of P(3HO) with PLA and PEG, caused no significant decrease in degradation rate, this could be mainly due the presence of second component, not affecting the rearrangement of the P(3HO) (Byrom *et al.,* 1994; Misra *et al.,* 2007; Reddy *et al.,* 2003).The studies on the rates of degradation of polyester films, in buffer solutions, reveal that copolymers containing 4-hydroxybutyrate (4HB) monomer units degraded more rapidly than P(3HB) or P(3HB-co-3-HV). The degradation rate also gets affected by environmental factors like temperature, moisture level, and pH (Veda*et al.,* 2003; Reddy *et al.,* 2003).

One of the main types of degradation that can occur in the body is enzymatic degradation. *In vitro* degradation studies are performed mainly in phosphate buffer saline (PBS) cell culture media and Dulbecco's Modified Eagle media (DMEM). The *in vitro* degradation studies of P(3HB) in buffer solution showed, no bulk mass loss after 180 days and a decrease in the molecular weight of the polymer was observed after 80 days. The i*n vitro* degradation of P(3HB-3HV) is

mainly dependent on the HV content, because the varying HV affects the molecular weight, crystallinity, and porosity of the polymer (Shishatskaya *et al.,* 2005).

1.5 Applications of Polyhydroxyalkanoates

SCL and MCL PHAs have been found to be useful in large number of applications. SCL-PHAs have replaced many conventional petrochemical products including moulded goods, paper coating, non-woven fabrics, food packaging, performance additives and diaphragms. The copolymer P(3HB-co-3HV) have been used for various commercial applications including the production of bottles.

Studies revealed that, SCL-PHA scaffolds enhance cell adhesion, migration, proliferation and differentiation, which leads to new tissue growths and eventually results in replacement by natural tissue. For example, P(3HB) has been used as surgical sutures. P(3HB) can also be used to construct composites for fixing fractured bones thereby enhancing bone growth as well as healing (Sudesh and Doi, 2000). P(3HB-co-3HV) with 3HV units more than 20 mol% have been used for manufacturing films and fibres to create wound patches. P(3HB-co-3HV) has been used for hard tissue regeneration like bone tissue engineering. *In vitro* studies conducted at the University of Westminster, proved that osteoblast cells could be successfully grown on P(3HB) scaffolds. Another copolymer, P(3HB-co-4HB) has shown good biocompatibility with fibroblast cells suggesting that it has potential to be used for cardiovascular and orthopaedic applications (Byrom *et al.,* 1994;Luengo *et al.,* 2003; Findlay *et al.,* 1983; Sodiun*et al.,*2000;Nirupama *et al.,* 2007).

MCL-PHAs are flexible polymers and can be processed to form appropriate shapes for tissue engineering applications. *In vitro* studies proved that MCL

PHAs have good degree of biocompatibility and biodegradability when they are grown with cell lines like fibroblast and endothelial cells. Thus, these polymers have been mainly used for biomedical applications like heart tissue engineering, in creating vascular grafting in order to repair malfunctioned blood vessels. MCL PHAs are promising candidate for soft tissue engineering because they are less crystalline and elastomeric in their properties as compared to SCL-PHAs. The copolymers of MCL PHAs like Poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate), P(3HHx-co-3HO), have been used for vascular grafting in order to repair or replace malfunctioned blood vessels in the arterial or venous systems due to damage or disease (Steinbuchel and Fuchtenbush, 1998). Further, P(3HO) have been used as scaffolds for the regeneration of nerve axons (Bian*et al.,* 2009; Cathryn *et al.,* 2005; Misra *et al.,* 2006). Due to their biodegradable and biocompatible properties PHAs have become ideal candidates for drug delivery. These polymers are extensively used as nano particles, microcapsules, matrices and micro porous powders as matrices for delivery of drugs and hormones. For example, SCL-PHAs such as P(3HB co-3-HV) microspheres, have been used for release of low molecular weight drugs. Here, the release is dependent on porosity, molecular weight and percentage of drug loading. MCL-PHAs have low melting points and are considered to be much more suitable for slow drug delivery applications due to it's low porosity. For e.g, blend polymers of PEG-P(3HO) have been used for controlled drug delivery system for ibuprofen as a model drug (Julian*aet al.,*2009; Kim*et al.,*2005;Misra *et al.,*2006).

1.6 Tissue engineering a major medical application for PHAs

Tissue Engineering is an emerging field that aims to regenerate natural tissues and create new tissues using biological cells, biomaterials and biotechnology. In the common tissue engineering strategy a highly porous biodegradable scaffold

is seeded with the relevant type of cells and the growth is carried out *in vitro*. This is followed by implantation where the scaffold acts as a support on which new tissue develops (Durner *et al.,*2001).The materials are generally being used to make scaffolds on which cells can be seeded so as to induce new tissue growth. The scaffold must be biocompatible, provide a suitable surface for the cells to adhere. It must be able to support cell growth, maintaining cells in a viable state by proper diffusion of nutrients without cell release. Once the new tissue replacement is formed, the scaffold must be able to degrade and the degradation products must be non toxic and well tolerated (David *et al.,* 2003; Williams *et al.,* 1999).

PHAs are ideal to be used as scaffolds in tissue engineering applications due to their biocompatibility, ability to support cell growth, cell adhesion and biodegradability. Also, the hydrolytic degradation of PHAs is less acidic and inflammatory, compared to synthetic materials like poly (lactic acid (PLA). For example, Wang and Cai (2010) and his group have found that P(3HB-co-3HHx) is a more suitable biomaterial for osteoblast attachment, proliferation and differentiation for bone marrow cells when compared to poly(lactic acid) (Wang et al' 2004).PHAs have also been already explored for hard tissue engineering. P(3HB) and composites of P(3HB) with bioactive 45S5 Bioglass® have been extensively studied for bone tissue engineering. The composite P(3HB)/45S5 Bioglass® showed good biocompatibility with the seeded human osteoblast cell line (MG-63) (Misra *et al.,* 2007; Misra *etal.,* 2007; Misra *et al.,* 2009).Sodian *et al.,* (2000) and hisgroup fabricated a trileaflet heart using an elastomeric P(3HO) for the fabrication of a trileafletheart valve scaffold. Vascular cells were seeded onto the heart valve scaffold. The study concluded that tissue engineered P(3HO) fabricated heart valve can be used for implantation in the pulmonary position (Sodian *et al.*, 2000). Studies have been carried out to explore PHA scaffolds for skin regeneration and wound healing. Tang *et al.*, (2008) used copolymers of poly(3HB-co-5mol%3HHx) and poly(3HB-co-7mol%-4HB) to fabricate electrospun scaffolds for skin tissue regeneration.

The tests conducted on the mechanical properties of the scaffolds, tensile strength and Young's modulus had values between 5-30 MPa and 15-150 MPa, which were comparable to those of the human skin. Hence their study concluded that the scaffolds are mechanically stable in supporting regenerated tissues. Li *et al.*,(2008) prepared nanofibrous matrices using a blend of P(3HB)/P(3HB-co-3HHx) and P(3HB)/P(3HB-co-4HB). The human keratinocyte cell line, (HaCat) was seeded on nanofibrous scaffolds of this blend. The cell morphology, adhesion ability and viability was found to be better on this nanofibrous matrices.

1.7 Neural Tissue Engineering

The physiology of the nervous system presents challenges to bioengineering research addressing nerve injuries. The recovery of the nervous system is a challenge because once impaired it is difficult to repair. It may also lead to malfunctions in other parts of the body because mature neurons don't undergo cell division. Neural tissue engineering has mainly focused on the discovery of new ways to recover nerve functionality after injury. Researchers in neural tissue engineering mainly focus on designing "nerve guidance channels" or "nerve conduits" to increase the prospects of axonal regeneration and functional recovery (Huang *et al.,* 2007) ;Yi *et al.,* 2006; Stephanie *et al.,* 2007).A variety of biomaterials have been investigated for their suitability in nerve tissue engineering application. Polymers like poly(lactic acid) and poly(ε-caprolactone) have previously been shown to biodegrade and to be biocompatible in order to act as a support for nerve regeneration. Poly(lactic-co-glycolic acid)(PLGA) scaffolds (75:25) copolymer ratio of lactic acid to glycolic acid) have already been successfully used in an attempt to guide peripheral nerve regeneration. Another important advancement has been the development of a tube using polyglycolic acid (PGA) with inner space filled with collagen sponge. This tube has been successfully used to enhance peripheral nerve regeneration applications (Jiang*et al* 2010., Stephanie *et al.*, 2007;Hall*et al.*, 1986).

Polyhydroxyalkanoates (PHAs) have also been shown to have biodegradability and biocompatibility as well as good mechanical properties for nerve tissue engineering applications. For example, P(3HB) conduits has been tested for superficial radial nerve repair in cats. This study showed that axonal regeneration could be carried out using P(3HB) with low inflammatory response. In another experiment, the use of nerve guides made up of P(4HB) was found to facilitate increased cell regeneration in peripheral nerve repair. Further, Bian*et al.,* studied the application of the copolymer poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), P(3HBHHx) for repairing damaged nerves in rats. The result of the study proved that the foetal mouse cerebral cortex cells were able to grow well on P(3HB-co-3HHx) films All these studies emphasise the feasibility of PHAs in nerve regeneration.(Bian *et al.,* 2009).

1.8 Aims and Objectives

The overall aim of this research project was the production of MCL-SCL PHA blends as novel material for tissue engineering. The detailed objectives of the work carried out in this context include.

1. Production and characterization of the MCL and SCL polymers using *Pseudomonas mendocina* and *Bacillus cereus* SPV.

This involved, the production of P(3HO), from *Pseudomonas mendocina* using sodium octanoate as the sole carbon source, and production P(3HB) from *Bacillus cereus SPV* using sucrose as sole carbon source. Characterisation of both of SCL and MCL polymers were carried out involving the determination of their monomeric compositions (Chemical structure analysis: Gas chromatography and Nuclear magnetic resonance (NMR); Thermal analysis: Differential Scanning Calorimetry (DSC); Mechanical analysis: Dynamic Mechanical Analysis (DMA); and Biocompatibility analysis.

2. Preparation of blends of SCL/MCL and MCL/SCL polymer films

The P(3HB) /P(3HO) and P(3HO) /P(3HB) blend polymer films were prepared in 5:1 ratio and films casted using the solvent casting method. These were also characterised using all the techniques mentioned in objective-1.

3. *In vitro* biocompatibility studies

In vitro biocompatibility tests were carried out using the HaCat cell lines. Cell adherence, viability, morphology and proliferation on SCL, MCL, and SCL/MCL blend polymer films were analysed.

Chapter-2 Materials and Methods

2.1 Bacterial strains

The *Pseudomonas mendocina* used in this study for the production of medium chain length polyhydroxyalkanote was bought from National Collection of Industrial Marine Bacteria (NCIMB). The *Bacillus cereus* SPV used for the production of small chain length polyhydroxyalkanote was obtained from University of Westminster Culture Collection.

2.2 Cell line

Cell culture studies were done using HaCat (keratinocyte cell line) obtained from the University of Westminster's cell line collection.

2.3 Media and Chemicals

All analytical grade and chromatographic reagents were obtained from Sigma-Aldrich Company and BDH. Readymade media was used for general bacterial media preparations. Chromatography grade reagents were used for Gas chromatography and nuclear magnetic resonance (NMR) analysis. Cell culture studies were done using cell culture grade media and reagents from Sigma.

2.4.1 Inoculum growth medium

Nutrient broth media was used for the seed culture preparation, according to the manufacturers directions. The medium contained the following concentration of nutrients.

Chemicals	Composition per litre
Yeast extract	2.5g
Peptone	5g
Sodium Chloride	5g
Agar	2g

Table: 2.1 Chemical composition of inoculum growth media

2.4.2. Short chain length PHA production media

2.4.2.1 Kannan and Rehacek Media

B. cereus SPV was grown in Kannan and Rehacek Media (Kannan and Rehacek, 1970).

Chemicals	Composition per litre
Glucose	20.00g
Yeast extract	2.50g
Potassium chloride	3.00g
Ammonium sulphate	5.00g
Soybean dialysate	100 mL

Table:2:2 Chemical composition of Kannan and Rehacek media

(Soybean dialysate was prepared from 10 g of defatted soybean flour in 1000 mL of distilled water for 24 hrs at 4 ^{o}C).

2.4.2.2 Modified Enrichment media (MEM)

A previously reported *Bacillus cereus* enrichment media was modified to be used for PHA production under potassium and magnitium deficient conditions. The medium contained the following concentration of nutrients.

Chemicals	Composition per litre
Ammonium phosphate	1.0g
Potassium chloride	0.5g
Magnesium sulphate	0.5g
Sucrose	20 g
Yeast Extract	2 g
Trace element solution	1 ml

Table: 2.3 Chemical composition of Modified Enrichment media.

Trace element solution potassium and sulphur deficient media had the following concentration of nutrients:

Chemicals	Composition per litre
$CoCl_2$	0.22 g
$FeCl_3$	9.70 g
$CaCl_2$	7.80 g
$NiCl_3$	0.12 g
$CrCl_6.H_2O$	0.11 g
$CuSO_4.5H_2O$	0.16 g

Table: 2.4The trace element solution was prepared by dissolving the chemicals in 0.1 N HCl.

2.4.3 Medium chain length (MCL) PHA production media

MCL-PHA production was done by growing the *Pseudomonas mendocina* in a defined MCL-PHA production media. This media contained sucrose at concentration of 20 g/L or fatty acid (sodium octonoate) at 20 mM as carbon feed. The composition of media used is listed below (Tian *et al.,* 2000).

Chemicals	Composition per liter
$(NH_4)_2SO_4$	0.50 g
$MgSO_4$	0.40 g
Na_2HPO_4	3.80 g
KH_2PO_4	2.65 g
Trace element solution	1 mL/L

Table: 2.5 Chemical composition of the MSM media

2.4.3.1 Trace element solution

Trace element solution of the MSM media had the same concentration as explained in Table 2.4.

The above mentioned PHA production media was adjusted to a final pH of seven using 1 M NaOH and 1 M HCl. The carbon sources (sodium octonoate) and magnesium sulphate were sterilised separately at 121^{o}C for 15 minutes. Except for sucrose, which was sterilized at 110^{o}C for 10 minutes. The remaining chemical components of the media were sterilized together at 121^{o}C for 15 minutes. The trace element solution was filter sterilised. The different components of the medium were then mixed together aseptically before the inoculation.

2.5 Production of PHAs

2.5.1 PHA production at shaken flask level

Batch PHA production using *B. cereus* SPV was carried out at shaken flask level using one stage seed culture preparation. Sterile nutrient broth, 30 mL in a 250 mL conical flask was inoculated using a single colony of *B. cereus* SPV. The organism was then grown in an orbital shaker (Stuart Scientific Orbital Shaker, S150) at 30^{o}C, at a speed of 200 rev min^{-1}. The growth of the organism was monitored by taking OD readings at 450 nm and when the seed culture reached a final OD of 3.0, it was then used to inoculate the final PHA production medium and grown for different time periods, at 30^{o}C and 200 rev min^{-1}samples were taken out for periodic analysis of pH, dry cell weight and PHA production. Throughout the study, while inoculating the production medium, (Kannan and Rehacek, 1970)the inoculum volume used was 10% of the final working volume of the production medium.

Similarly, batch PHA production using *Pseudomonas mendocina* was carried out at shaken flask level. Unlike the one stage culture of *Bacillus cereus,* two stage seed culture preparation were carried out. The first seed culture was prepared by inoculating 30 mL of sterile nutrient broth in a 250 mL conical flask, using a single colony of the *Pseudomonas mendocina* and was grown for 24 hrs in an orbital shaker at 30^{o}C and at a speed of 200 rev min^{-1}. This inoculum was used for inoculating sterile PHA production medium, 300 mL in a 1L flask to prepare the second stage seed culture and the organism was again grown under the same culture conditions of 30^{o}C and 200 rev min^{-1}. The growth of the organisms was monitored by measuring optical density (OD) readings at 450 nm. For OD values above 0.8, a tenfold diluted culture was used. The seed culture at an OD of 3.0 was used throughout the study in order to inoculate PHA production medium Tian *et al.,* 2000)and the inoculum volume was 10% of the final working volume of the final production medium. The organism was grown for different time periods, at 30^{o}C and 200 rev min^{-1}, and the samples were withdrawn for periodic analysis of pH, dry cell weight and PHA production.

2.5.2 Batch cultivation studies on PHA production

Batch cultivation of *B. cereus* SPV and *Pseudomonas mendocina* were carried out in a 2L fermentor in which pH of the media was adjusted to 7.0 and dissolved oxygen (DO) was initially set at 1 vvm and agitation speed was set to 250 rpm per minute. One stage seed culture preparation was done for *Bacillus cereus* SPV and in the case of *Pseudomonas mendocina*a two stage seed culture preparation were carried out when sodium octonate was used as carbon source. When *Pseudomonas mendocina* was grown on sucrose as a sole carbon source, only one stage inoculums preparation was done. In all fermentations inoculum volume was 10% of the final working volume of the final production medium. PHA production media grown at 30^{0}C for different time periods and samples were taken out for periodic analysis of pH, dry cell weight and PHA production.

2.5.3 Fed-Batch cultivation studies on PHA production by *Pseudomonas mendocina*

Additional supply of nutrients to the organism in the production medium was investigated in order to further improve dry cell weight and PHA accumulation by *Pseudomonas mendocina*. Fed bath cultivation of this organism was done in shaken flask experiments in which the pH of the media was initially adjusted to 7.0. In this fermentation the polymer was initially grown on glucose, subsequently sodium octaonoate was added. When the pH rose above 7.1, the feeding solution (15 mM sodium octonate) was then added intermittently to the flasks up to 48 hours. One stage seed culture preparation was done and the fermentation inoculum volume was 10% of the final working volume of the final production medium. The seed culture at an OD of 3.3 was used to inoculate PHA production medium and grown for different time periods at 30^{o}C and 200 rev min^{-1}.Samples were taken out for periodic analysis of pH, dry cell weight and PHA production.

2.6 Extraction of PHAs

The biomass was recovered by centrifuging the cultures at 4600 g (Sorval, centrifuge) for 30 minutes and then lyophilised. The polymer was extracted from this dried or wet bacterial biomass using various methods as described below.

2.6.1 Extraction using dispersion method

In this method of extraction, two different hypochlorite concentrations, hypochlorite to chloroform ratios and incubation times were used. In the first method the dried bacterial biomass of *Bacillus cereus* SPV was incubated (orbital shaker from Stuart Scientific Orbital Shaker, S150) in dispersion with 30% NaOCl and $CHCl_3$in a 1:1 ratio at 30^{o}C for two and half hours and 200 rev min-1.

In the second extraction method the dried bacterial biomass of *Pseudomonas mendocina* was incubated in a dispersion containing 80 % NaOCl and HCl_3in a 1:4 ratio at 30° C for one hours and 200 rev min^{-1}. It was then centrifuged at 4600 g for 15 minutes. There were three layers formed after centrifugation. The bottom layer was the $CHCl_3$containing the dissolved polymer, the middle layer contained the cell debris and topmost layer was that of hypochlorite. Polymer was then precipitated by introducing this $CHCl_3$layer into 10 volumes of ice cold methanol (Rai *et al* 2011)

2.6.2 Soxhlet extraction

The freeze dried biomass was incubated with 80% NaOCl at 37°C for half an hour. The lysed cells were then centrifuged at 4600 g, for 5 minutes. Then the cell residue was washed twice with 30 mL each of distilled water, acetone, and ethanol. The biomass obtained was freeze dried and then refluxed using $CHCl_3$for 24 hrs. The polymer was finally precipitated using ten volumes of chilled methanol (Rai *et al* 2011)

2.6.3 Chlorofom extraction

The dried cell biomass mixed with chloroform. This was then incubated at 37°C for 24 hours. Polymer was then precipitated from chloroform using 10 volumes of ice cold methanol (Vallapil et al 2007).

2.6.4 NaCl-Triton X-114 based recovery of PHAs

Different concentrations of NaCl (5-25%) and Triton X-114 (1.5%) was dissolved in deionised water (200mL) and pH was adjusted to alkaline (pH-13.5) using 5 M NaOH. The wet biomass containing PHA was added to this cold solution and stirred for 15 mins. The solution was heated up to 37°C, then treated with boiling n-hexane and the solution was again stirred for 30 minutes, and centrifuged for 10 minutes at 4600g.

Four layers were formed after centrifugation. The topmost layer was that of, n-hexane containing dissolved polymer, a second consists of cellular protein with Triton X-114, followed by NaCl solution layer, and the bottom layer, contained the cell debris pellet. Then the n-hexane layer was then taken out and concentrated using rotary evaporator and polymer was precipitated by adding four times volume of ethanol and methanol mixture (1:1 ratio).

2.7 PHA purification

The polymer was purified by repeated precipitation process. At first the polymer was dissolved in chloroform then precipitated using methanol and ethanol (1:1 ratio). The precipitated polymer was then repeatedly dissolved in acetone and again precipitated using the same methanol to ethanol mixture (Rai *et al* 2011).

2.7.1 Triton based polymer purification

The polymer was purified using Triton based purification method. The polymer was dissolved in hot n- hexane (60^0C). Then 1% Triton was added to polymer dissolved in the solvent. This solution was kept in the shaker for 15 minutes. Then the solution was centrifuged to remove the Triton layer, followed by precipitation using a mixture of ethanol and methanol (1:1).

2.8 Biomass estimation

Estimation of the biomass was done either by measuring the cell dry weights of the freeze dried cells, or by taking absorbance readings at 450nm using a Novespec II spectrophotometer.

2.9 Nuclear Magnetic Resonance Spectroscopy

Structural elucidation of the PHA monomers, accumulated by *Pseudomonas mendocina* and *Bacillus cereus SPV* were done using ^{13}C and ^{1}H NMR. For this 20 mg of purified polymer was dissolved in 1 mL of the deuterated chloroform ($CDCl_3$) and analysed on a Bruker AV400 (400 MHz) spectrometer. Spectra were analysed using the Mest Rec software package. The specturm was measured at the Department of Chemistry, University College London, UK.

