Production of Polyhydroxyalkanoates by Pseudomonas mendocina using vegetable oils and their characterisation

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Production of Polyhydroxyalkanoates by *Pseudomonas mendocina* using vegetable oils and their characterisation

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ABSTRACT

Synthesis of Polyhydroxyalkanoates (PHAs) by *Pseudomonas mendocina*, using different vegetable oils such as, coconut oil, groundnut oil, corn oil and olive oil, as the sole carbon source was investigated for the first time. The PHA yield obtained was compared with that obtained during the production of PHAs using sodium octanoate as the sole carbon source. The fermentation profiles at shaken flask and bioreactor levels revealed that vegetable oils supported the growth of *Pseudomonas mendocina* and PHA accumulation in this organism. Moreover, when vegetable oil (coconut oil) was used as the sole carbon source, fermentation profiles showed better growth and polymer production as compared to conditions when sodium octanoate was used as the carbon source. In addition, comparison of PHA accumulation at shaken flask and fermenter level confirmed the higher PHA yield at shaken flask level production. The highest cell mass found using sodium octanoate was 1.8 g/L, whereas cell mass as high as 5.1 g/L was observed when coconut oil was used as the feedstock at flask level production. Moreover, the maximum PHA yield of 60.5% dry cell weight (dcw) was achieved at shaken flask level using coconut oil as compared to the PHA yield of 35.1% dcw obtained using sodium octanoate as the sole carbon source.

Characterisations of the chemical, physical, mechanical, surface and biocompatibility properties of the polymers produced have been carried out by performing different analyses as described in the second chapter of this study. Chemical analysis using GC and FTIR investigations showed medium chain length (MCL) PHA production in all conditions. GC-MS analysis revealed a unique terpolymer production, containing 3-hydroxyoctanoic acid, 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid when coconut oil, groundnut oil, olive oil, and corn oil were used as the carbon source. Whereas production of the homopolymer containing 3-hydroxyoctanoic acid was observed when sodium octanoate was used as the carbon source. MCL-PHAs produced in this study using sodium octanoate, coconut oil, and olive oil exhibited melting transitions, indicating that each of the PHA was crystalline or semi-crystalline polymer. In contrast, the thermal properties of PHAs produced from groundnut and corn oils showed no melting transition, indicating that they

were completely amorphous or semi-crystalline, which was also confirmed by the X-Ray Diffraction (XRD) results obtained in this study. Mechanical analysis of the polymers produced showed higher stiffness of the polymer produced from coconut oil than the polymer from sodium octanoate. Surface characterisation of the polymers using Scanning Electron Microscopy (SEM) revealed a rough surface topography and surface contact angle measurement revealed their hydrophobic nature. Moreover, to investigate the potential applicability of the produced polymers as the scaffold materials for dental pulp regeneration, multipotent human Mesenchymal stem cells (hMSCs) were cultured onto the polymer films. Results indicated that these polymers are not cytotoxic towards the hMSCs