2.10 Mechanical properties of neat and blend films

2.10.1 Dynamic mechanical analysis

Tensile testing was carried out using a Perkin Elmer dynamic mechanical analyzer at room temperature. The test was carried out on polymer strips of 10 mm length and 4 mm width cut from solvent casted polymer films. Stress and strain was recorded during the test. The test was carried out on 3 repeats of the samples.

2.10.2 Contact angle study

Water contact angle measurements were carried out to evaluate the hydrophilicity of the fabricated films. The experiment was carried out on a KSV Cam 200 optical contact angle meter (KSV Instruments Ltd., Finland). A gas tight micro-syringe was used to place an equal volume of water (<10 μl) on every sample by means of forming a drop. Photos (frame interval of 1 second, number of frames = 100) were taken to record the shape of the drops. The water contact angles on the specimens were measured by analysing the record drop images (n=3) using the Windows based KSV-Cam software. An image was recorded by the KSV-Cam software every second for 30s in order to monitor the contact angle as a function of time. The analysis was carried out at the Department of Biomaterials and Tissue engineering, Eastman Dental Institute, University College London, UK.

2.10.3 Surface study of the polymer

2.10.3.1 Scanning electron microscopy

Scanning electron microscope was used to study the micro structural studies of the neat and blend polymer films. The samples were placed on 8 mm diameter aluminium stubs and then coated with gold using the gold spluttering device (EMITECH-K550). The SEM images were taken with an acceleration voltage of 15 kV. The samples were analysed using a JOEL 5610LV scanning electron microscope at the Department of Biomaterials and Tissue engineering, Eastman Dental Institute, University College London, UK.

2.10.3.2 White light interferometry study

White light interferometry was used to quantify the roughness and topography of the surfaces. 2D and 3D plots of the surface topography was obtained by analyzer ZYGO (New View 200 OMP 0407C) at Imperial College London, UK. Three analyses per sample were performed to get an average rms (root mean square average) measurement.

2.11 Thermal properties

The thermal properties of the polymer (glass transition temperature (T_g) and melting temperature (T_m) were measured by differential scanning calorimetry, (DSC) using a Perkin Elmer Pyris Diamond DSC (Perkin Elmer Instrument). The amount of the polymer used for the study ranged from 5-7 mg. The samples were placed in standard aluminium pans and all tests were performed under nitrogen atmosphere. The samples were heated/cooled/heated at a heating rate of 20^{o}C min^{-1}between -50^{o}C and 250^{o}C. The analysis was carried out in triplicates at the Department of Biomaterials and Tissue engineering, Eastman Dental Institute, University College London, UK.

2.12 Fabrication of P(3HB) and P(3HO)neat films

The homopolymer P(3HO) and P(3HB) were solvent casted to form circular film discs of about 20 mm diameter. To make the film 0.5 grams of the polymer was well dissolved in chloroform after which the polymer solution was filtered and the films made, by casting the polymer solution into glass petridishes. The solution was then left to air dry at room temperature for 1 week followed by freeze drying for 10 days.

2.13 Preparation of blends of P(3HB) and P(3HO)

Preparation of P(3HB)/P(3HO) and P(3HO)P(3HB) blends were prepared in 5:1 ratio and films casted using the solvent casting method. This involved, blending of both P(3HB) and P(3HO) polymer in 10 mL of chloroform then heated up to boiling, the solution was then sonicated for three minutes and this process was repeated for 3-4 times. Then this blend solution was poured in to a clean glass petrti dish, and closed until the film dried.

2.14 Cell culture study

The *in vitro* cell culture studies were carried out on the P(3HO), P(3HB) neat films and P(3HB)/P(3HO) and P(3HO)/P(3HB)5:1 blends using the keratinocyte cell line (HaCaT). The cells were cultured in DMEM supplemented with 10% foetal calf serum, 1% penicillin and 1% streptomycin solution and the media warmed at 37^{o}C for about 15 minutes before use. The cells were incubated at 37^{o}C in a humidified atmosphere (5% CO_2in 95% air). The culture medium was changed every 2 days.

2.14.1 Cell seeding on polymer discs

The neat and composite polymer film samples were cut in to small discs (1 cm^2 size). These discs were UV sterilised for 30 minutes on each side for 12 hrs prior to seeding the cells. The cells were trypsinised to get detached and new medium was added to the cell suspension and pelleted by centrifugation at1500 rev $min^{-1}$for 10 minutes. The pellet was then seeded on polymer discs in well. A cell number of 20,000 cells were used to seed the polymer discs kept in 24 well plates. The samples were held onto the surface of the wells using the circular crown TM disk obtained from Scaffdex, Finland, which are placed on top of the polymer disc. The cell seeded films were again incubated at 37^oC in a humidified atmosphere with 5%CO_2in 95% air. The medium was changed every 2 days and the cells were analysed after 3, 5 and 7 days for cell adhesion and proliferation. Three cell culture assays were performed to get an average reading.

2.14.2 Cell adhesion and proliferation studies

Cell adhesion and proliferation studies on neat and blend films were carried out in triplicates using the Neutral Red assay (NR). The cells were incubated in DMEM medium containing 60 μg/mL Neutral Red for 3 hrs to allow the viable intact cells to take up the dye. After incubation, the samples were transferred to new 24 well plates, and washed twice with 2 mL of solution A (fixative: containing 1%$CaCl_2$, 0.5% formaldehyde). 300μl of solution B (1% acetic acid and 50% ethanol solution) was then added to each sample to leach out the dye. The absorbance of the dye was read at 540nm using a microtitre plate reader (Thermomax) using SoftMax Pro version 4.8. Both a positive control (cells + medium in the tissue culture plates) and a negative control (medium + samples, no cells) were done alongside each experiment. Background absorbance of the negative control was deducted from that of the test samples. The total NR uptake was a measure of the cell's viability and proliferation as the percentage of NR uptake is directly proportional to the number of live cells.

2.14.3 Scanning electron microscopy of HaCaT cells

The neat and composite polymer film samples with HaCaT cells were analysed under scanning electron microscopy (JEOL 5610LV, JEOL, USA) in order to observe the HaCaT cell attachment on the surface of the samples. The specimens were fixed using a fixing solution consists of 0.1 M cacodylate buffer and 3% glutaraldehyde for 12 hrs at 4^{o}C. Subsequent dehydration process was done using a series of graded ethyl alcohols 50%, 70%, 90% twice and 100%. The samples were then left to air dry for half an hour in the fume cupboard for subsequent drying. The dried samples were then attached to aluminium stubs, gold coated and examined under SEM at a voltage of 10-15 kV.

Chapter 3: Production & Characterisation of PHAs

Medium chain length (MCL) PHAs consist of 6-14 carbon atom containing monomers. They are synthesised and accumulated in a wide variety of Gram-negative bacteria, mainly *Pseudomonas* and *Aeromonas*. *Pseudomonas oleovorans* and *Pseudomonas putida* are able to grow on a wide variety of different substrates ranging from *n*-alkanoic acids, *n*-alkanals, *n*-alkanols, and *n*-alkanes in order to produce MCL-PHAs. They also grow on carbohydrates and other structurally unrelated carbon sources like glucose, sucrose and fructose to accumulate the polymer. MCL PHAs produced by these bacteria normally contains 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) and 3-hydroxydodecanoate (3HDD). MCL-PHAs are elastomers with a low degree of crystallinity and a low melting temperature due to an increase in the length of the side chain in the structure (Yao *et al* (1999).

Pseudomonas mendocina is a Gram negative organism first isolated by N J Paleroni from soil and water samples collected from Argentina (Palleroni *et al.,* 1970). Like other *Pseudomonas sp,* it is also able to accumulate MCL-PHAs under nutrient limiting conditions. Nevertheless, not many studies have been carried out on this organism, and it remains relatively unexplored for PHA production. This chapter includes the works that was carried out with an objective of biosynthesising MCL-PHA using *Pseudomonas mendocina*. The experiments were conducted to improve the productivity of the polymer in batch and fed batch fermentations.

Small chain length polyhydroxyalkanotes (SCL-PHAs) are successfully produced by many Gram negative bacteria like *Alcaligenes latus, Azotobacter vinelandii* and *Cupriavidus necator*. The lipopolysacaride (LPS) found in the cell wall of Gram negative bacteria can co-purify with P(3HB). The presence of LPS in the polymer can induce strong immunogenic reactions. So, studies have been

carried out to produce PHAs from Gram positive organisms like *Bacillus sp*. For example, Valappil *et al* achieved P(3HB) production in Kannan and Rehacek medium using *Bacillus cereus* SPV, fed with glucose as carbon source. This chapter describes the production of the SCL-PHA using *Bacillus cereus* SPV and sucrose as carbon source. Experiments were also conducted to improve the productivity of the polymer using a novel media (Valappil*et al.,*2006).

The yield, molecular weight, process ability and lippopolysacharide contamination of the extracted polymer depends on the combined effect of fermentation conditions, and downstream processing like biomass pre-treatment and extraction methodology used. Therefore, different extraction methods such as extraction using a dispersion of $CHCl_3$and NaOCl (Vallapil *et al.,* 2007), soxhlet extraction (Ramsay *et al.,* 1994) and extraction using chloroform (Hahn *et al*., 1995) have been studied for the extraction of the polymer from bacterial biomass. In this study extraction of the polymers were initially carried out using dispersion and soxhlet extraction methods. A new PHA recovery method based on the osmotic and detergent based lysis and purification was also developed for MCL-PHA purification in this study. This chapter also reports the structural, thermal and mechanical characterisation of the neat SCL and MCL PHAs.

3.2 Results

3.2.1 Production of MCL PHA using *P.mendocina* in shaken flask cultures.

*P.mendocina*was studied for the production of MCL-PHAs using shaken flask cultures. The organism was grown using octanoate as the sole carbon source and the results obtained are shown in Figure 3.1. The dry cell weight achieved was between 0.14-2.3 g/L.The dcw increased steadily up to 48 hrs at which

highest dry cell weight of 2.3 g/L was reached after which it decreased to about 1.69 g dcw/L at 60 hrs. PHA yield was analysed at 12 hours intervals. The organism had already started to accumulate PHAs by 24 hrs however, the highest accumulation of polymer, which was 29.43% dcw, was observed at 48 hrs. The PHA yield decreased to about 23.80% dcw at 60 hrs. The pH of the culture broth increased as the fermentation progressed and by 54 hrs the pH had reached 7.7 after which it began to drop at 60 hrs.

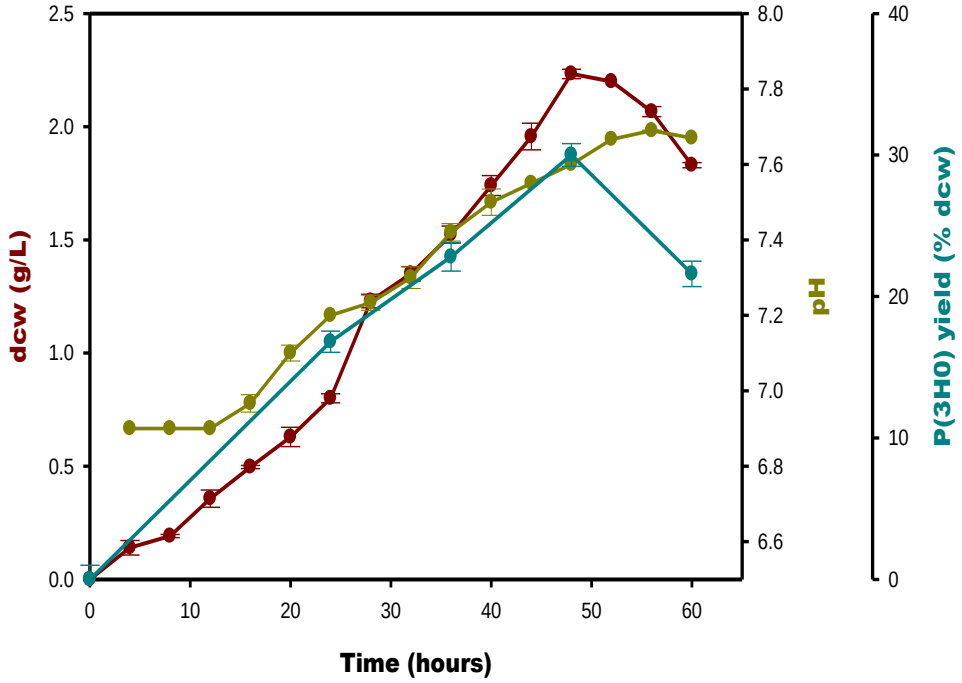


Figure 3.1: Fermentation profile for PHA production by *P. mendocina* using octanoate as the carbon source.

3.2.2 Production of MCL-PHA using *P.mendocina* in 2L batch fermentations

Additional investigations on the effect of the nitrogen source and carbon concentrations were performed in 2L fermentor in which pH and airflow rate was initially set at 7.0 and 1 vvm respectively. The agitation speed was kept constant at 250 rpm through out the fermentation. The results that were obtained are shown in Figure 3.2. The dry cell weights observed for the organism ranged between 0.68 to 2.4 g dcw/L. At 48 hrs the highest dcw of 2.4 g

dcw/L was achieved after which a decrease was observed and at 60 hrs, the dcw had decreased to about 1.99 g dcw/L. The polymer accumulated by the organism ranged between 22.5 to 32.7% of the dry cell weight. The PHA yield was analysed at 12 hours intervals and the polymer was accumulated to a maximum value of 33.0% dcw at 48 hrs. At the beginning of the fermentation the pH was set at 7 however, the pH increased as the fermentation progressed and reached up to 7.74, after which it began to drop at 60 hrs.

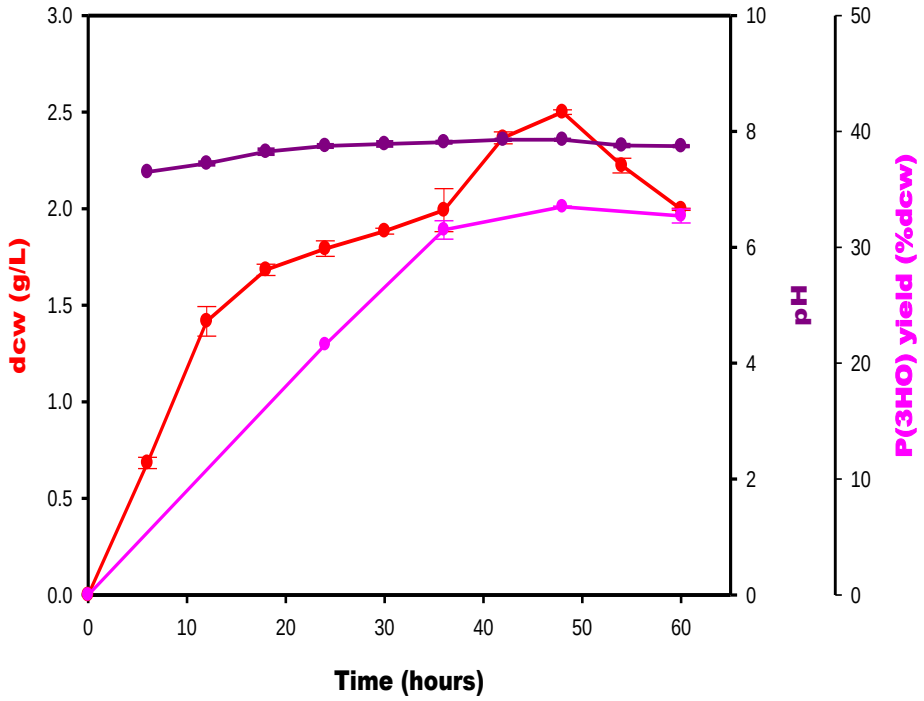


Figure 3.2: Fermentation profile for PHA production by *P. mendocina* using sodium octanoate as the carbon source.

3.2.3 Production of MCL-PHAs using *P.mendocina* in fed batch cultures.

In order to find optimum conditions for cell growth and MCL-PHA accumulation by *P. mendocina*, the influence of various nutrients and their possible combinations in the production medium were investigated. The structurally

unrelated carbon source like glucose was provided in the lag phase of the growth, which resulted in sufficient growth of the organism (Figure 3.3). This was followed by intermittent addition of ocatanoate every four hours as the major carbon source for polymer accumulation. The intermittent addition of octonate led to an increased polymer accumulation in the organism. The highest cell mass of 2.8 g L^{-1} was achieved in this fermentation, which simultaneously resulted in much higher PHA accumulation of 37.09 wt% dcw at 48 hours. The organism entered stationary phase after 36 hrs of fermentation and this phase lasted until 54 hrs of the fermentation. The dry cell weight of the organism ranged between 0.245 to 1.79g dcw/L with maximum accumulation of 2.8g/L. The pH of the medium was set at 7 as the fermentation progressed, reached up to 7.67 by 60 hrs.

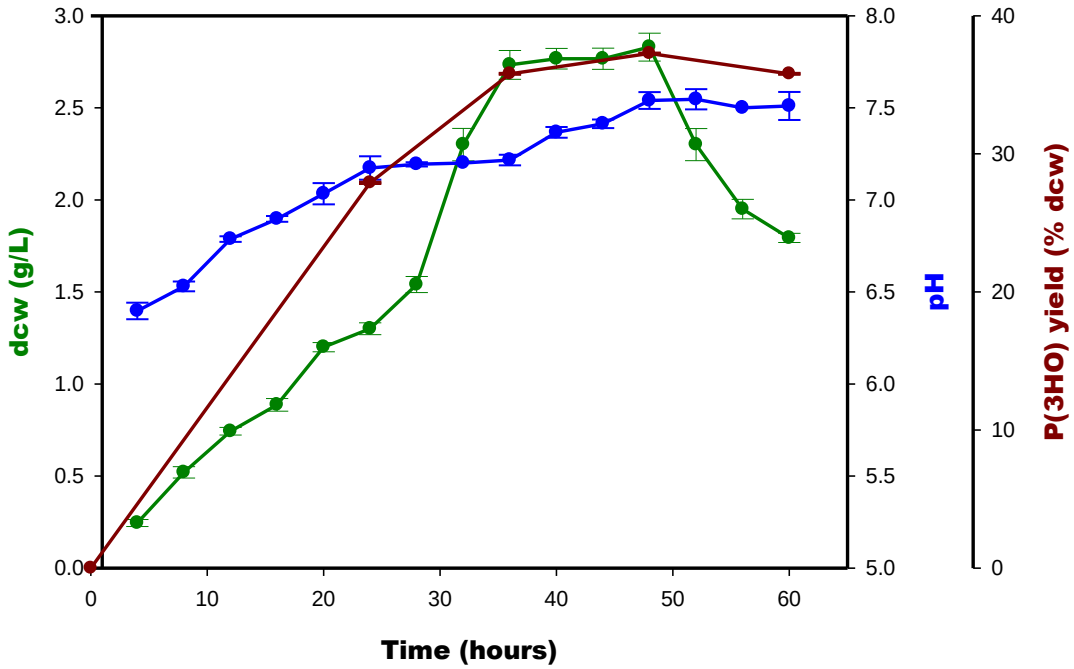


Figure 3.3: A fed bath fermentation profile for PHA production by *P. mendocina* using glucose and octonoate as the carbon source.

3.2.4 Production of PHA from *P.mendocina* by shaken flask cultures using sucrose as carbon source

P.mendocina was next grown in sucrose as the carbon source and results obtained are shown in Figure 3.4.The dry cell weights achieved were between 0.84-1.81 dcw/L. The dcw increased steadily up to 48 hrs at which highest dry cell weight of 1.81 g/L was reached after which it decreased to 1.74 g dcw/L at 60 hrs. PHA yield was analysed at 12 hours intervals. The organism had already started to accumulate PHA by 24 hrs however, the highest accumulation of polymer, which was 27.19% dcw, was observed at 48 hrs and the PHA yield decreased to about 18.180% dcw at 60 hrs. The pH of the culture broth decreased as the fermentation progressed and by 60 hrs and the pH had reached to a value of 5.66.

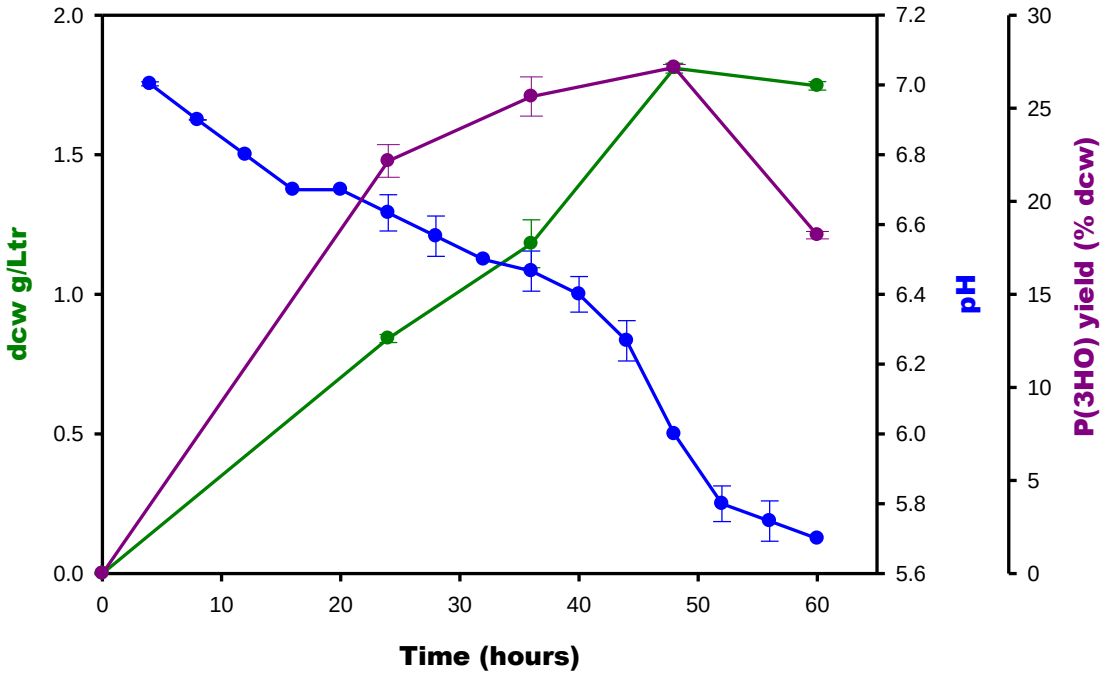


Figure 3.4: Fermentation profile for PHA production from *P. mendocina* using sucrose as the carbon source

3.2.5 Production of SCL-PHA using *Bacillus cereus* SPV

Bacillus cereus SPV was used for the production of SCL PHA in shaken flask experiments. The organism was grown on sucrose as sole carbon source and the results obtained are shown in Figure 3.5. The dry cell weight of the organism ranged between 0.81 to 3.06 g dcw/L. The highest dry cell weight (3.06 g dcw/L) was observed at 48 hrs and at 60 hrs the dcw had decreased to about 2.7g dcw/L. The PHA produced by the organism ranged between 35.6 to 37.80% dcw. PHA yield was analysed at 12 hours intervals. The polymer yield increased up to 48 hrs with the highest PHA accumulation of 41.50% of dcw after which the yield decreased to 37% of dcw by 60 hrs. As the fermentation progressed, the pH of the culture medium, which was initially set to 7, had decreased to a minimum value of 5.1 by 60 hrs.

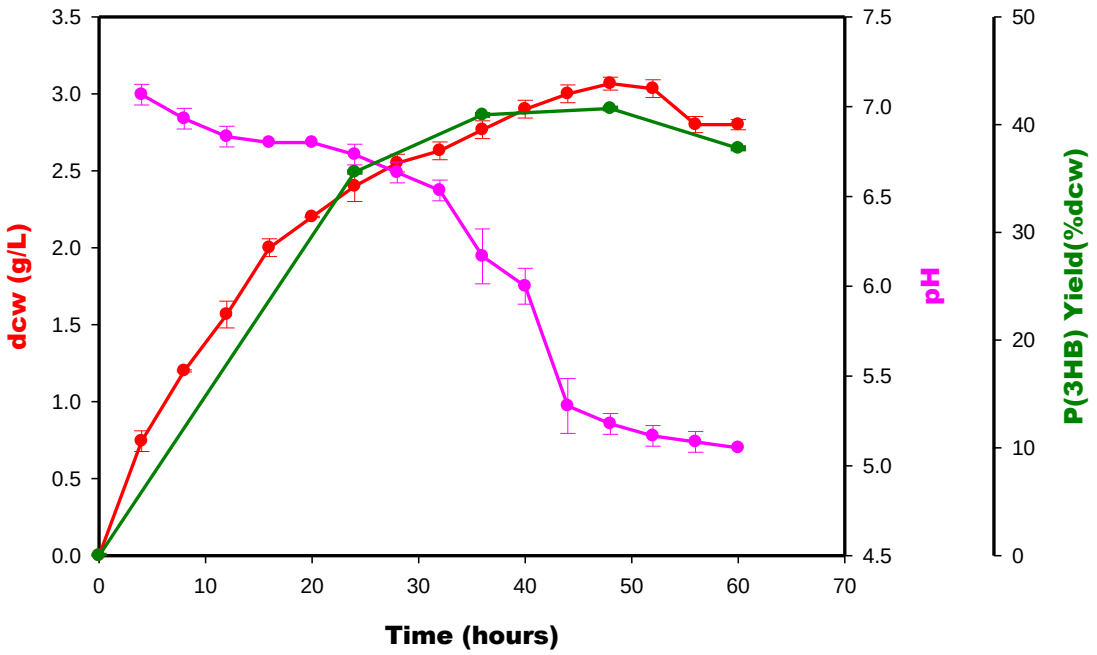


Figure 3.5: Shaken flask studies on PHA production using Kannan and Rehaek medium.

3.2.6 Production of SCL PHA using *Bacillus cereus* in batch cultures

Bacillus cereus was studied extensively for the production of SCL-PHAs in a two litre fermentor, in which pH and airflow rate was initially set at 7.0 and 1 vvm respectively. The agitation speed was kept constant at 250 rpm during the fermentation. The results that were obtained are shown in Figure 3.6. After 36 hrs the organism had entered the stationary phase of growth. The dry cell weight observed for the organism ranged between 0.65 to 3.12 g dcw/L. At 48 hrs the highest dcw of 3.12 g dcw/L was achieved after which a decrease was observed. In fact, by 60 hrs, the dcw had decreased to about 2.81 g dcw/L. PHA yield was analysed at 12 hours intervals and the polymer accumulated to a maximum yield of 44.60% dcw at 48 hrs, after which the yield decreased to 43% dcw by 60hrs. At the beginning of the fermentation the pH was set at 7 however, the pH decreased as the fermentation progressed and reached 4.2 at the end of the fermentation.

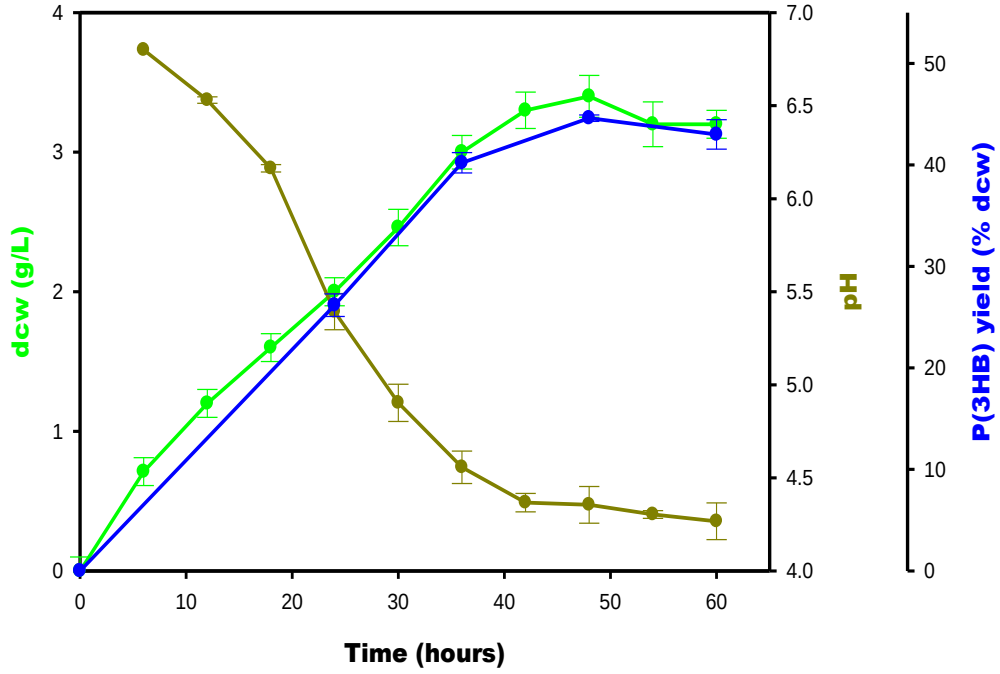


Figure 3.6:Batch fermentation studies on PHA production using Kannan and Rehaek media

3.2.7 Production of PHAs from *Bacillus cereus* using a Modified Enrichment Media (MEM).

Shaken flasks studies were done to analyse the effect of multiple nutrient limitations on the production of PHA using a modified enrichment media (MEM). Figure 3.7 shows a typical fermentation profile for the organism, when grown in the nutrient limited conditions. The dry cell weights of the organism ranged between 0.72 to 4.30 g/L. At 48 hrs the highest dcw of 4.30 g dcw/L was achieved after which a decrease was observed and by 60 hrs, the dcw had decreased to about 3.60 g dcw/L. PHA yield was analysed at 12 hours intervals. The polymer yield increased up to 48 hrs with the highest PHA accumulation of 52.64% dcw after which the yield was decreased to 48.40% dcw by the 60hrs. As the fermentation progressed, the pH of the culture medium, which was initially set to 7, had decreased to a minimum value of 5.9 by 60 hrs.

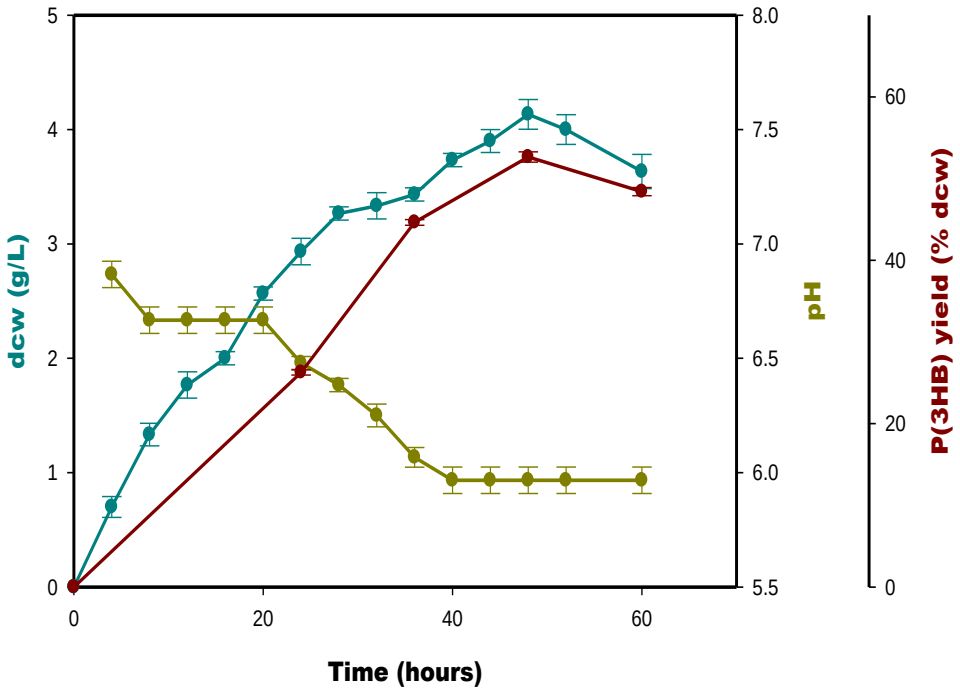
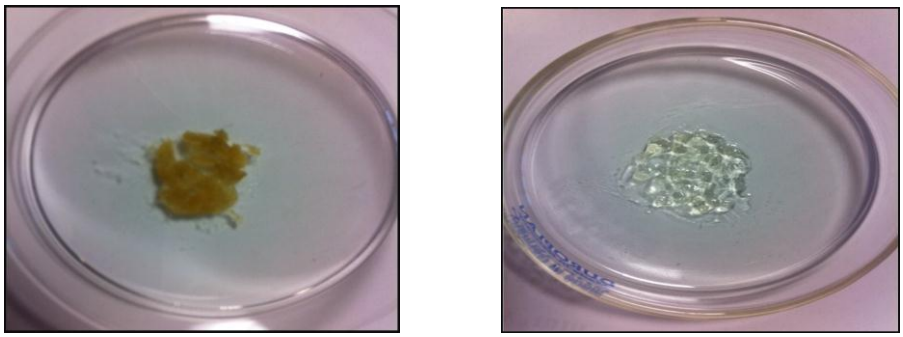


Figure 3.7: Shaken flask fermentation studies on PHA production using a defined media

4.1 Downstream processing

Extraction of the polymer using the dispersion of sodium hypochlorite and chloroform (method described in section 2.6-1 and 2.63) gave the highest yield of polymer, which was 29.43% dcw. The yield obtained from other extraction methods was found to be, 20.38% dcw using chloroform, and soxhlet extraction, 12.64% dcw.A new recovery method based on the osmotic and detergent based lysis was also developed in this study. This process involved the use of NaCl-Triton X114 aqueous solution to achieve cell lysis and polymer extraction. The key factors that influenced the recovery and purity of the polymer by this method were the concentrations of NaCl and Triton X114. In this method lysis of the cells were achieved by different concentration of NaCl. Among different concentrations, (Table- 3.8.1) 20% NaCl gave cell lysis within fifteen minutes of incubation. In this extraction method the pH, temperature, and concentration of Triton X114 was kept constant at pH 12, 37^0C and 1.5% respectively. Extraction of the polymer using this method resulted in a highest polymer yield of 28% dcw. The purity of this polymer was confirmed by GC analysis. Equal amount of P(3HO) extracted using the dispersion method and osmotic and Triton X114 based method subjected to methalolysis reaction. The area under the peak observed was measured. The polymer extracted by the Triton based method found to 25% more pure compared to the dispersion method Figure (3.7.).



A **B**

Figure 3.7: Extraction of P(3HO) from *P .medocina* A) Dispersion method B) Osmotic and Triton X114 based method.

NaCl (%)	PHA – Yield (% dcw)
5	18
10	20
15	28
20	30
25	30

Table 3.8.1:Differnet NaCl concentration used for cell lysis

5.1 Thermal analysis

PHAs are partially crystalline polymers and therefore their thermal and mechanical properties are usually expressed in terms of the glass transition temperature and the melting temperature. These parameters were measured using differential scanning calorimetry. The thermal analysis of this polymer showed that the polymer sample has a low glass transition temperature, T_g of 2.43°C, high melting temperature, T_m of 167.39°C and showed a T_c value of 54.33°C (Figure 3:8). All these results showed that the extracted polymer has the characteristic thermal properties of the SCL-PHA family.

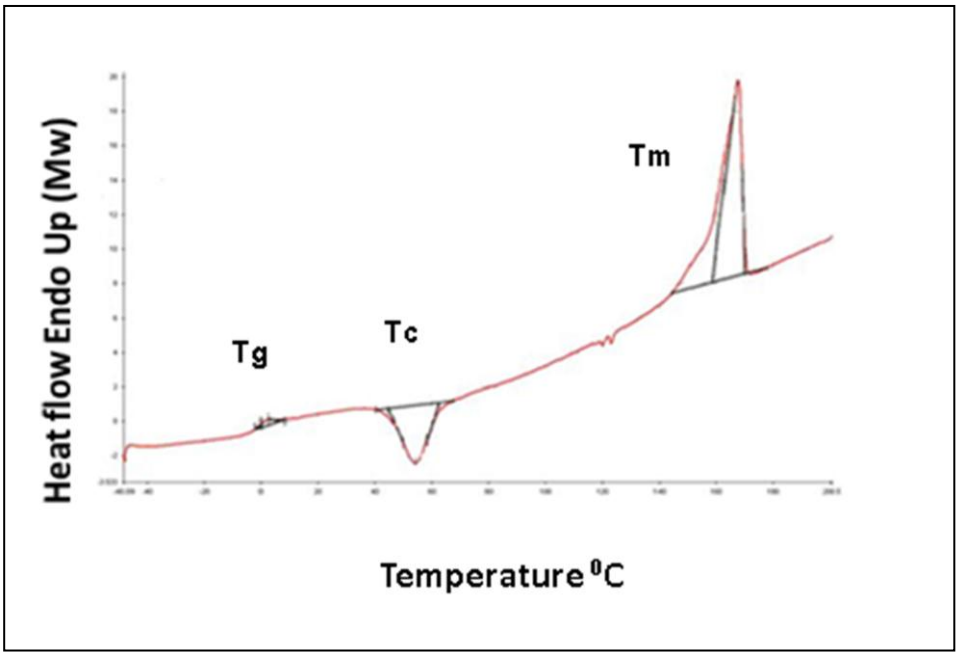


Figure 3.8: Thermal profile of the polymer extracted from *Bacillus cereus SPV* grown on sucrose.

Studies were also carried out to determine the thermal properties of the polymer, extracted from the lyophilized *P. mendocina* cells grown on sodium octanoate as carbon feed. The thermal analysis results showed that, the polymer exhibited a very low glass transition temperature, T_g of -32.86^{0}C and melting temperature T_m of 50.36°C (Figure 3.9).

Interestingly, no T_c was observed for the polymer produced from sodium octonate. All these results showed that the extracted polymer had the characteristic of the MCL-PHA family. Medium chain length (MCL) PHAs have low melting temperatures and have a much lower level of crystallinity as compared to SCL.

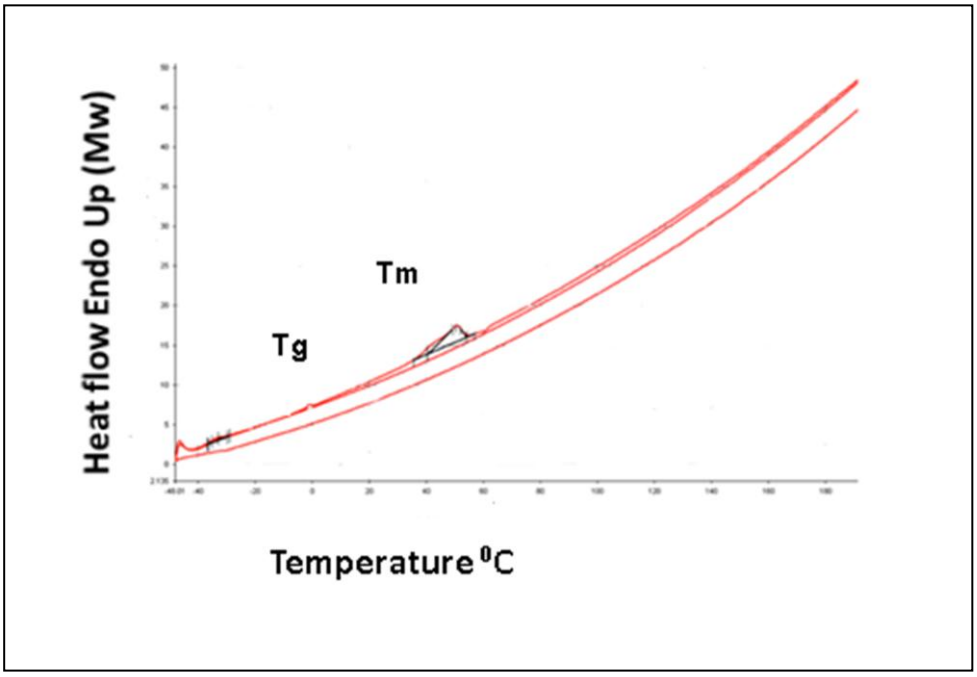
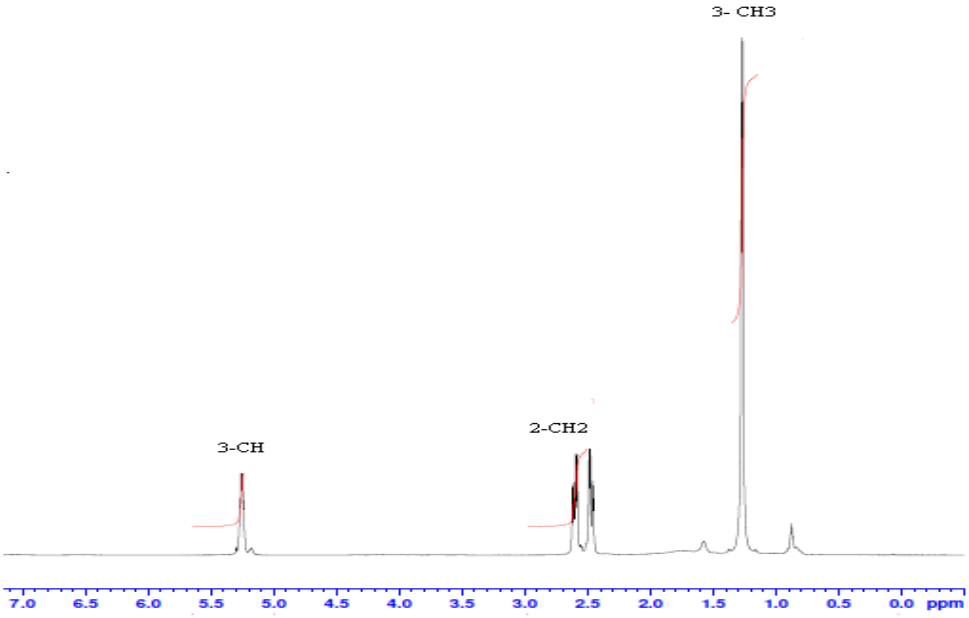


Figure 3.9: Thermal profile of the polymer extracted from *P. mendocina* cells grown in sodium octaonate.

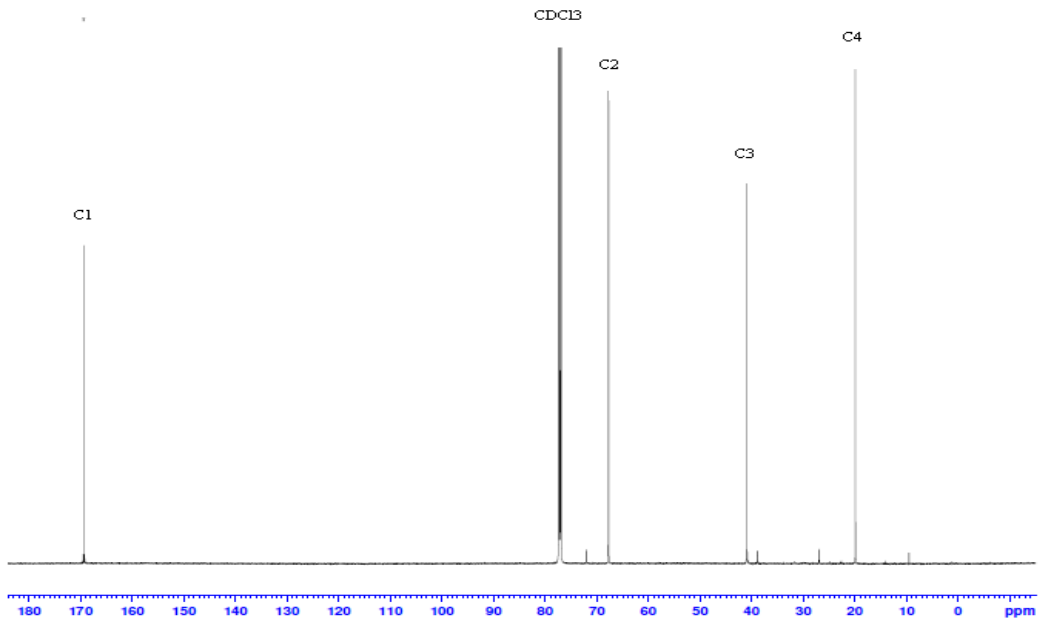
6.1 Nuclear magnetic resonance spectroscopy

Polymer produced from *Bacillus cereus* SPVwhen grown in sucrose was analysed using ^{1}H, and ^{13}C NMR.In the proton spectrum, different environments for the hydrogen in the polymer appeared with very strong

intensities. The peak 2.6 corresponded to protons bonded to C_2(-CH_2group), 5.2ppm to CH groupFigure 3.10(A). In the ^{13}C NMR eight different peaks were obtained corresponding to the four different environments for the carbon in the molecule chemical shift at 67.72 corresponded to methine CH group, 40.88-methylene CH_2, group169.54, C=O group, methyl (CH_3-19.88 ppm)Figure 3.10(B).^1H and ^{13}C NMR thus confirmed the presence of homopolymer of P(3HB) in the extracted polymer.^1H and ^{13}C NMR thus confirmed the presence of homopolymer of P(3HB) in the extracted polymer.



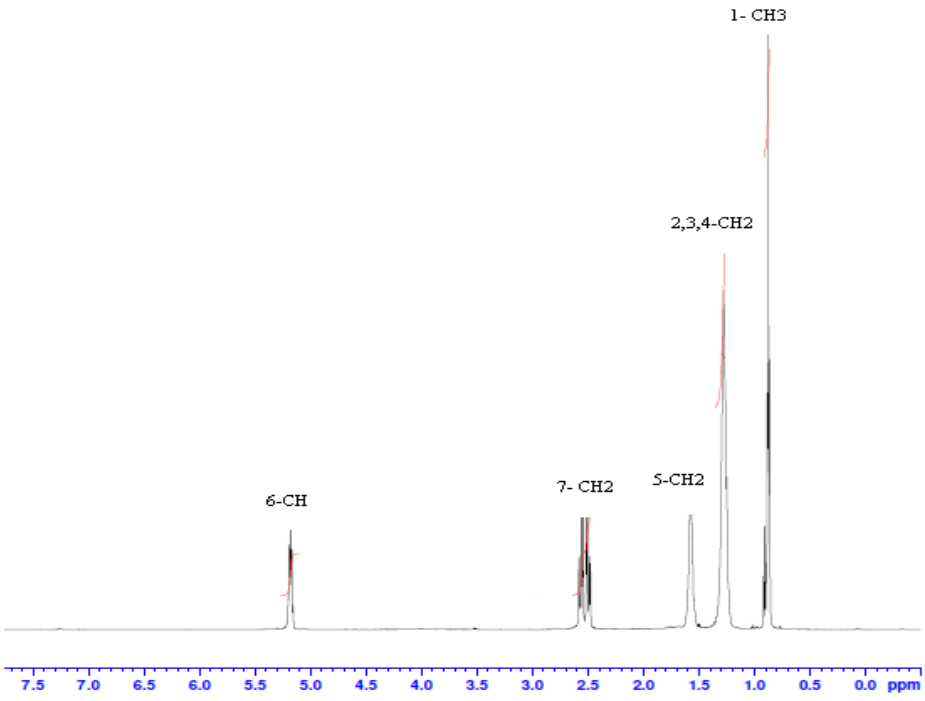
(A)



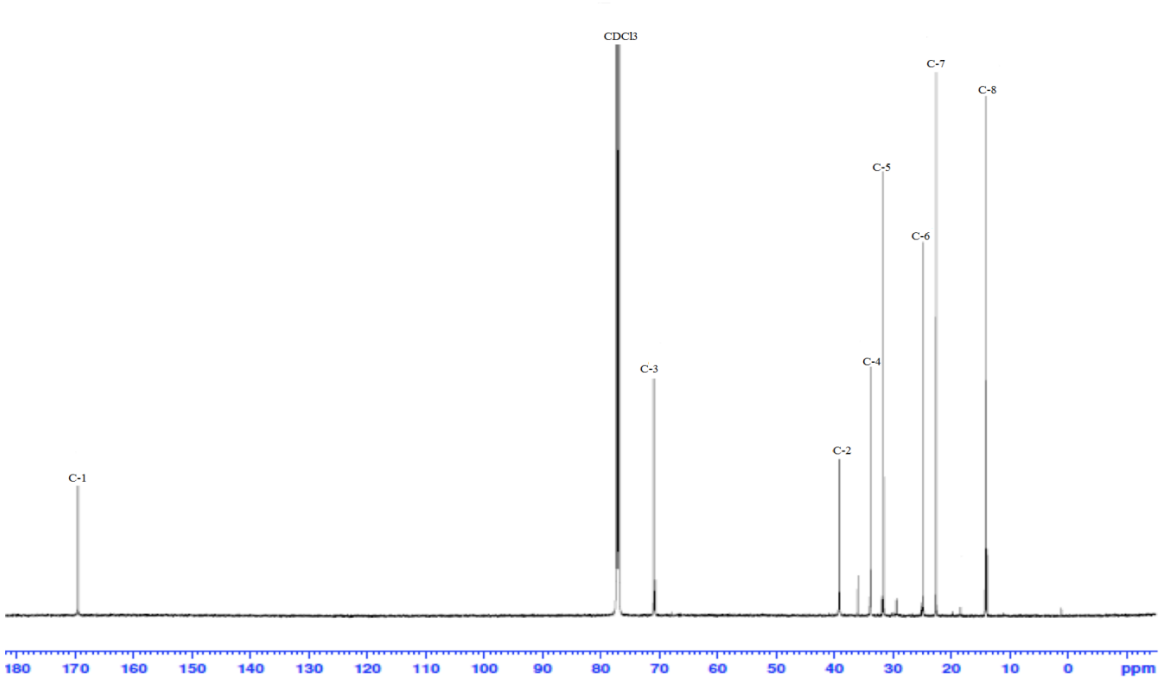
(B)

Figure 3.10: NMR spectra of the extracted homopolymer produced from *B.cereus SPV* when grown in sucrose (A) ^{1}H NMR (B) ^{13}C NMR

The polymer produced from *P. mendocina* when grown in sodium octanoate was analysed using ^{1}H, and ^{13}C NMR as shown in Figure 3.11 (A) and (B) respectively. In the proton spectrum, five different environments for the hydrogen in the polymer appeared with very strong intensities. The peak 2.5 corresponded to protons bonded to C_2(-CH_2group), 5.18 ppm to C_3(-CH group), 1.57 ppm to C_4(-CH_2group), 1.2 ppm to C_5, C_6, C_7(-CH_2group), and 0.8ppm to C_8(-CH_3group).The ^{13}C spectrum, Figure 3.11(B) shows 8 peaks, corresponding to different environments for the carbon in the extracted polymer. In the ^{13}C NMR eight different peaks were obtained corresponding to the eight different environments for the carbon in the molecule. The chemical shift at 169.38 ppm corresponded to C_1(C=O group), 70.82 ppm to C_3(-CH group), 39.08 to C_2(-CH_2group), 23 - 35 ppm to C_4, C_5, C_6, C_7(-CH_2group) and 13.95 ppm to C_8(-CH_3).


1- CH3
2,3,4-CH2
5-CH2
7- CH2
6-CH
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0 ppm

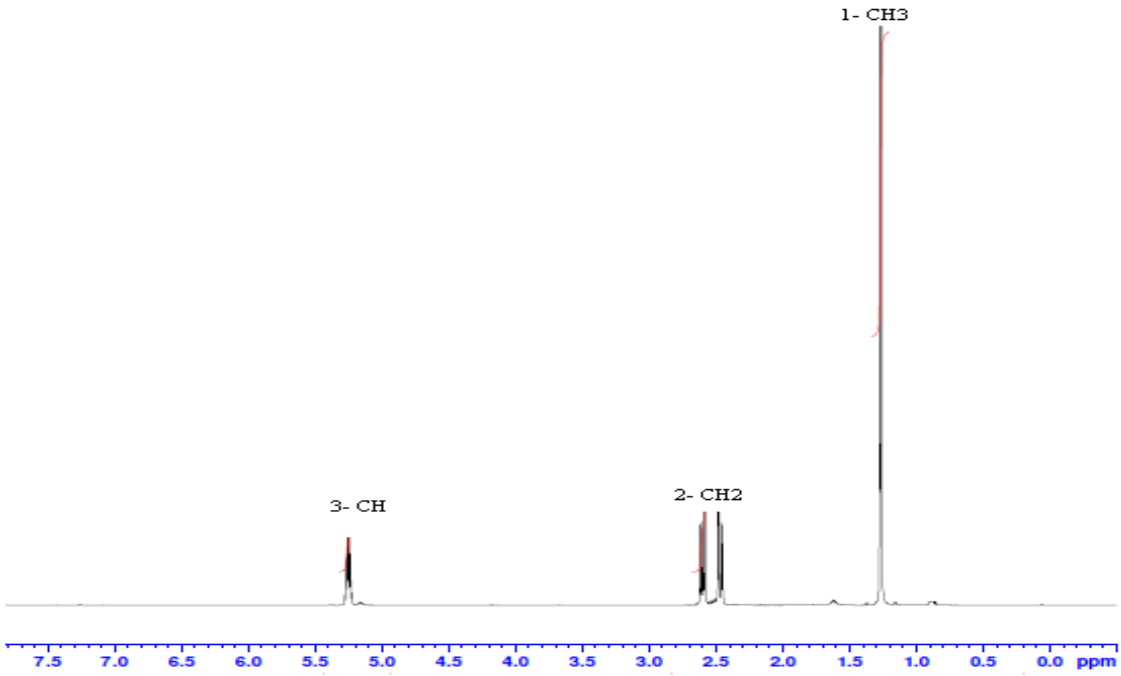
(A)



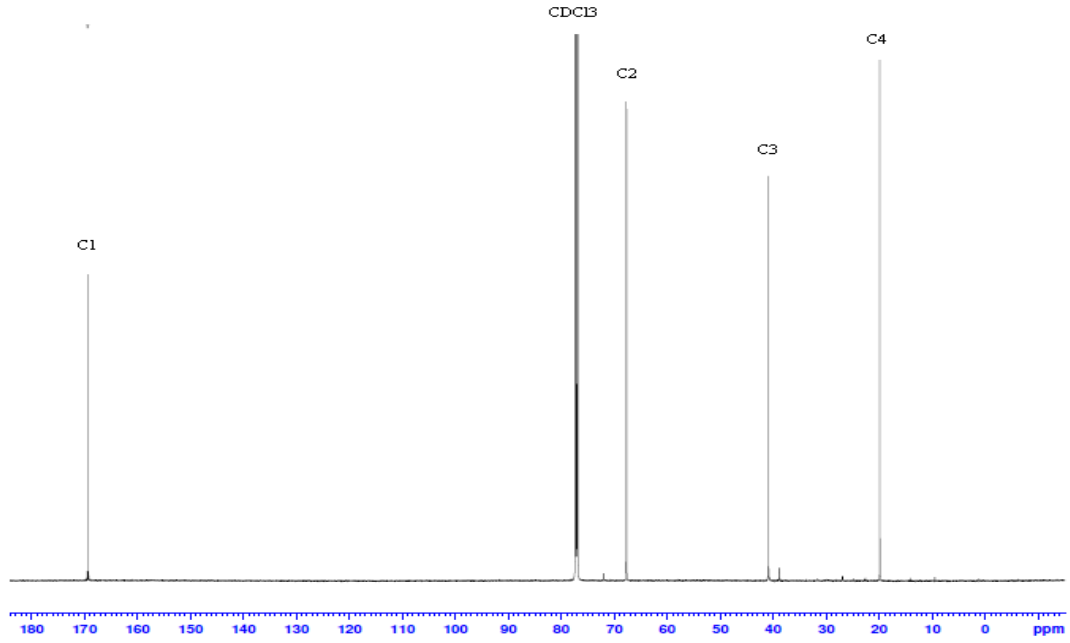
(B)

Figure 3.11: NMR spectra of the extracted homo polymer of P(3HO) produced from *P. mendocina*when grown in octanoate: (A) ^{1}H NMR, (B) ^{13}C NMR

The polymer extracted from the freeze dried cells of *P. mendocina* grown in sucrose was analysed using the ^{1}H and ^{13}C NMR. In the ^{1}H NMR four different peaks corresponded to the different environments for the hydrogen in the molecule.In the proton spectrum, different environments for the hydrogen in the polymer appeared with very strong intensities. The peak 2.6 corresponded to protons bonded to C_2(-CH_2group), 5.2ppm to CH group, 1.27ppm –CH_3(Figure 3.2(A). In the ^{13}C NMR eight different peaks were obtained corresponding to the four different environments for the carbon in the molecule. The chemical shift at 67.72 corresponded to methine CH group, 40.88- methylene CH_2, group,.169.54, C=O group, methyl (CH_3-19.88 ppm)Figure 3.2(B). ^{1}H and ^{13}C NMR thus confirmed the presence of homopolymer of P(3HB) in the extracted polymer.^{1}H and ^{13}C NMR thus confirmed the presence of homopolymer of P(3HB) in the extracted polymer.



(A)



(B)

Figure 3.12: NMR spectra of the extracted homopolymer of P(3HB) produced from *P. mendocina* when grown in sucrose (A) ^{1}H NMR, (B) ^{13}C NMR

7.1 Discussion

P. mendocina was mainly used for the production of MCL-PHAs in this study. This organism was initially grown in the production media for growth and accumulation of PHAs. The organism was grown in both structurally related (sodium octanoate) and unrelated (sucrose) carbon sources using a two stage seed culture preparation. The second stage seed culture was carried out, to facilitate improved acclimatisation of the organism in the media. This would enable better growth and adaptation by the organism in the final PHA production media. In this present study the maximum yield of PHA accumulated by *P. mendocin a*cells was 29% dcw when grown in sodium octonate as the sole carbon source. Similar observation of MCL-PHA accumulation (31.38% of dcw) has been made when *P. mendocina* was grown in octanoate within 48 hours in mineral salt media (Rai *et al.*, 2011). Media selection for the study was important because the media composition and in particular the carbon source plays an important role of supporting both their

organism's growth and the accumulation of PHAs. It has already been established *Pseudomonas sp* shows selective preference for different media to support its growth and PHA accumulation. For example, according to previous studies conducted at University of Westminster, different species of *Pseudomonas* exhibited selective preference for different mineral base media for PHA production. For example, *P. mendocina* showed higher yields of polymer accumulation when MSM (mineral salt medium), was used along with different carbons source while *P. putida* preferred E2 medium and *P. aeruginosa* the ME2 medium for PHA accumulation.Hence, in this study the MSM media was used for the production of MCL-PHAs using *P. mendocina* (Rai *et al.*, 2011).

In similar studies, *P.nitroreducens* when grown on octanoate was observed to accumulate PHA of up to 34.3% dry cell weight (Yao *etal.,* 1999). In another study, *P. putida* GPO1 was observed to accumulate PHA up to 37%dcw when grown in octanoate. Similar observations were also made with*Pseudomonas oleovorans* when the organism was fed with octanoic acid (Durner *et al.*, 2000). These results therefore confirm that different *Pseudomonas sp,* like *P. putida*, *P. nitroreducens* and *P.oleovorans* accumulate MCL-PHAs with a yield ranging from 34-37% dry cell weight, when grown on the structurally related carbon sources, octanoate. *P.mendocina* when grown on structurally unrelated carbon sources like sucrose as sole carbon source, it was observed to accumulate PHA up to 27.19% dcw of its dry cell weight. Similar studies carried out by Haywood *et al.*, (1990) showed that *Pseudomonas* sp NCIMB 40135 when grown on carbohydrates such as glucose and fructose were found to accumulate polymer up to 8 and 16% dcw respectively. *P. putida* KT2442, when grown in ME2 medium using glucose and fructose at a C/N ratio of 46, was found to accumulated polymer up to 16.9% dcw and 24.5% dcw respectively (Huijberts and Eggink.,1992).It was also observed that the media composition and in particular the amount of carbon and nitrogen present in the production medium have an effect on the PHA monomers accumulated and also on the yield of the

polymer. The above observation indicates the yield observed in this study was one of the highest yields of polymer obtained from *Pseudomonas sp.*

In the media used in this study, the amount of nitrogen 'N' was much less than the amount of carbon, 'C' leading to high C/N ratio. Thus a high C/N ratio was used for growing *P. mendocina* for polymer production. In this present study the maximum PHA accumulated by the *P. mendocina* cells were 29% dcw and 27.19% dcw for, sucrose and sodium octonate at a C/N ratio of 53 and 20 respectively. Similar high yield of PHA, i.e. 31.38% dcw, was obtained when *Pseudomonas mendocina* was grown in octanoate at a C/N ratio of 20 by Rai *et al.,* 2011. They also found that maximum PHA accumulated by the *P. mendocina* at C/N ratios of 58, 67 and 53 were 16.12% dcw, 23% dcw and 4.6% dcw for glucose, sucrose and fructose respectively.

Studies carried out by Huijberts *et al.*, (1992) showed that, *P. putida* KT2442, when grown in ME2 medium using glucose at a C/N ratio of 46, accumulated polymer up to 16.9% dcw (Huijberts *et al.*, 1992). In this present study the highest yield of the polymer from sodium octonoate and sucrose was achieved at 48 hrs during stationary phase of fermentation. After 48 hrs the polymer yield dropped for all the carbon sources possibly due to the utilisation of PHA for growth under carbon deficient conditions. PHAs are accumulated as energy resources, utilise the accumulated PHAs to sustain its growth. This utilization of accumulated PHAs by the organisms has been well studied (Anderson and Dawes, 1990). The synthesis of PHA from *P.medocina* was carried out in batch fermentations. In batch fermentation the media is not replenished therefore, the organism has higher possibility of utilizing the accumulated PHA granules when faced with nutrient limitation.

The nature of the carbon source could be also a reason for the productivity (29% dcw) of the polymer in batch fermentations. According to previous studies, MCL PHAs are mainly biosynthesized by *Pseudomonas sp* using substrates such as linear and branched alkanes, 1-alkenes, and alkanoates. When these are used as carbon feed in the growth medium, they are either immiscible or toxic to the bacteria even at low concentrations. (Yao *et al.*, 1999) (Tian *et al.,*2000). A similar problem was encountered in this project when sodium octoanate was used as a carbon source. It was found that higher concentrations of this carbon source could not be used because of the toxic nature, which hindered the growth of the organism. So in the present study, the highest amount of sodium octonate used was 20 mM and no further increase in carbon concentration which prevented any possibility of a further increase in the yield of the polymer (Durner *et al.,* 2000).It was also noted that, a higher oxygen demand of *Pseudomonas sp* has often been an obstacle in obtaining high yield of MCL-PHAs in shaken flask experiments. So, in the present study additional investigations were also performed to determine the productivity of the polymer under controlled conditions in 2L fermentors provided with increased aeration and agitation in the MSM media. The fermentation was performed in a pH of 7.0 and aeration set at 1 vvm respectively. In this study it was found that there was a 13.9% increase in the yield obtained as compared to the shaken flask experiment. This increase in the production was most likely due to the availability of increased oxygen level and agitation in the fermentation media. Sun*et al.,* (2007) made a similar observation during the production of MCL-PHA from *Pseudomonas putida* KT2440 using nonanoic acid. They concluded that the increase in the polymer yield was due to higher oxygen demand of the organism (Sun*et al.,* 2007).

In the present study, experiments were conducted to achieve higher polymer production. When sodium octanoate was used as the sole carbon source to grow *P. mendocina*, it resulted in growth delay because the organism required time to

get acclimatised to the nutrient conditions. So, in this project studies were conducted in order to avoid this, glucose was provided along with sodium octanoate in the media to achieve rapid cell growth. Thus, the addition of glucose allowed rapid cell growth in the initial log phase. This resulted in the better utilization of sodium octanoate for the improved polymer accumulation of PHA of 37.09% dcw with 38%more yield compared to the shaken flask fermentation studies. Previous studies also been carried out to obtain higher yield of MCL-PHAs. One such approach was to carry out a two step fermentation, whereby a high cell concentration was achieved in the first step followed by limiting the organism growth in the second step in order to induce maximum PHA accumulation. For example Kim*et al.,* (1997) carried out two step fermentation of *P. putida* by combining the use of glucose and octanoate. They first grew the organism in glucose, which is a favorable carbon source for cell growth, to obtain
a high cell concentration. Next, the organism was grown on octanoate for the PHA accumulation. Using this approach a PHA yield of up to 40% dcw were achieved. *P.mendocina* was able to accumulate P(3HB) when grown in sucrose. The synthesis of P(3HB) by a *Pseudomonas sp,* from a structurally unrelated carbon source, (sucrose) is a rare phenomenon and so far not reported in the available literature. *Pseudomonas sp* along with *P. mendocina* in all other known cases produce and MCL-PHA or a MCL-SCL copolymer. For example the strain *Pseudomonas* NCIMB 40135 produced PHA containing monomers of 3HO and 3HD when grown on glucose (Haywood *et al.,* 1990). Similarly, *P. putida*KT2440 also accumulated monomers of 3HO and 3HD when grown in glucose (Sun *et al.,* 2007).

The synthesis of P(3HB) by *Pseudomonas* can be attributed to broad range in substrate specificity of the two *phaC* genes. For example, *P. nitroreducens* accumulated monomeric units of P(3HB) when grown on octanoic acid (Yao *et al.*, 1999). In another study, *Pseudomonas* sp 61-3 produced copolymers of

MCL monomers, when grown in glucanoate (Kato *etal.,* 1996). *P. mendocina* has two *phaC* genes encoding for the PHA synthase. Hein *et al*., (2002) found that *phaC1* codes for the major synthase in *P. mendocina* for PHA production and *phaC2*encodes an enzyme with a minor role in the accumulation of polymer. Similarly, in the studies carried out by Conte *e al*., (2006) it was found that in *P. corrugate*, *phaC1*activation occurred with any carbon sources but *phaC2*gene expression occurred only in the presence of glucose or sodium octanoate (Conte *et al.,* 2006). So, the production of P(3HB) could be attributed to the broad substrate specificity of PHA synthases and perhaps *phaC-2* is specifically involved in P(3HB) production in *Pseudomonas sp*. The fatty acid de novo biosynthetic pathway known to be used often by produced for the production of 3-hydroxybutyryl-Co-A producers like *Pseudomonas sp,* use when grown on structurally unrelated carbon sources, for example, glucose, sucrose, fructose. It is possible that the *de novo* biosynthesis pathway in *Pseudomonas mendocina* in the presence of glucose is regulated in such a manner that the direct conversion of sucrose to 3-hydroxybutyl-CoA is preferred rather than further synthesis of longer chain derivatives.

In this present study the highest yield of the polymer from both octanoate and sucrose was achieved at 48 hrs of the fermentation. After reaching maximum accumulation, the yield of the polymer was observed to decrease possibly due to the utilization of PHAs for growth under carbon deficient conditions. *P. mendocina* was able to grow and accumulate PHAs when grown on both structurally related and unrelated carbon sources. However, the yields of the PHAs were better in the structurally related carbon source as opposed to the structurally unrelated carbon sources. The reason attributed for the higher yield for fatty acids against carbohydrates could be because of the ease of the utilisation of structurally related carbon sources by the organism. The relative load on the biosynthetic machinery is relatively less, as these carbon feeds do

not need as many modifications as the structurally unrelated carbon source, leading to a higher yield.

Another factor that might have led to the differences in the yields could have been the pH of the media. When *P. mendocina* was grown in fatty acids the pH of the medium, which was set at 7 at the beginning of fermentation, increased progressively reaching 7.7. On the other hand for sucrose, the pH of the medium, which was also set at 7, dropped gradually and reached a value of 6.76. Kim *et al.,* (2002) observed an increased yield of MCL-PHA production in pH stat fermentations at 7 compared to non pH stat study.In the studies carried out by Philip *et al.,* on the effects of pH on P(3HB) accumulation by *B. cereus* SPV they found that among the three pH stat fermentations at 3, 6.8 and 10, a maximum accumulation of 23% dry cell weight P(3HB) yield was observed at a pH of 6.8.Hence, another approach to increase the yield of PHA production is to control the pH of the culture media, which has an important effect on the accumulation and degradation of PHAs. In this study the initial pH of the nutrient limiting media used was to grow *Bacillus cereus* set at 7.0. As fermentation progressed the pH of the culture media was decreased gradually reaching a value of 5. It is possible that this low pH in the media prevented PHA degradation.(Valappil *et al.,* 2006). Kominek and Halvorson also made a similar observation in 1965 where low pH environment resulted in the inhibition of polymer degradation and spore formation. The production of P(3HB) using *B. cereus* SPV was carried out in 2 L fermentor using Kannan and Rehacek media. The pH and airflow rate was initially set at 7.0 and 1 vvm respectively. In this study an increased yield of 3.1% was observed compared to the shaken flask experiment. The reason for this observation of increased yield is possibly due to controlled fermentation conditions like improved agitation in the media. Previous studies by Philip *et al.,*2009 already reported that, improved agitaion resulted in enhanced PHA yield.

To study the effect of multiple nutrient limitations on the accumulation of PHA by *B. cereus* SPV, the organism was grown in a modified enrichment media (MEM). In this study the combined effect of the nutrient limitation such as nitrogen, potassium, and magnitium, on the accumulation of PHA was studied for the first time. In this study by limiting nutrients we obtained improved PHA accumulation (52.64 % dcw) within 48 hrs. According an earlier study conducted by Wakisaga *et al*., (1982) on *B. cereus* IFO 3466 showed the production of P(3HB) possible under potassium limiting conditions.

They observed an improved production of P(3HB) under this nutrient deficient medium. Hosseini *et al.,* 2009 optimized media components and observed that deficiency of nitrogen and magnesium is crucial for P(3HB) accumulation *Methylobacterium extorquens* DSMZ 1340. In another study, (Kim and Lenz, 2001) potassium sulphate was found to be the limiting nutrient, leading to PHA formation in *Bacillus thuringiensis*. These studies showed that both magnitium and potassium were limiting factors that could led to the PHA production in *Bacillus* sp. In this project it was found that multiple nutrient limitations led to improved PHA accumulation in *Bacillus cereus* SPV.

7.2 Downstream processing

Studies were also carried out on the effects of different extraction methods on the yield, purity, and lipopolysaccharide content of P(3HO) produced from *P. mendocina* using octanoate as sole carbon source. It was found that the extraction method does have an effect on these parameters. Cellular proteins and lipopolysaccharides (LPS) are the main component of the Gram negative bacterial cell wall that gets coextracted with the PHAs. These protein contaminants and LPS have to be effectively removed to obtain a contaminant free pure polymer. LPS is pyrogenic in nature and therefore, the PHA extraction methods employed must remove this contaminating LPS. Among different extraction methods, extraction of polymer using the dispersion of NaOCl and

$CHCl_3$ gave the highest yield of polymer, which was 29% dcw. This could be due to the cell disruption caused by the sodium hypochlorite, which then provided increased access of the chloroform to the accumulated intracellular granules resulting in better solubilisation of the polymer. The simple $CHCl_3$extraction resulted in a polymer yield of 28% dcw. This low yield compared to the dispersion method could be because in this method the cells were incubated with the solvent. In this case the solvent mostly dissolve the cell wall and the penetration of the cells by the solvents may not be efficient. Hence, less PHA granules get dissolved in the extracting solvent, resulting in lower amounts of polymer getting extracted leading to relatively lower yields. The soxhlet extraction, gave the lowest yield of 12.0% dcw as opposed to the dispersion method. This could be due the degradation of the polymer by incubating it with sodium hypochlorite and repeated washing using acetone. MCL-PHAs are soluble in acetone, therefore; polymer could have been lost in these processes (Furrer *et al.,* 2007; Vallapil *et al.,* 2007).

In this project a new method of MCL-PHA extraction successfully developed based on the sodium chloride and detergentbased lysis and purification. Using this method efficient lysis, removal of protein contaminants and polymer extraction has been achieved. Among different concentrations tested 20% NaCl solution was found to be most effective in inducing maximum cell lysis. This provides exposure of the intracellular polymer to the n-hexane in which it dissolves. In this method osmotic lysis was used followed by membrane protein extraction using a non-ionic detergent, Triton X-114 which disrupt the cells. This detergent has been known to be an effective reagent for the isolation of membrane proteins. For example, Triton X-114 is a unique detergent not only able to solubilise membrane proteins but also separates them from hydrophilic proteins via phase partitioning at physiological temperature. Bordier *et al.,* (1981) demonstrated that Triton X-114 could be used to extract membrane proteins and when the temperature of this mixture was raised above 22 °C, the

solubilised membrane proteins separated out into the two phases according to their hydrophobicity. The most hydrophobic proteins tend to concentrate in the lower phase, whereas hydrophilic proteins appear mainly in the upper phase. Quoronfleh *et al.,* (2002) andLuis*et al.,* (2009)introduced the application of thermally induced phase separation of proteins using Triton X-114. The area under the peak observed was measured showed that the polymer extracted by the Triton based method found to 25 % more pure compared to the dispersion method. Hence Triton X-114 was used to effectively remove protein impurities from the PHAs. The removal of endotoxin from the PHA was an another important advantages of the using Triton X-114 for PHA extraction. Aida *et al.,* successfully removed endotoxin contamination of protein solutions by a phase separation technique using the detergent, Triton X-114. Their study concluded that, the phase separation technique provided a rapid and gentle method for removing endotoxin from protein solutions. Another advantage of the new extraction method there was nosodium hypochlorite mediated digestion and loss of molecular weight of polymer.Less time for the extraction is another advantage of the new method, which takes only one hour while the dispersion method takes two hours. In the dispersion method after centrifugation process the polymer dissolved in the solvent was found as a bottom layer below the cell debris and sodium hypochlorite solution, which make it difficult to remove the solvent layer. Hence, there is more chance of cell debris contamination in to the polymer while removing the top layers. In the new extraction method easy removal of the polymer dissolved in the solvent was possible since the n-hexane layer found always as a top layer after the centrifugation process. Hence, the top solvent layer could be easily removed without any disturbance of the bottom layers. Also, n-hexane used in the extraction method is not carcinogenic while chloroform is known to be carcinogenic in nature.

7.3 Thermal properties

The thermal properties of these polymers like glass transition temperature (T_g) melting temperature, (T_m) and crystallinity (T_c) were studied using the differential scanning calorimetry analysis. When *P. mendocina* was grown on sodium octonoate, the polymer produced showed T_m of 50.36^{o}C with -32.86^{o}Cfor the T_g polymer, and no peak was observed for crystallintiy. This result is consistent with the study conducted by Choi and Yoon (Choi and Yoon, 1994) in which they observed no melting peak in the polymer obtained from *P. citronellois* using heptanoate as the carbon source. The absence of any T_m peak for the polymer produced using heptanoate indicated the lack of a crystalline phase. So, MCL-PHAs are polymers with low melting points that either crystallise very slowly or don't crystallize in nature. The higher T_m values correspond to higher crystallinity and the presence of low T_m peak for the PHA produced using octanoate indicates the low of crystalline properties of polymer.The polymer extracted from *P. mendocina* using octanoate showed a low T_g value of -32.86°C. This low T_g value agrees with previously reported values for P(3HO) (Kim *et al.,* 2007). The T_g value of the MCL-PHA was found to be lower due to the increased average length of the pendant group that end up in the increased mobility of the polymer chains (Vander Walle *et al.,* 2001).

The glass transition temperature (T_g)melting temperature (T_m) and crystalllinity of the polymer produced by *Bacillus cereus* were also studied using the differential scanning calorimetry analysis. The studies on the thermal properties on P(3HB) showed that, it has a low glass transition temperature, (T_g of 2.43°C) and high melting temperature of 167.39°C. The polymer showed a crystallisation temperature (T_c)of 54.33°C. These values were consistent with study conducted by Vallapil *et al.,* (2007). The observation of high melting temperature also confirms that the crystallinity is much higher compared to P(3HO).The high crystallinity and rigidity of the polymer is due to short methyl side chain and the polymer chains are found to arrange themselves in of ordered

structures called spherulite. Due to the formation of these spherulites the polymer was found to be relatively hard and brittle in nature.

7.4 Nuclear magnetic spectroscopy

The ^{1}H, and ^{13}C NMR analysis of the polymer produced from octanoate confirmed that the polymer produced by *P. mendocina* using octanoate as the sole carbon source is a homopolymer poly(3-hydroxyoctanoate) P(3HO). The production of homo polymer of P(3HO) is an unusual observation because according to the known literature copolymers of P(3HO) containing other monomers have been produced from *Pseudomonas sp*. For example, *Pseudomonas oleovorans* when grown on octane produced polymer consisting of both 3-hydroxyhexonate and 3-hydroxyoctonoate (Ronald *et al.,* 1988). Similarly when *Pseudomonas aeruginosa* ATCC 27853 grown on octanoic acid to accumulate PHA consisting three different repeating units and the most predominant units were 3-hydroxyoctanoate and 3-hydroxydecanoate. The production of the copolymer of P(3HO) and poly(3-hydroxydecanoate) was observed in *P. mendocina*0806 when grown on octanoate (Tian *et al.,* 2000). In the present study, a homo polymer of P(3HO) was obtained from octanoate instead of any copolymer. This observation confirms the observation made by Rai *et al.,* 2011.

Interestingly ^{1}H, ^{13}C NMR analysis confirmed that the polymer produced by *P. mendocina* using sucrose as the sole carbon source is the homopolymer poly(3-hydroxybutyrate) P(3HB). Accumulation of SCL monomers from structurally unrelated carbon source like sucrose is very rare and so far not reported in the literature. *P. nitroreducens* AS 1.2343 has been found to accumulate P(3HB) when grown on octanoic acid (Yao *et al.,* 1999). The production of P(3HB) could be mainly due to the broad substrate specificity of PHA synthesis in the *Pseudomonas sp*. For example, *Pseudomonas sp*. A33 was able to accumulate the polymers containing 3 (HB) and monomers like

3-hydroxyhexadecanoate, 3(HHD), 3-hydroxydodecenoate, 3(HDDE), 3-hydroxytetradecenoate, 3(HTDE) and 3-hydroxyhexadecenoate, 3(HHDE) when grown using both fatty acid and glucose (Lee *et al.,* 1995). *Pseudomonas* sp. 61-3 accumulated copolymers of 3HB and MCL monomers when grown in glucanoate as well as alkanoic acids (Kato, 1996). Diversity in the PHA biosynthetic capability of *Pseudomonas* is attributed to the difference in substrate specificity of the two *pha* genes coding for the PHA synthase in the organism.

The NMR analysis was used to determine the structure of the isolated polymer from *B. cereus* SPV. These four peaks were assignable four different groups. These four narrow lines appeared were identical to the ^{13}C NMR spectra of P(3HB) reported previously (Doi *et al.,* 1989). The four peaks were assignable to the methyl(CH3 ; 19.88 ppm),methy-lene (CH2 40.88ppm), methane (CH;67.72 ppm) and carbonyl (C = O; 169.54 ppm) carbon resonance of PHB (Doi et al., 1986, 1989). Similar results were also observed by Vallapil *et al.,* 2009 in the NMR spectra used to determine the polymer isolated from *Bacillus cereus*. The peaks observed in the ^{1}H spectra coincide, corresponding to the different types of carbon atoms presented in the P(3HB) structure. Similar results were observed by Jan *et al.,* 1995 in the study of ^{1}H NMR spectroscopic determination of P(3HB) extracted from *Rhizobium meliloti*. Similar results were also obtained by Yoshie *et al.,* 1992 in their study on the biosynthesis and NMR studies of deuterated poly(3-hydroxybutyrate) produced by *Alcaligenes eutrophus* H16.

Chapter-4 Production Characterisation &Application of PHA blend polymers

4.1 Introduction

PHAs have attracted a lot of interest in a number of agricultural, industrial and particularly for medical applications. The physical properties of PHAs greatly affect their possible applications. Hence, before using PHAs for various applications one has to consider the properties of the PHAs. The properties of the PHAs vary depending on the different monomer units in their structure. Depending on the number of constituent carbon atoms present in their monomer units, PHAs exhibit properties ranging from hard to brittle and flexible to elastomeric in nature.

As discussed in Chapter1, MCL-PHAs produced from *P. mendocina* are found to be flexible and elastomeric which makes them suitable for soft tissue engineering such as cardiovascular and skin tissue engineering (TE) however, they lack strength. On the other hand, SCL-PHAs produced from *B. cereus* SPV have higher tensile strength, but are found to be brittle and stiff and have been studied for hard tissue engineering like bone TE. These properties of both MCL and SCL PHAs limit the range of application of these polymers. Therefore, in this project studies were carried out on blends of SCL and MCL PHAs. Such blends are expected to be elastomeric and strong, i.e to have the combined the desirable properties of SCL-PHAs and MCL-PHAs.

This chapter describes the work that was done with an objective to study the properties of neat and blend polymer films made up of the MCL-PHA, poly (3-hydroxyoctanoate),P(3HO) and the SCL-PHA, poly(3-hydroxybutyrate), P(3HB). The neat polymers as well as the blends were processed into two dimensional films, using the solvent casting method described in Chapter 2.This chapter also describes the work that was done with an objective to study the detailed thermal, physical and mechanical properties of the neat and blend PHA films. These fabricated blend films were then studied for the possible application as scaffolds for the tissue engineering with a particular interest in the nerve tissue regeneration.

4.1.1 The P(3HO)/P(3HB) blends as a potential biomaterial for the nerve conduit structures.

Neural tissue engineering has mainly focused on the recovery of nerve functionality after injury. This is a long term medical challenge that requires suitable nerve guides for bridging nerve injury gaps for restoring nerve functions. A nerve guidance conduit is an artificial means of guiding axonal regrowth to facilitate nerve regeneration. In this approach a biodegradable nerve conduit is populated *in vitro* with Schwann cells and implanted onto the damaged region. Schwann cells are involved in many important aspects of peripheral nerve biology, including the conduction of nerve impulses along axons, nerve development and regeneration, trophic support for neurons, production of the nerve extracellular matrix, and modulation of neuromuscular synaptic activity.

Moreover, Schwann cells are essential for healthy maintenance of axons by producing growth factors known as neurotrophins, which are involve in the survival, development and function of neurons. The bioengineered nerve grafts have been developed from polymeric materials for peripheral nerve regeneration. To achieve desired nerve regenerative functions it is important to understand the intrinsic properties of these polymers. The structural thermal and mechanical properties of these polymers, and their fabrication methods are important, as these aspects are critical for the performance of fabricated nerve conduits (Jiang *etal.,* 2010).

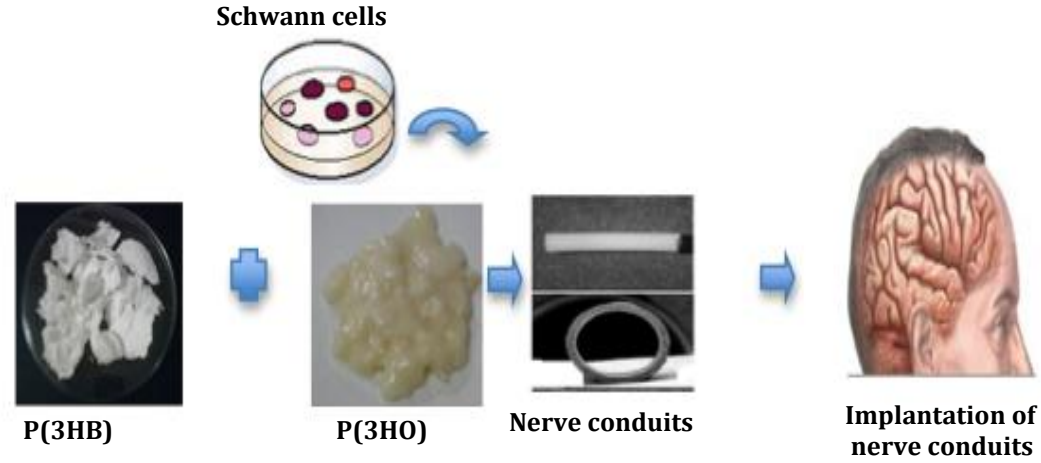


Figure 4.1: Nerve conduits made using P(3HO)/P(3HB) blends can be seeded with Schwann cells *in vitro*, allowed to proliferate, then implanted into the damaged region.

In this study blends consisting of P(3HB) and P(3HO) were used to prepare nerve conduits(Figure 4.1).A variety of biomaterials have been investigated for their suitability in nerve tissue engineering application. For example, polymers like poly(lactic acid) and poly(ε-caprolactone) have previously been shown to be suitable biodegradable and biocompatible materials to support nerve regeneration. However, their brittle nature and low tensile strength are major limitations for these polymers (Shanfeng and Lei., 2010). Pioneering experiments have been carried out to confirm that the damaged nerve cells can be repaired using PHAs. For example, P(3HB) conduits have been tested for superficial radial nerve repair in cats. This study showed that axonal regeneration could be carried out using these conduits. From this work it has been concluded that the addition of tubular P(3HB) to the damaged nerve region could have important effects on fast nerve regeneration, as these structures help guide the proliferation of the nerve cells allowing faster regeneration. However, P(3HB) is rigid with tensile strength of 40 MPa and an elongation to break value of 6% (Lee *et al*., 1995). On the other hand P(3HO) is flexible with tensile strength of 11 MPa and elongation at break value of 350% (Marchessault *et al* 1990; Rai*et al.,* 2011;David and Simon, 2003).Hence, both of these polymers are not preferred for preparation of nerve conduit as they do not have required mechanical properties.

Yucel *et al.,* 2010 proposed P(3HBV)/PLCA (Poly(L-lysine citramide) blend for nerve conduits preparation and found that the ideal tensile strength of the conduit was 1.05 MPa. This study showed that that the conduit must have good tensile strength and should not be too brittle in nature. Therefore, in this work studies were carried out for the first time to produce blends consisting of rigid P(3HB) and flexible P(3HO) to be used to prepare tubular nerve guide material as shown in Figure 4.1.

4.2 Results

4.2.1 Fabrication of neat and blend films.

The fabrication of blends was carried out as described in Chapter-2. Two types of blend films were cast, P(3HO)/P(3HB)(5:1) g/g and P(3HB)/P(3HO)(5:1)g/g. The thickness of the P(3HO)/P(3HB)(5:1) blend films were 0.3 mm whereas that of P(3HB)/P(3HO)(5:1) blend was 0.223 mm, P(3HO) neat film was 0.15 mm and P(3HB)neat film was0.2 mm (Figure 4.2).

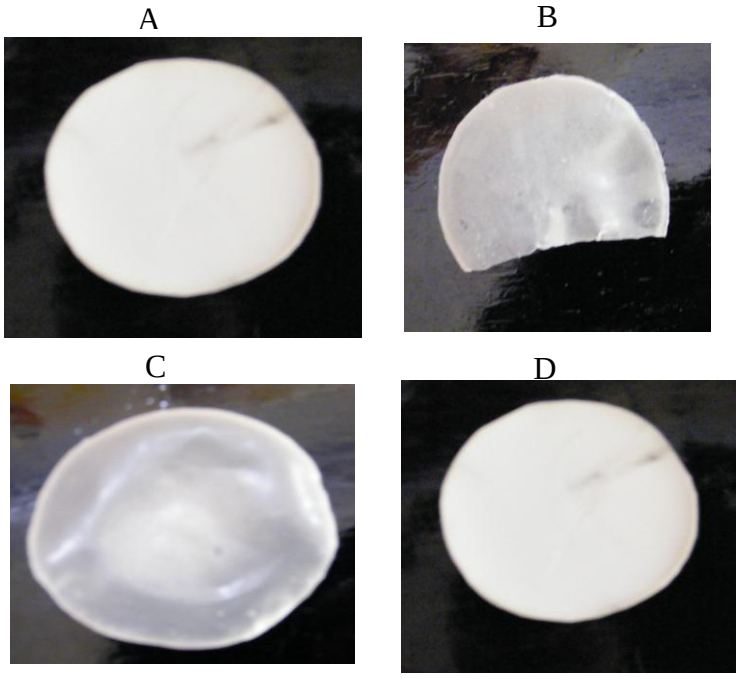


Figure 4.2: Fabricated (A) P(3HB) (B) P(3HO) (C) P(3HO)/P(3HB)(5:1) and (D) P(3HB)/P(3HO)(5:1) blend films made using the solvent casting method

4.2.2 Micro structural studies

The surface morphology and microstructure of the films were observed by SEM. The SEM images of the neat P(3HB) film (Figure 4.3 (A) showed reasonably rough surface compared to P(3HO). P(3HO) film however revealed smooth surface properties of as seen in Figure 4.3 (B).In the case of P(3HB)/P(3HO) (5:1)blend, a rougher surface topography was observed compared to that of neat P(3HB) film (Figure4.3 (C).The P(3HO)/P(3HB)(5:1)film on the other hand showed a completely changed surface morphology with small protrusions on the surface (Figure 4.3 (D).

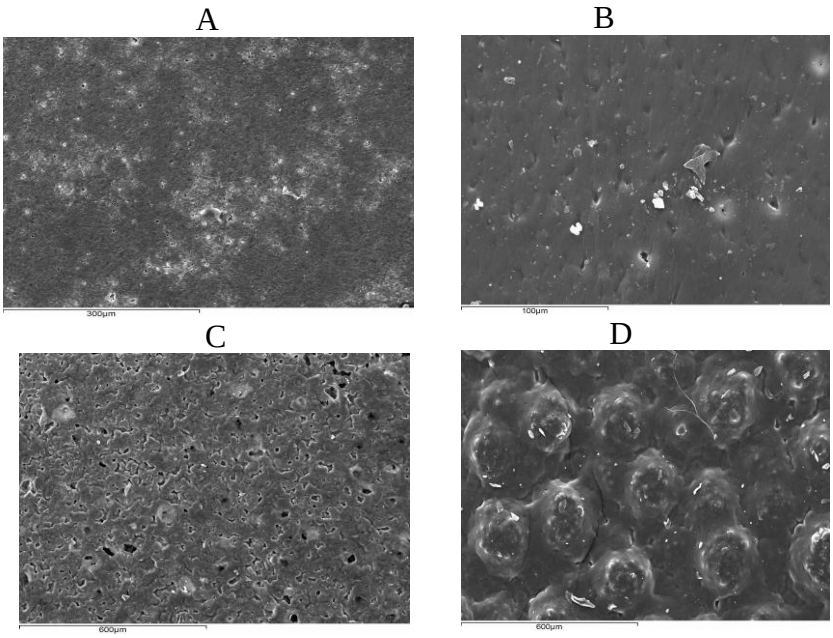


Figure 4.3: SEM images of the PHA neat and blend films: (A)P(3HB)(B) P(3HO) neat films(C) P(3HB)/P(3HO) (5:1)(D) P(3HO)/P(3HB) (5:1) blend films.

Surface analysis of the neat and blend films were carried out using white light interferometry using ZYGO® to visualise the topography of the films as shown in the Figures 4.4. The blending of P(3HB) with P(3HO) in the ratio of 5:1 had increased the roughness of the film with respect to neat P(3HO) and P(3HB). This was confirmed by the white light interferometry analysis whereby, a typical root mean square average (RMS) value of roughness was measured to be 2 µm for the P(3HB/P(3HO)(5:1) blend film as opposed to 1.105 µm for the neat P(3HB) and 0.547 µm for the neat P(3HO)film. For the P(3HO)/P(3HB) (5:1) blend the RMS value was 2.907µm as opposed to 0.547µm for the neat P(3HO) and 1.105 µm for the neat P(3HB) neat films. However, the blend was so rough and the RMS value went beyond the maximum value measurable by the equipment.

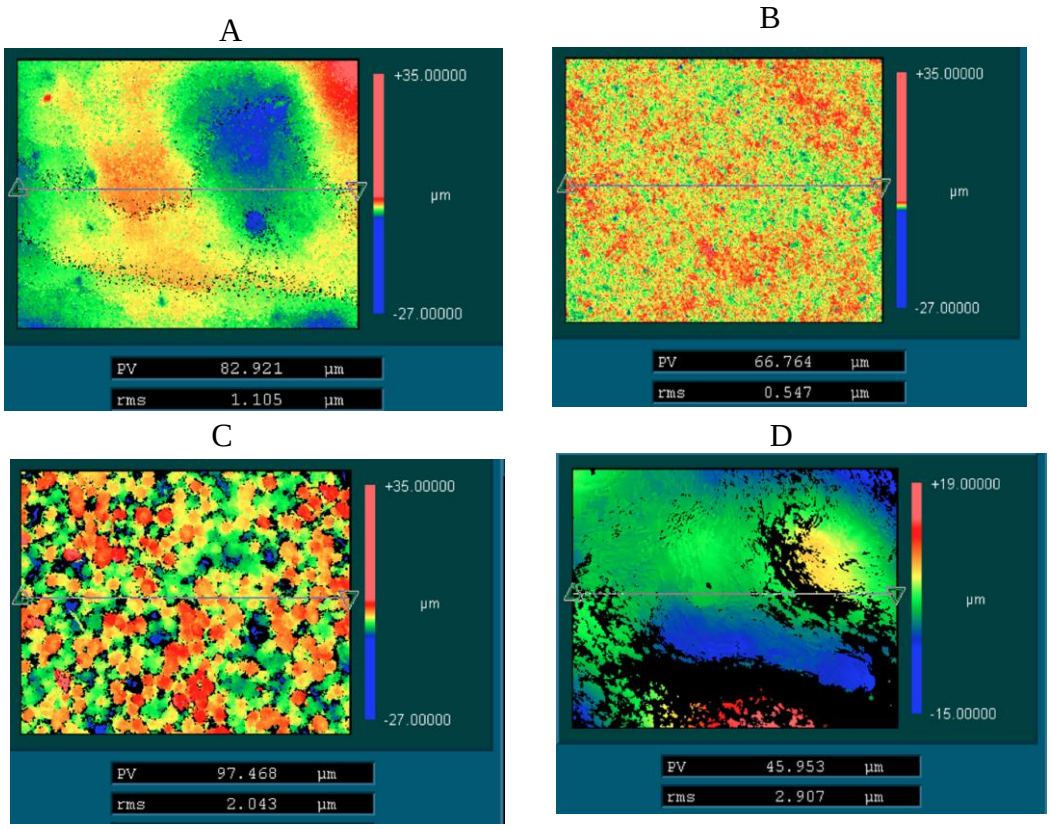


Figure: 4.4: White light interferometry analysis of the surface topography of the fabricated films: (A) P(3HB),(B)P(3HO) neat films(C) P(3HB)/P(3HO) (5:1), D) P(3HO)/P(3HB) (5:1) blend films.The surface variation topography (depth or roughness) is represented by different colors.

4.2.3 Water contact angle study of neat and PHAblend films

Water contact angle measurements were carried out on both the surfaces of the P(3HO),P(3HB) neat films and the P(3HO)/P(3HB) (5:1), (P(3HB)/P(3HO) (5:1) blend films to assess their wettability. The water contact angle (θ_{H2O}) is a measure of the hydrophilicity or hydrophobicity of a material surface. According to Peschel *et al.,* 2007 surfaces with θ_{H2O} less than 70°are considered to be hydrophilic and θ_{H2O} greater than 70°are considered to be hydrophobic. The contact angle measurements of the fabricated films are shown in Figure 4.5. The water contact angle value for the neat P(3HB) film was 70.37° and for P(3HO) was 99.94°. In the case of blend films P(3HO)/P(3HB) (5:1) and P(3HB)/P(3HO)(5:1), the θ_{H2O} was 80° and 90.39° respectively. Therefore, surfaces of the fabricated neat and blend films were hydrophobic in nature.

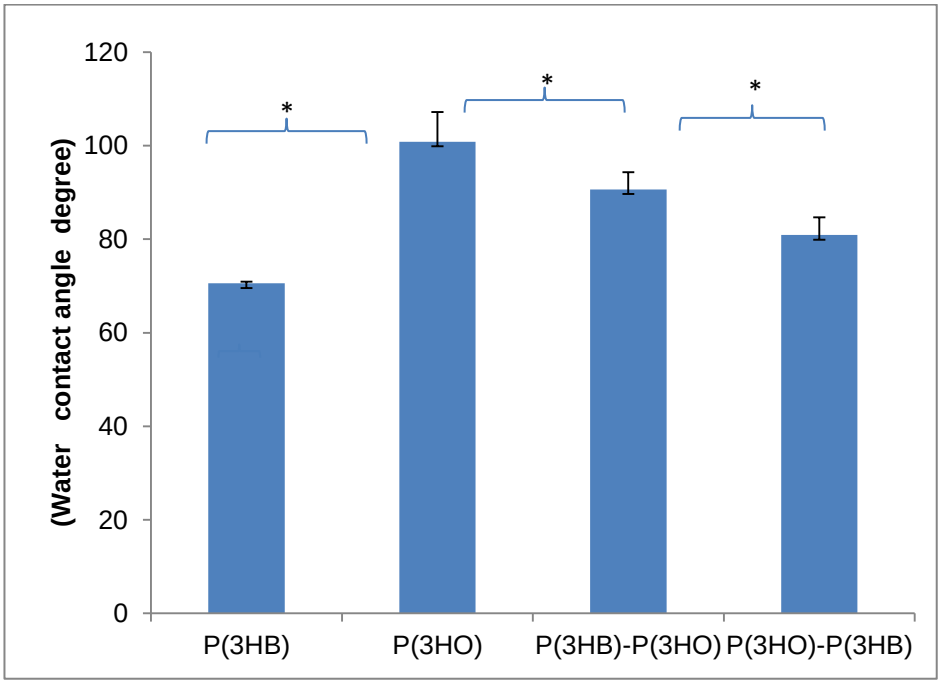


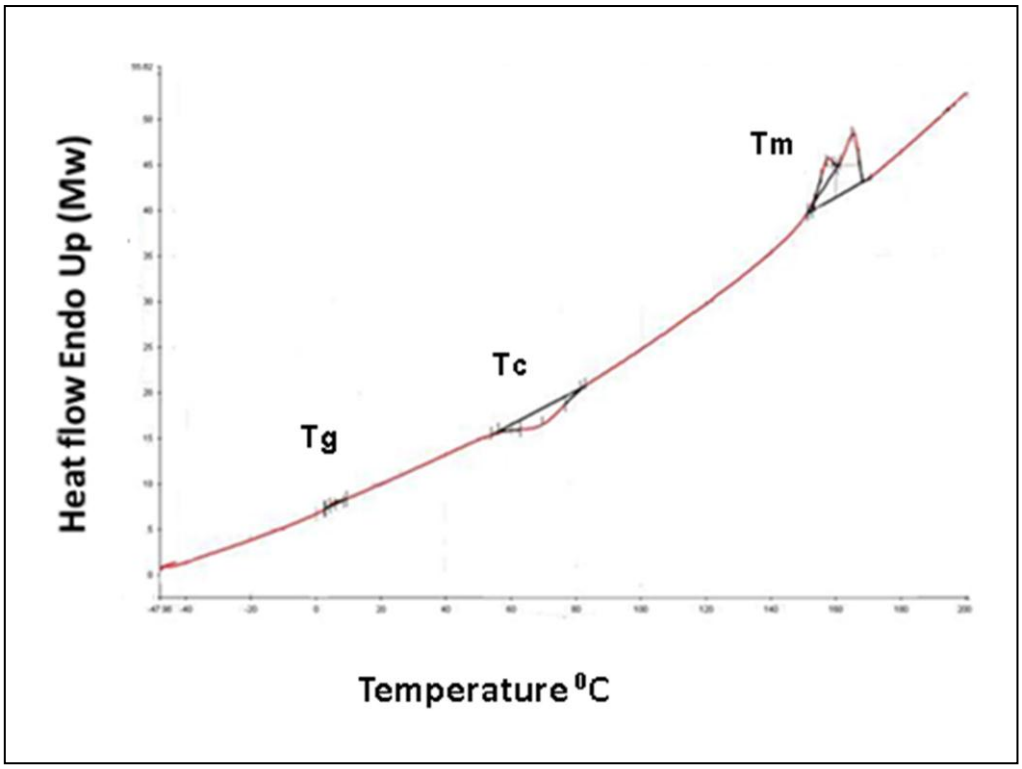
Figure 4.5: Contact angle measurement for theP(3HB), P(3HO) neat films and P(3HB)/P(3HO) (5:1) D) P(3HO)/P(3HB) (5:1) blend films. The data were compared using the students t-test and the difference were considered significant when *p<0.05

4.2.4 Thermal characterization

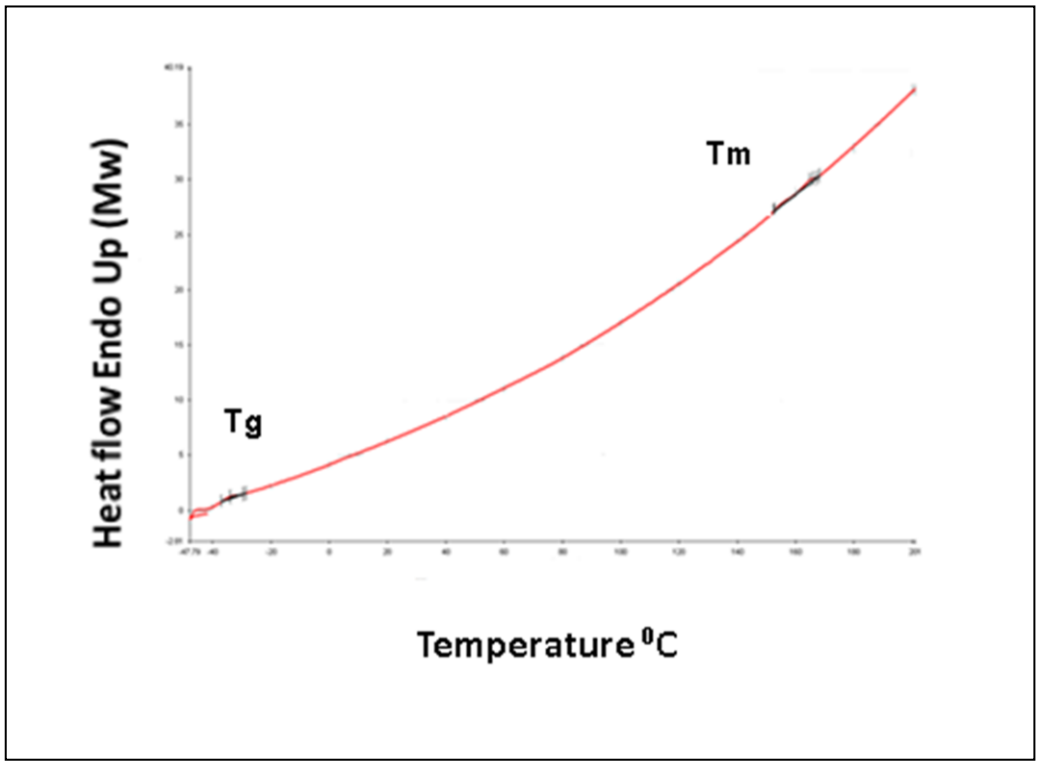
Thermal characteristics of a material are an important property that defines the stability of a material and it has an important implication on the materials process ability and end goal application. The thermal analyses of the fabricated films were carried out using differential scanning calorimetry (DSC) to characterise the thermal transitions corresponding to the melting, glass transition and crystallisation temperatures. Figures 4.6 (A&B) show the thermal profiles of the fabricated films. All the fabricated neat and blend films showed the endothermic transition of the amorphous to the glassy state (T_g) and the melting of the crystalline(T_c) phase (T_m) during the heat scan. The results of the analyses are summarised in Table 4.1.

Polymer blends	T_g (^{0}C)	T_c (^{0}C)	T_m (^{0}C)
P(3HB)/P(3HO) 5:1	5.84	69.58	164.91 157.22
P(3HO) /P(3HB) 5:1	-36.99	*	164.85
P(3HB)	2.43	54.33	167.39
P(3HO)			50.36

Table 4.1: Compilation of the thermal properties of the fabricated neat and blend PHA films.* not observed



(A)



(B)

Figure 4.6: Thermo gram of the fabricated films (A) P(3HB)/P(3HO) (5:1) (B) P(3HO)/P(3HB) (5:1)

4.2.5 Mechanical properties of neat and blend PHAs

Static tests were carried out the neat and blend films to understand their mechanical properties at the Faculty of Engineering, University of Southampton by John Clarke and Dr. Atul Bhaskar. The tensile tests were carried out on thin strips of the films as described in materials and method section. The results of the analyses are summarised in Table 4.2.

PHAs	**Young's modulus E (MPa)**	**Tensile strength (MPa)**	**Elongation at break (%)**
P(3HB)	250	27.4	2.56%
P(3HB)/P(3HO) (5:1)	136	4.99	35.81%
P(3HO)/P(3HB) (5:1)	37	1.5	160%
P(3HO)	20	1	200%

Table 4.2: Compilation of the mechanical properties of the fabricated neat P(3HB) and P(3HO) neat films and P(3HB)/P(3HO) (5:1)P(3HO)/P(3HB)(5:1)blend films

4.2.6 *In vitro* biocompatibility study

In vitro biocompatibility studies on neat and blend polymers were carried out using the HaCaT, a keratinocyte cell line. The cell attachment and proliferation studies were carried out using the Neutral Red assay. The HaCaT cells were seeded on the films and the proliferation of the cells was studied over a period of 3, 5 and 7 days. The proliferation studies were carried out using the standard tissue culture plate as the control. The cells were able to attach and proliferate on both the neat and blend films. Figure 4.7shows the attachment and proliferation

of the HaCaT cells on the fabricated films. The growth of HaCaT cells on all the films increased over the studied time duration.

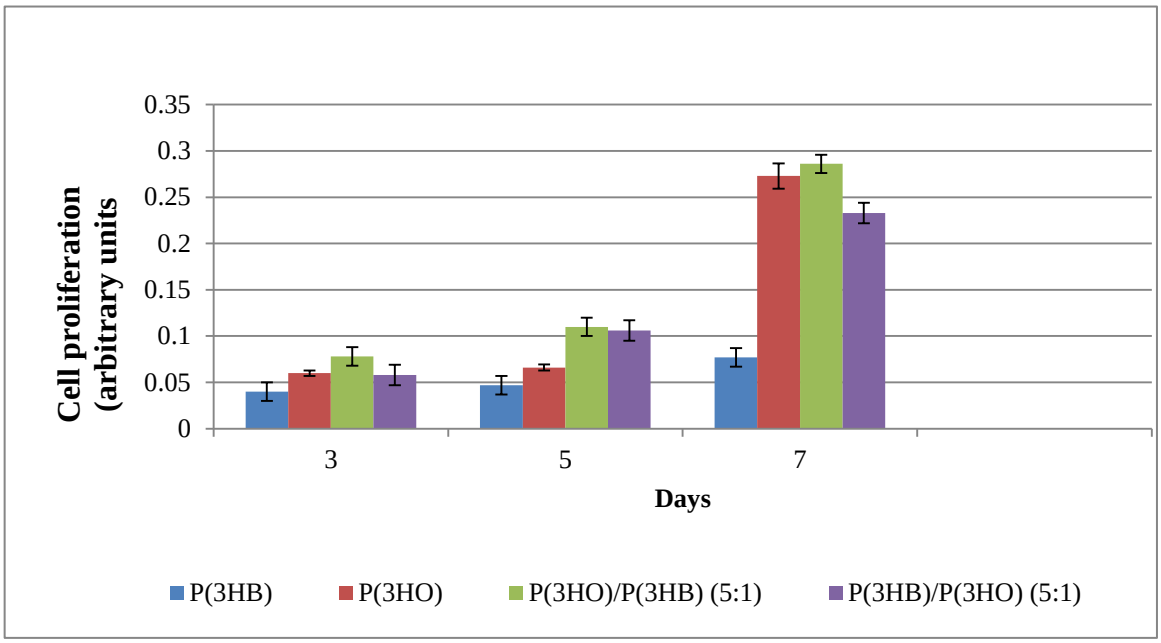


Figure 4.7: Proliferation study of the seeded HaCaT cells on the P(3HB),P(3HO) neat films and P(3HO)/P(3HB) (5:1), P(3HB)/P(3HO)(5:1) blend films on day 3, 5 and 7. The data were compared using the students t-test and the difference was considered significant when *p<0.05

The growth of HaCaT cells on all the films increased over the studied time duration. However, compared to the neat films, the cells showed better attachment and proliferation on the blend films. From Figure 4.7 it can be observed that the OD values at 540nm indicating cell proliferation showed a difference between neat and blend films on day 3 of cell culture. A significantly higher cell growth was observed on the P(3HO)/P(3HB) 5:1 blend films when compared to the neat P(3HO),P(3HB) films and the P(3HB)/P(3HO) 5:1 blend. Cell proliferation further increased on the day 5. Here too the OD value of cells (0.15) on the P(3HO)/P(3HB) 5:1 was significantly higher than that on the neat P(3HB) film (0.047) neat P(3HO) film (0.066) and the P(3HB)/P(3HO) 5:1 blend film (0.106).On day 7 as expected the growth of HaCaT cells was highest on the P(3HO)/P(3HB) 5:1 blend film as opposed to neat P(3HB) and neat P(3HO) films. Infact the OD values of the cells on the P(3HO)/P(3HB) 5:1

increased relative to day 5 (0.286) while that of the cells on the P(3HB) neat film (0.077), P(3HO) neat film (0.22), and the P(3HB)/P(3HO) 5:1 (0.230) had comparatively decreased.

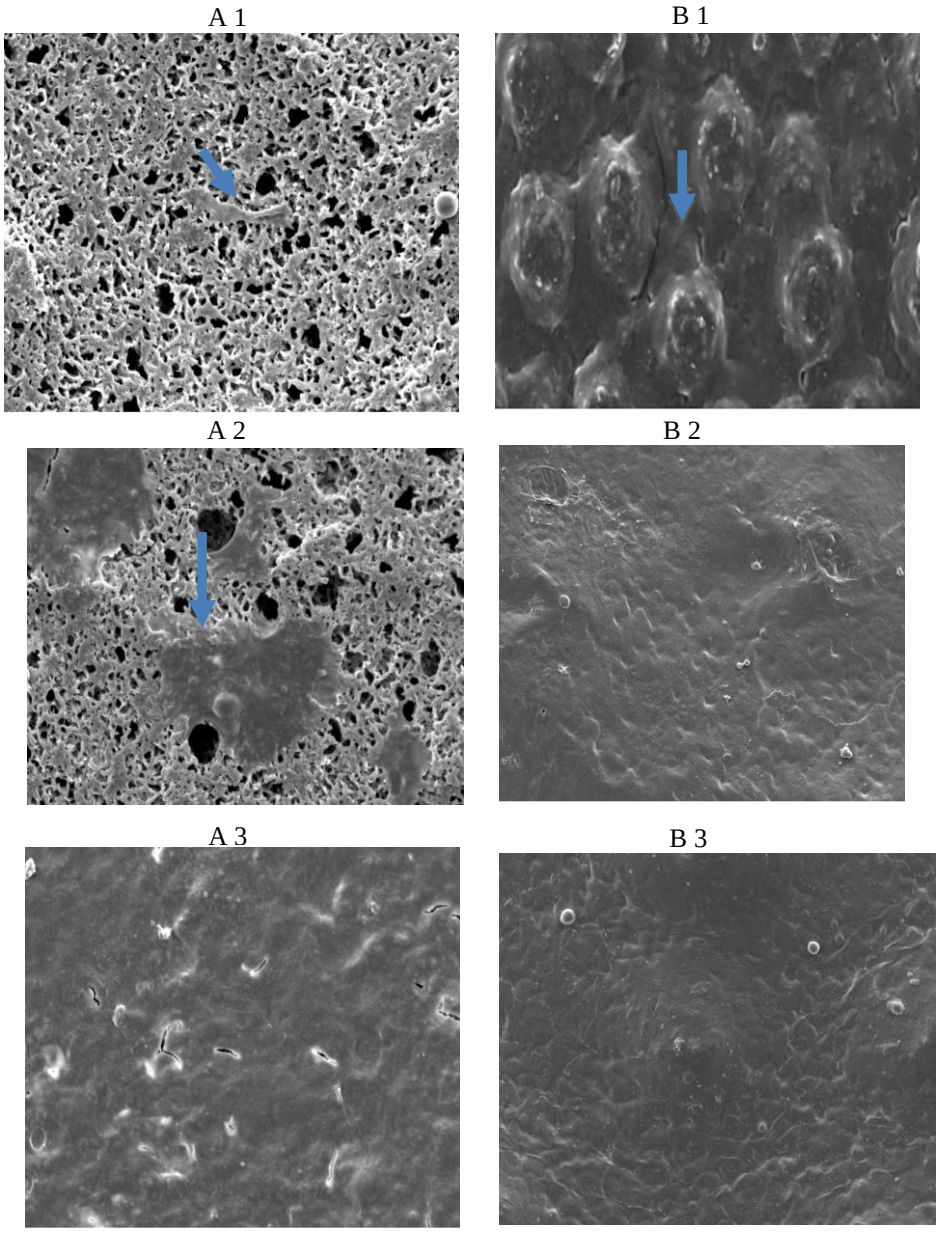


Figure 4.8: SEM images of the seeded HaCaT cells on the fabricated neat and blend films (A) P(3HB)/P(3HO) (B) P(3HO)/P(3HB). (A1,B1) Seeded HaCaT cells on day 3 showing its attachment and proliferation on the blend films. (A 2)Seeded HaCaT cells at day 5, on the P(3HB)/P(3HO) film; (B 2) confluent growth of the horn cells observed on P(3HO)/P(3HB); (A 3, B3) arrangement of cells in horn sheets on day 7on the blend films. (1) Uncovered PHA matrix, (2) Cell layer and (3) Spreading of the cell on PHA film.

The growing HaCaT cells seeded onto the fabricated P(3HB)/P(3HO) 5:1 and P(3HO)/P(3HB) 5:1 blend films were also analysed using SEM to observe their the morphology of the cells and their attachment on the films. The cells were able to attach well on both the blend films as seen in Figure 4.8. HaCaT cells are known to form four distinct layers. This arrangement of cell layers from the bottom to the outermost layer is as follows: (1) stratum basale (basal layer), (2) stratum spinulosum, (3) stratum granulosum and (4) stratum corneum (horn sheet layer) (Schoop *et al.,*1998).The horn sheets are the outermost and most mature or differentiated stage of the HaCaT cell line. In both the neat films, P(3HO) and P(3HB) confluent growth was observed by day 5, however it was speculated that arrangement of the cells into mature coherent horn cells occurred only by day 7.

In the blend films too, the cells showed good growth and proliferation. The covering of the cell layer (Figure 4.8(A2-B-2) was however observed by day 5 in both blend films. As discussed above the horn sheets are the most mature stage of the HaCaT cell line. Hence, these observations imply that the cells had successfully attached, proliferated and grown on the fabricated blend films. However the P(3HO)/P(3HB) 5:1 showed relatively better (Figure 4.8(B3)growth compared to the P(3HB)/P(3HO) 5:1 film. In conclusion SEM observations show that the HaCaT cells were able to proliferate better on the P(3HO)/P(3HB) 5:1 blend films with the presence of horn sheets on the 5 th day as opposed to the P(3HO)/P(3HB) 5:1neat and P(3HB)/P(3HO) 5:1 blend films. These observations correlated well with the proliferation data observed using the Neutral Red Assay.

4.3 Discussion

In this section the microstructural, mechanical, thermal properties, and cell proliferation studies on the PHA blends were discussed.

4.3.1 Properties of blend films

In this project the micro structural, mechanical, thermal properties and biocompatibility of the fabricated blends films were investigated in order to assess if the properties of the films make them suitable for the proposed applications.

4.3.2 Microstructural properties

Surface and micro structural features of a biomaterial have serious implications on it's biocompatibility and area of applications. Hence, to evaluate the micro structural properties the fabricated films were analysed using scanning electron microscopy (SEM), white light interferometry and contact angle study. The incorporation of a small amount of P(3HB) into the P(3HO) polymer has indeed changed the surface morphology by introducing a rough topography on the surface for P(3HO)/P(3HB) (5:1) blend films. Likewise, in the case of P(3HB/P3HO) (5:1) incorporation of a small amount of P(3HO) into P(3HB) matrix also caused a rough surface topology compared to neat P(3HB). Similar results of increasing roughness were found by Reis *et al.,* when starch was blended with (P3HB-co-3HV). In this study the SEM analysis revealed a rough topology on the (P3HB-co-3HV)/starch blend surface with respect to (P3HB-co-3HV). Likewise, Rossana *et al*., (2006) also studied the compression moulded P(3HB)/starch blend and found rough topology on the blend surface compared to neat P(3HB).Estelle *et al.,* (2004) observed a plain surface for the pure P(3HO) film. However the blend film composed of P(3HO)/PLA was found to be clearly rough on its surface. They concluded that the PLA was embedded in the P(3HO). Studies conducted by Bian *et al.,* (2009) found that the copolymers of P(3HB-co-HHx) and P(3HB-co-3HV-co-3HHx) films had

rough surface topology compared to neat P(3HB) films. These results suggest that the copolymer of SCL-MCL also exhibited a rough surface topology, comparable to the neat polymers. Interestingly these results reveal that copolymer of P(3HB-co-3HHx) as well as blend of P(3HO)/P(3HB) (5:1) exhibited a property of increased roughness on the surface.

4.3.3 Thermal properties of the blends

Polymer blends constitute a mixture of two polymers in the amorphous phase and may exist as a single phase of intimately mixed segments of the two components. Such a blend is homogeneous and is considered to be miscible in the thermodynamic sense. When a mixture of two polymers separate into two distinct phases of the individual components, the blend is heterogeneous and is considered to be immiscible in the thermodynamic sense. Miscibility between any two polymers is detected by the presence of a single glass transition temperature (T_g) and melting point ($T_{m)}$) intermediate between those of the two component polymers of the blends. Immiscibility of two polymers is demonstrated by the retention of the T_g and T_m values of both the individual components in the blend (Shanfeng and Cai., 2010;Verhoogt *et al.,* 1994). For example, immiscibility of P(3HB)/PBA (polybutyle nadipate)and P(3HB)/EPR(ethylenepropylene rubber) blends have been demonstrated by the presence of the T_g values of the individual components in the DSC analysis of the blend (Won., *et al* 2001). In a contrasting example, the miscibility ofP(3HB)/polymethacrylate) (PMA) blends have been observed by DSC by An *et al.*,1999. They concluded that P3HB) and PMA are miscible with each other due to the presence of single glass transition and melting point of the blend.

According to Verhoogt *et al.,* a miscible blend containing a semi crystalline polymer should possesses two important characteristics in addition to the single T_g of the mixed amorphous phase. The first characteristic is that a change is observed

in the crystallization behaviour of the semicrystalline polymer. A change is observed in the growth rate of spherulites when a miscible polymer constituent is added. The other characteristic demonstrating miscibility of blends containing a semi crystalline polymer is the change in the melting point of the polymer blend when an amorphous polymer is present.

The thermal analysis of the P(3HB)/P(3HO) (5:1) blend showed a single glass transition temperature (T_g) value of 5.8^0C followed by melting of the polymer blend at 57.22^0C and T_m 164.91^0C. This observation indicates that P3HB) and P(3HO) in 5:1 are partially miscible with each other due to the presence of two melting point of the blend. There are two reasons that indicates the blends are not immiscible with each other. The first reason is the presents of T_m at 157.22 which indicates, the depression of melting point of P(3HB) (167^0C). The second reason was the presence of a single glass transition temperature (T_g) of the blend indicates the miscibility of the blend. However, the present of an another peak which was more similar to peak of P(3HB) indicated the partial miscibility of the blend. The presence of a single glass transition temperature (T_g) and melting point ($T_{m)}$) was observed by studies conducted by Xing *et al.,* (1998) with P(3HB)/PVAc-co-VA)(polyvinyl acetate-co-vinyl alcohol) blend. They found that the PVAc-co-VA)s with vinyl alcohol content of 15mol% formed a miscible blend with P(3HB) in the solution cast samples. However, P(3HB) and PVAc-co-VA) with 15 mol%, vinyl alcohol did not form miscible blends in 20/80 composition, instead the blend showed a partially miscibility in the melt state.In an another similar study Lotti *et al.,* (1993) investigated the miscibility and isothermal crystallization behaviourof the P(3HB) and polymethyl methacrylate (PMMA). They observed a single phase glass transition temperature for the P(3HB)/PMMA blends containing up to 20 wt% P(3HB), which indicated the miscibility of P(3HB) in PMMA. When the concentration of P(3HB) exceeds 20 wt%, partially miscibility of P(3HB) with PMMA was observed which represents the solubility limit of P(3HB) in PMMA. In a contrasting example for immiscible blends, Estelle*et al.,*(2004) observed two T_m

peaks for P(3HO)/PLA blends at 51^0C and 177^0C. P(3HO) is a semi crystalline polymer with a melting point of 57^0C and PLA is also a semi crystalline polymer with a melting point increasing from 164^0C to 180^0C. Hence, the crystallinity of P(3HO) remained unaffected by the presence of PLA in the blend. They thus concluded that the two polymers P(3HO) and PLA were immiscible.

In the case of the P(3HO)/P(3HB) (5:1) blend a single glass transition temperature of -36.99^0C and single T_m was observed at 164.85^0C. Hence, it was indicated that P(3HO)/P(3HB) (5:1) blend miscible with each other. The addition of a small amount of semicry stalline polymer P(3HB)to an amorphous P(3HO)expected to increase in the overall crystallinity of the P(3HO)/P(3HB) 5:1 blend. However, no T_c was observed for the blend. The melting temperature of (164.85^0C) was mostly observed due to the presence of P(3HB) which is crystalline in nature. These results are consistent with the studies conducted by Bhatt *et al*., (2007) which involved that the blending of MCL-PHA with rubber leading to the formation of a polymer blend with single melting point of 90^0C higher that that observed for MCL-PHA. Their results thus confirmed that the two polymers had successfully interacted leading to a shift in melting point of the newly synthesized polymer blend.

4.3.4 Mechanical properties of blends

The modification of mechanical properties of P(3HO) and P(3HB) have also been investigated in this study. In this study the blend film with a higher amount of P(3HO) and a lower amount of P(3HB) i.e. P(3HO)/P(3HB) (5:1) blend showed an E value of 37 MPa and elongation at break of 160%. The film exhibited such low stiffness because of the high P(3HO) content which has a low E value. Also, since P(3HO) is very flexible and elastomeric in nature as illustrated by the high elongation to break value of 200% the blend had a high elongation at break.

Increasing amount of P(3HB) as in the case of P(3HB)/P(3HO) (5:1) blend increased the stiffness of the film as reflected in the value of high value of Young's modulus, 136 MPa. Also as expected the percentage of elongation was reduced to just 35.81%, since P(3HB) is quite brittle in nature, with an elongation at break value of 2.56%. These observations are consistent with mechanical properties previously reported by Lianlai *et al.,*1996 for P(3HB)/PCL (polycaprolactone), blends. They observed a gradual decrease in the crystallinity and stiffness the P(3HB)/PCL blend, when the percentage of PCL in the blend was increased. They concluded that the blends exhibited low stiffness and increased elongation at break compared to neat P(3HB).Similar observations were also made in the study conducted by Rasal and Douglas, (2008) where it was observed that blending of 10% P(3HHx) to PLA led to the loss of stiffness of PLA and the corresponding elongation at break of the blend was increased. P(3HHx) is a flexible polymer with low tensile strength and higher elongation to break compared to PLA. The incorporation of P(3HHx) in to the PLA led to a decrease in the stiffness and increase of elongation at break of the blend. Sun *et al.,* (2009) studied the mechanical properties of a blend made up of silk fibroin (S/F) and P(3HB-co-HHx). In this study they found that SF/P(3HB-co-HHx) films exhibited a maximum tensile strength of 11.5 MPa and the an elongation at break of 175%, which was lower than P(3HB-co-HHx) film with an tensile strength of 11.7 MPa and elongation at break of 204%. In another study conducted by Reis *et al.,*(2008) on a P(3HB-co-3HV)/starch blend, they found that the tensile strength and elongation at break decreased with an increase in the starch content in the blend with P(3HB-co-3HV). Hence, in all cases including the observations made in this study, the final property of the blend is found to be intermediate to that of the individual neat PHAs.

4.3.5 Water contact angle studies

Water contact angle studies of the neat and blend PHA films were carried out to assess their hydrophobicity. As expected the studies showed that, the blending of MCL and SCL PHAs had an effect on the water contact angle on the PHA blend films. The blend films (P(3HO)/P(3HB) (5:1)= 80^{o}(P(3HB)/P(3HO) (5:1)= 90.39^{o}indeed showed increased water contact angle as opposed to the neat P(3HB) (70.37^{o}) film. However, neat P(3HO) (99.94^{o}) showed highest degree of contact angle compared to other neat and the blend PHA films. The water contact angle (θ_{H2O}) is a measure of the hydrophilcity or hydrophobicity of a material surface. According to Peschel *et al.,* 2007 surfaces with θ_{H2O} less than 70^{o}are considered to be hydrophilic and θ_{H2O} greater than 70^{o}are considered to be hydrophobic.

The contact angle values (θ_{H2O}) observed for other PHAs in literature are 90^{o} for poly(3-hydroxybutyrate-co-3-hydroxyvalerate), (Ji *et al.,* 2008) and the 98^{o} for P(3HO-co-3HU) poly(3-hydroxyoctanoate-co-3-hydroxy-10-undecenoate (Furrer *et al.,* 2006). Hence, both neat and blend PHA films are hydrophobic in nature even though, the neat P(3HB) (70.37^{o}) observed to be moderately hydrophobic in nature. Also, according to Zisman*e*, 1964 and Namen *et al.,* 2008 the contact angle on a solid surface decreases as the surface becomes rougher. Hence, the P(3HO)/P(3HB) 5:1 blend which showed a high surface roughness exhibited a decreased water contact angle of 80^{o} compared to compared to neat P(3HO) and P(3HB)/P(3HO) 5:1blend which showed higher water contact angles of 97^{o} and 90^{o} respectively. The fabricated neat P(3HO) showed the highest water contact angle probably due to its very smooth surface topology. Hence, it was observed that the smooth surface of the P(3HO) led to higher degree of contact angle. The decrease in the water contact angle for the blend films with respect

to neat P(3HB) were consistent with the observations made by studies conducted by Namen *et al* on the surface properties of dental polymers Solitaire 2, Ariston® , pHc, and Tetric Ceram. According to their studies the roughness caused a decrease in the contact angle sof unfinished dental polymers compared to finished polymers. In an another study the decrease in water contact angle of the P(3HBV)/HE(poly[(R)-3-hydroxybutyrate]-alt-poly(ethyleneoxide) blend compared to neat P(3HBV) was observed by Li *et al*., 2009. The SEM analysis showed a rough topology of the P(3HBV)/HE blend compared to neat P(3HBV) film. These results therefore show that contact angles of the fabricated material can be affected by the surface roughness.

The increase in the surface roughness of the film is governed by the method of fabrication method of the film. It was also noted that there is no change occurred in the innate hydrophobicity of the PHAs. However, the change in the surface roughness in fact affected the water contact angle there by also the hydrophobicity of the fabricated film. Hence, it was observed that that water contact can be affected by surface roughness of the both neat and blend PHAs, which could have resulted in the decrease in the hydrophobicity of the rough surfaced P(3HO)/P(3HB) 5:1 and P(3HB) films compared to smooth surfacedP(3HO).

4.3.6 The *in vitro* cell biocompatibility studies

Biocompatibility is an important requirement for any tissue engineering application for a suitable polymer. The cell proliferation studies using HaCaT cells showed that the cell proliferation increased progressively on both the neat and blend films. The optical density (OD) value obtained from Neutral Red assay was proportional to the live cell numbers. Hence, we can measure the OD values to evaluate the relative cell amount on the films. The optical density (OD-540) value obtained from the Neutral Red assay was proportional to the

number of live cells. It was found that the OD values showed a significant difference between neat and blend films in 3rd day of cell culture. On day 5, the OD values of cells on the P(3HO)/P(3HB) 5:1 are significantly higher than that on the other neat and P(3HB)/P(3HO) 5:1 blend. This indicated that the cells on the P(3HO)/P(3HB) 5:1 blend proliferated faster. At day 7, the OD values corresponding to the cells on the P(3HO)/P(3HB) 5:1 kept increasing while P(3HB), P(3HO), and P(3HB)/P(3HO) 5:1 films comparatively decreased. This demonstrated that the P(3HO)/P(3HB) 5:1 blend can support the cell growth for longer time than the other neat and blend films, and hence is better suited for tissue regeneration.

It is known from the previous work that a rough surface provides a better matrix for cell attachment and proliferation (Xi *et al.*, 2008). Therefore, the increased proliferation of the HaCaT cells on the P(3HO)/P(3HB)(5:1) films can be attributed to rough topography of the surface of the film. The presence of irregular protrusions on P(3HO)/P(3HB)(5:1) film provided increased surface area thereby, increasing the available surface area for cell attachment. A similar observation of increased chondrocyte proliferation was observed for P(3HB)/P(3HBHHx) blend as opposed to neat P(3HB) scaffolds by Zhong *et al.*, (2005). Their studies also concluded that the increased cell proliferation on the blend scaffold was due to the increased roughness of the film. Zha*et al.*,(2006)studied the biocompatibility of fibroblast cells on the poly(hydroxybutyrate)/poly(ethylene glycol) P(3HB)/PEG blend films. In this study they found that the addition of PEG improved the cell compatibility of P(3HB). Their studies also concluded that the increased cell proliferation on the blend polymer was due to the increase in the hydrophilicity and roughness of the film. Sun *et al.,* (2009) studied blends made up of silk fibroin (S/F) and P(3HB-co-3HHx). In order to characterize the cell affinity of the blend, endothelial cells were seeded on the SF/P(3H-co-3BHHx) films. The histo chemical analysis demonstrated that cells attached and reached nearly

100% confluence on the SF/P(3HB-co-HHx) films, which was much faster than that on the pure P(3HB-co-HHx) film (Min *et al.,* 2009).

In addition to Neutral Red assay, SEM analysis of the HaCaT cells also confirmed that the polymer matrix was able to support cell growth. HaCaT cells are known to form four distinct layers as the cells divide and differentiate as described in 4.2.6. The outermost layer is reported as stratum corneum (horn sheet layer).It was speculated that that the HaCaT cells had already arranged themselves into horn sheets (the most mature stage) by day 5on P(3HO)/P(3HB)(5:1) film as seen on the SEM.However, on the neat films, and P(3HB)/P(3HO)(5:1), the horn sheet arrangement of the films appeared only on day 7.The arrangement of horn sheets in the P(3HO)/P(3HB)(5:1) blend film within day 5 indicates that the HaCaT cells have been able to grow and mature faster in this film as compared to neat and blend film. Similar observation of increased osteoblast MC3T3 proliferation was observed for a gelatin/P(3HB-co-3HHx) blend as opposed to neat P(3HB) scaffolds by Wang *et al*. (2004) Their studies concluded that the increased cell proliferation in the blend was due to the increase in the hydrophilicity and roughness of the film due to the incorporation of gelatin in the blend. According to Mei *et al.,* 2006, a rough surface provides a better matrix for cell attachment and proliferation. As discussed above the increased proliferation of the HaCaT on the blend films could be mostly due to the increased roughness. In the case of P(3HO)/P(3HB)(5:1) blend, confluent growth was observed on 5th day unlike that on P(3HB)/P(3HO)(5:1) blend. Like neat PHA film the horn sheet arrangement of HaCaT cells were observed only on day 7 for P(3HB)/P(3HO)(5:1) blend film. This is probably due to the relatively smoother surface of this blend compared to P(3HO)/P(3HB)(5:1) blend film.

4.3.7 P(3HO)/P(3HB) blend as a potential biomaterial for nerve tissue engineering applications

Among various strategies for better recovery of nerve functions, autologous nerve grafts are considered as most reliable for bridging long gaps, but the major disadvantage of using autologous grafts are limited tissue availability, donor site morbidity, and potential mismatch of tissue structure and size. Hence various bioengineered nerve grafts have been developed from polymeric materials to meet the requirements for peripheral nerve regeneration. According to Bian*et al*., the material used for construction of nerve conduits should not be too brittle or too flexible for obtaining optimum nerve regeneration and recovery (Bian*et al.,* 2009). Natural polymers already utilized for fabricating nerve conduits include chitosan, collagen, gelatine and hyaluronic acid (HA). These natural polymers offer excellent biocompatibility, support for cell attachment and functions, avoid serious immune response, and can degrade by naturally occurring enzymes. However, these polymers generally need extensive purification, characterisation and modification for the nerve tissue engineering application. Further, they lack adequate mechanical strength and degrade relatively fast *in vivo*. For example, chitosan, a copolymer of D-glucosamine and N-acetyl-D-glucosamine, is a well known biodegradable polysaccharide with high glass transition temperature (T_g of 203°C) and relatively low thermal stability. Chitosan is brittle in nature with an elongation at break of 5.4-11.4 MPa and a Young's modulus value of 14.8 (Camacho*et al.,* 2010). The brittle nature of the chitosan make it less suitable for the preparation of nerve conduits hence, the mechanical properties of chitosan scaffolds need to be improved. Similarly, gelatin and collagen have low tensile strength and elongation at break, which is considered as the main disadvantage of these polymers. (Shanfeng and Lei., 2010).

The biomaterials being studied for the preparation of the nerve conduits are either too stiff like chitosan, whose tensile strength value range in 81MPa or are very soft like collagen gel whose tensile strength value range in 0.0016 MPa. Hence, these natural materials may not be suitable for the construction of nerve conduits due to the lack of adequate mechanical strength and fast *in vivo* degradation. Similarly, some PHAs also suffer from lack of adequate mechanical strength for the preparation of nerve conduits and others are not flexible enough. P(3HB) is rigid with a reasonably high tensile strength of 40 MPa and a low elongation at break value of 6% (Lee *et al*., 1995). On the other hand MCL-PHAs like P(3HO) exhibit low tensile strength (17MPa) and high elongation at break (350%) (Marchessault *et al*., 1990). Hence P(3HB) has good tensile strength but is brittle and stiff and lacks ductile properties. On the other hand P(3HO) is flexible and elastomeric, however they lack enough strength for the construction of nerve conduits. Therefore, the main aim of this project was to improve the mechanical properties of both P(3HB) and P(3HO) by preparing blends of these two polymers.

It was found that the P(3HO)/P(3HB)5:1 blend had mechanical and thermal properties that are suitable for the preparation of the of nerve conduits. Itwas found to be flexible and showed good elongation at break value of 160%.Also, the blend showed the required tensile strength of 1.5 MPa which is comparable to values recommended for the preparation of nerve conduits.For example, Le *et al.,* 2007 studied PLGA/gelatin blend for the preparation of nerve conduits and observed that the tensile strength of the blend was between 0.2-3.75 MPa. In an another study, Yucel *etal.,* 2010 proposed the P(3HBV)/PLCA (Poly(L-lysine citramide) blend for nerve conduit preparation and found that the tensile strength of the film was 1.05 MPa. Bian *et al.,* 2009 prepared nerve conduits using dip coating and leaching method and observed that the tensile strength of conduits with non uniform wall porosity was 2.29MPa and with uniform wall porosity was 0.94MPa. In an another study Borkenhagen *et al.,*1998 studied *in*

vivo performance of biodegradable nerve guide channels based on the biodegradable urethane/ P(3HBV) blend and showed that 1.9 MPa was the ideal tensile strength of the nerve conduits. The flexible and strong mechanical properties of the P(3HO)/P(3HB) 5:1 blend makes it suitable as a biomaterial for nerve tissue engineering. Moreover, it also showed better biocompatibility to seeded HaCaT cells compared to other neat and the blend PHA. The P(3HO)/P(3HB) 5:1 blend can be further tailored by including neuro protective drugs such as Ginsenoside Rg1 so that, this could be used for enhanced neural regenerative performance of the blend (Ma *et al.*,2010).

Chapter:5 Conclusions and Future work

5.1 Conclusions

PHAs represent a class of polymers that have immense potential because of their biocompatibility and biodegradability, so these polymers have been used for a number of industrial, agricultural and medical applications. In fact, research on the medical applications of PHAs is increasingly becoming the focus of a lots of attention, because of the diversity and tailorability of these polymers. This present study was carried out with an aim to producing polyhydroxyalkanoates for biomedical applications, more specifically for the nerve tissue engineering. Mainly two different types of PHAs, P(3HB) and P(3HO) have been produced and used for the preparation of blends with an objective to make materials suitable for the tissue engineering application. This multidisciplinary project encompassed a wide range of techniques ranging from microbiology and materials science to cell biology. The most important conclusions from the chapters are summarised below along with some recommendations for the future investigations, which might further enhance the applicability of the blends as neural tissue engineering scaffolds.

The study was initiated with production of the SCL-PHA,P(3HB) from *Bacillus cereus* SPV. Previous studies on the *B. cereus* SPV at the University of Westminster has been proven that the it is able to accumulate P(3HB) when grown using glucose, (Valappil *et al.*,2006). In this study,*B. cereus* SPV was initially grown in the Kannan and Rehacek medium using sucrose as the carbon source in shaken flask cultures. Under the nitrogen limiting conditions *B. cereus* SPV grew well and accumulated maximum polymer yield of the (38.0% dcw). The study was continued with batch fermentation studies with monitored fermentation conditions in a 2 litre fermenter. Improved polymer yield was observed in this study with a maximum yield of 44.60% dcw at 48 hours of fermentation with an increased PHA accumulation of 6.6% dcw compared to the shaken flask experiment. From these studies, it was understood that nutrient limitations plays an important role in driving organisms to accumulate PHAs. In addition, controlled cultural conditions in fermentor allow better bacterial growth leading to significant yield.

Further the effects of different nutrient limitation on PHA accumulation was studied with an aim to improve the yield of P(3HB)by *B. cereus* SPV. Hence, the organism was subjected to multiple nutrient limitations. An enrichment media for *B. cereus* SPV was modified, with three simultaneous nutrient limitations including nitrogen, potassium, and magnitium, for the production of P(3HB)using sucrose as the carbon source. An improved yield of the polymer production (52.64% dcw) was observed in this novel medium compared to Kannan and Rehacek medium. In this study by limiting nutrients we obtained improved PHA accumulation of 14.64% dcw within 48 hrs compared to shaken flask studies using Kannan and Rehacek medium. This observation thus revealed the importance of multiple nutrient limitations for the enhanced production of PHAs by *B. cereus* SPV. The analysis of the extracted polymer produced was carried out using NMR, and it was concluded that the organism had accumulated a homo polymer of P(3HB).

Studies were also carried out for the biosynthesis of P(3HO) using *Pseudomonas mendocina.* Previous studies conducted on *P.mendocina* at the University of Westminster showed that, nutrient limitations play an important role in driving the organism to accumulate PHAs. In these studies it was found that, the organism has a selective preference for a mineral salt media (MSM) to grow and accumulate PHAs. Hence, in the present study *P. mendocina* was grown using MSM medium for the production of PHA. It was found that the organism was able to grow in the MSM media to accumulate PHAs, when grown on both structurally related (sodium octanoate) and unrelated (sucrose) carbon sources. When the organism was fed with sodium octanoate as the carbon source, production of the P(3HO) was achieved and the maximum yield obtained for the polymer was 29.43% dcw of it's dry cell weight, at 48 hours of growth, in shaken flask fermentation. A batch fermentation study on the production of this polymer was also carried out using 2 litre fermenters using the same mineral salt medium. In this experiment an improved yield of polymer (33.5 wt % dcw)

was noted with a 3.57% increased yield compared to the shaken flask fermentation studies carried out in this study. The structural analysis using NMR proved that the organism mainly accumulated a homo polymer of 3-hydroxyoctanoate, P(3HO). According to the current available literature, this production of a homopolymer of 3-hydroxyoctanoate, P(3HO) is a very unusual result, till date most of the MCL-PHA production by *Pseudomonas sp* have shown the production of copolymers of MCL-PHAs e.g, (P(3HB-co-3HHx) and P(3HO) with varying degrees of other monomeric units (Ronald *et al.,* 1988, He *et al*., 1998). This result was a confirmation of the observation made by Rai *et al.,* 2011.

Although *P. mendocina* was able to accumulate MCL-PHAs, the yield was found low in shaken flask experiments (29.43% dcw) and (33.5 wt % dcw) in bath fermentation. So, in order to achieve rapid cell growth, and higher yield, fed batch fermentation was done, in which glucose was provided in the initial stage of the fermentation. This was followed by intermittent addition of sodium octanoate. This strategy increase the polymer accumulation in the organism, giving a maximum yield of 37.09 % dcw at 48 hours of fermentation with 7.66%more yield compared to the shaken flask fermentation in this study. *P.mendocina* was also grown using the structurally unrelated carbon source such as sucrose for the production of PHAs. The polymer obtained from this fermentation was structurally analysed by NMR and identified as the homopolymer of P(3HB). Such a production of SCL-PHA by *Pseudomonas mendocina* from a structurally unrelated carbon source like sucrose has never been reported previously in the literature. The maximum yield obtained for this polymer was 27.19% dcw at 48 hours of fermentation. The ability to produce P(3HB) can be related to the unusual broad substrate specificity of the PHA synthase present in *P. mendocina.*

The downstream processing study showed that, the kind of extraction method employed had an effect on the yield of the polymer, molecular weight and lipopolysaccharide LPS) content of the P(3HO). Among different extraction

methods used in this study, the dispersion method was found to be an easy method to extract the polymer from the bacterial biomass with a maximum yield of 30 % dcw. But the main disadvantage of this method is the digestion of the PHA by sodium hypochlorite solution, which mainly affects the average molecular weight of the polymer. The $CHCl_3$extraction also showed reasonably high polymer yield of 28% dcw. The soxhlet extraction, gave the lowest yield of 12% dcw as opposed to the dispersion method and that with $CHCl_3$.By subjecting the extracted polymer to repeated purification process the LPS content of the polymer was reduced. In addition in this study a novel PHA recovery and purification method based on the osmotic and detergent based lysis and purification was successfully developed. Among different NaCl concentration used, 20% was found to be efficient in facilitating improved cell lysis. The lipopolysaccharide contamination of the polymer during the extraction processes was the main problem related to other extraction methods and this was effectively alleviated by 1.5 % Triton X-114 used in this extraction method. Thus the lippopolysacharide and protein impurities could be effectively removed by this novel method to obtain very pure polymer.

In this study the blending approach has been used, in order to improve the mechanical properties of P(3HB) and P(3HO). Blending of the flexible and soft P(3HO) with the brittle and stiff P(3HB) was carried out in two ratios 5:1 and 1:5. The blends were prepared by a repeated heating and sonication method. The incorporation of P(3HB) into the biopolymer matrix of P(3HO) or P(3HO) into a predominantly P(3HB) matrix resulted in improved the mechanical properties with respect to the neat polymers. P(3HB) served the purpose of increasing the tensile strength of the blend, whereas P(3HO) served the purpose of increasing the elasticity of the material. The neat and blend films of P(3HB) and P(3HO) were fabricated by the solvent casting method. The characterisation of the neat and blend revealed that blending resulted in improved mechanical and thermal properties of the neat polymers.

The surface and micro structural features of the blend and neat polymers were analysed in detail using scanning electron microscopy and white light interferometry. The SEM analysis of the fabricated films revealed that the P(3HO) neat film had a smooth surface topography. In the case of neat P(3HB) the surface topology was found to be less smooth compared to the neat P(3HO). The surface topology of both the blends were found to be rough. However, the P(3HO)/P(3HB) (5:1) blend film was found to be more rough with minute protrusions on the surface with respect to P(3HB)/P(3HO) (5:1) blend. The surface roughness analysis was further confirmed by the white light interferometry. The increased roughness of the blend film was confirmed, where typical RMS roughness observed for the P(3HO)/P(3HB) (5:1) blend film was 2.9μm as opposed to RMS roughness value of 0.54μm for the P(3HO) neat film. In the case of P(3HB)/P(3HO) (5:1) blend film the RMS value of roughness was 2.04μm as opposed to 1.105μm for the P(3HB) neat film. It was observed that the surface properties of the blend films were greatly affected by the amounts of P(3HB) or P(3HO) incorporated in the blend.

Studies were also carried out to determine the thermal properties of P(3HB) extracted from the *B. cereus* SPV grown on sucrose as carbon feed. The thermal analysis of P(3HB) showed that the polymer sample has a high melting temperature, T_m of 167.39 °C and low glass transition temperature, T_g of 2.43°C and a T_c value of 54.33 °C. The results showed that the extracted polymer has the characteristic properties of the SCL-PHA. Studies were also carried out to determine the thermal properties of P(3HO) extracted from the *P. mendocina* grown on sodium octonoate as carbon feed. Thermal analysis results showed that the polymer exhibited a low glass transition temperature, (T_g of -32.86°C) and a low melting temperature of 50.36°C. The analysis showed no T_c value for the polymer. All these results proved that the extracted polymer has the characteristic properties of the MCL-PHA family.

The thermal properties of the blend PHAs were also studied by carrying out by the differential scanning calorimetry. The P(3HB)/P(3HO), 5:1 blend showed an increase in the glass transition temperature (T_g of 5.84°C), compared to the neat P(3HB) (T_g 2.43). Incorporation of P(3HO) in the P(3HB) (1:5) proportion resulted in a slight decrease in the melting temperature (164.91°C) of the blend compared to the neat P(3HB) (167.39°C). Both the blend P(3HB)/P(3HO) 5:1 blend and neat polymer P(3HB) films showed a T_c values 69.58°C and 54.33°C respectively. The P(3HO)/P(3HB) 5:1 blend showed a lower glass transition temperature of (T_g) value of -36.99^{0}C compared to the neat P(3HO) (-32.86°C).Incorporation of P(3HB) in the P(3HO) matrix resulted in two melting temperatures,164.85°C and 157.22°C compared to neat P(3HO) (50.36°C). Both neat P(3HO) and P(3HO)/P(3HB) (5:1)showed no T_c value for the polymer. The thermal analysis of the blend polymer showed that P(3HO)/P(3HB) (5:1) was miscible with each other since only one value was observed for the T_m and the T_c. P(3HB)/P(3HO) (5:1) observed to be partially miscible.

In this study the blend film with a higher wt% of P(3HO) and a lower wt% of P(3HB) i.e. P(3HO)/P(3HB) (5:1) had a Young modulus value of 3.7 MPa with a tensile strength of 12 MPa and elongation to break of 160%. The film exhibited such low stiffness because of the high P(3HO) content as P(3HO) is very flexible and elastomeric material in nature. Increasing the amount of P(3HB) as in the case of P(3HB)/P(3HO) (5:1) increased the stiffness of the film ie, 4.99 MPa with a Young's modulus value of 136MPa. The elongation at break of the P(3HB)/P(3HO) (5:1) was reduced to just 35.81%. This could be because P(3HB) is hard and brittle in nature with an 2.56% of elongation at break compared to P(3HO).

The water contact angle value for neat 5wt% P(3HB) film was 71.04^o and for P(3HO) was 97.57^o. In the case of blend films P(3HO)/P(3HB) (5:1) and P(3HB)/P(3HO)(5:1), the θ_{H2O} was 92.34^o and 88.08^o respectively. The water contact angle studies revealed that both the neat and blend polymers are hydrophobic in nature.

Biocompatibility analysis of the blend polymers using HaCaT cells proved that the special topological features of P(3HO)/P(3HB) 5:1 was desirable for the improved cell proliferation and attachment for the cells. It was observed that there was an increased growth of the cells on the blend films when compared to the neat films. The highest growth of the HaCaT cells on the P(3HO)/P(3HB) 5:1 was observed as opposed to neat and P(3HO)/P(3HB) 5:1 blend films. Also, the growth of the cells on the P(3HB)/P(3HO) 5:1 blend film was higher than the P(3HB) neat film. These results were further confirmed by the SEM analysis by observing the morphology and attachment of the cells onto the neat and blend films. The SEM analysis revealed that the arrangement of horn sheets of HaCat cells on the P(3HO)/P(3HB)(5:1) blend film appearedwithin 5 days of cell seeding. This indicated that the HaCaT cells had been able to grow and mature faster on P(3HO)/P(3HB)(5:1) blend film compared to P(3HB)/P(3HO) 5:1 blend.At the same time the appearance of horn sheets on P(3HO), P(3HB) and P(3HB)/P(3HO) 5:1 blend films appeared on the 7^{th} day of seeding of cells. This showed that, although HaCaT cells were growing and maturing relatively slowly on these films. These materials were biocompatible in nature.

The P(3HO)/P(3HB) 5:1 blend films fabricated in this study exhibited better biocompatibility for the seeded HaCaT cells. The blending method used in this study led to the formation of small protrusions on the surface of P(3HO)/P(3HB) 5:1 blend which was found to be advantageous for the cell attachment and proliferation. In addition this observation indicated that these blends might be better suitable for tissue engineering applications. The mechanical properties of the fabricated P(3HO)/P(3HB) were found to be suitable to be used for nerve tissue engineering.

Hence, in conclusion, This study has helped to provide an insight into the accumulation of P(3HB) by *B. cereus* SPV under multiple nutrient limitations. Also the PHA accumulation behaviour of the *P. mendocina* was studied and the homopolymer of P(3HO) was produced. *P. mendocina* was also able to accumulate the homopolymer of P(3HB)which has never been reported in the literature previously. Blends were created for the first time and studied for possible tissue engineering application.

5.2 Concluding points

- Homopolymer of P(3HB) was produced using *Bacillus cereus* SPV using sucrose as sole carbon source.
- A novel modified essential media (MEM) for *Bacillus cereus* SPV was developed with three simultaneous nutrient limitations for the improved production of the P(3HB).
- Homopolymer of P(3HO) was produced using *Pseudomonas mendocina* using sodium octanoate as sole carbon source.
- Fed batch fermentation was achieved for improved production of P(3HO) using *Pseudomonas mendocina.*
- Homopolymer of P(3HB) was produced using *Pseudomonas mendocina* using sucrose as sole carbon source, a very unusual result.
- A novel PHA recovery and purification method based on the osmotic and detergent based lysis and purification was successfully developed.
- Blends of P(3HB) and P(3HO) were successfully made.Chemical, thermal, mechanical and microstructural analysis of the neat and the blend PHAs were carried out.
- The P(3HO)/P(3HB) 5:1 blend was found to have suitable mechanical properties, high biocompatibility and hence recommended by future use in tissue engineering, more specifically, nerve tissue engineering.

5.3 Future work

The results obtained during this study have given an understanding on the biosynthesis of P(3HO) by *Pseudomonas mendocina* and P(3HB) by *Bacillus cereus* SPV. The blends of these polymers were assessed for their application as a biomaterial for the medical applications specifically, the nerve tissue engineering. Therefore, based on these results it is possible to identify the areas of future research. The following experiments are suggested which could be carried out in the future.

5.3.1 Production of SCL/MCL copolymer

The results showed that *P. mendocina* was able to grow and accumulate P(3HO), when grown on structurally related carbon sources like sodium octanoate. At same time the same organism, when grown on structurally unrelated carbon feed like sucrose as sole carbon source, the organism was able to grow and accumulate P(3HB). These results indicate a fundamental diversity in the metabolic pathway involved in the polymer production in this organism. So, the synthesis of copolymers containing short and medium (SCL-MCL) poyhydroxyalkanoates by *P. mendocina* could be investigated by growing the organism in a medium containing a mixture of sodium octanoate and sucrose.

5.3.2 Optimisation of P(3HO) and P(3HB)

In this present study, it was noted that, one of the major limiting factors for using *P. mendocina* for the synthesis of P(3HO) and *Bacillus cereus* SPV for the synthesis of P(3HB) have been the low yield of the polymer. In this study initial studies have been carried out to find better fermentation conditions for both P(3HO) and P(3HB) production. However, in the future, studies could be carried out for finding optimum fermentation conditions, considering pH, carbon nitrogen (C/N) ratio and fermenter agitation (rpm) as main varying parameters. This analysis could be done using Response surface analysis methodology.

5.3.3 Construction of electro conductive scaffolds for nerve regeneration

In neural tissue engineering, electrical stimulation has been shown to enhance the nerve regeneration process. Therefore, suitable electrically conductive polymers could be incorporated into the polymer matrix in order to improve the electro conductive properties of the blend. The electrical and nerve regeneration properties of this blend will have to be studied in detail to assess for the nerve tissue engineering applications. Long term degradation studies need to be carried out in order to have a better understanding of the degradation behaviour, and the physical and chemical changes taking place in the polymer undergoing degradation.

5.3.4 Construction and characterisation of nerve conduits

The porous tubular nerve conduits could be prepared using polymer blends. Polymer conduits could be manufactured by dipping and leaching technique as described by Bian *et al.,* 2009. The microstructure features of the prepared conduits have to be studied using Scanning Electron Microscopy. Thermal characterisation of the conduits has to be studied by DSC analysis. In addition, mechanical characterisation of the conduits will also be carried involving the measurement of tensile strength (using DMA), and elongation at break. Biocompatibility analysis for the tubular nerve conduits will be conducted using a neural cell line.

5.3.5Biocompatibility studies of the nerve conduits

For neural tissue engineering application the studies must also be carried out to check the biocompatibility of the fabricated conduits with the neural cell lines.

The conduits must be evaluated for the cell attachment, proliferation and maturation of the seeded nerve cells. The potential to use the P(3HO)/P(3HB) 5:1 blend for the nerve conduits could be assessed through an *in vitro* biocompatibility test using the Schwan cell line.

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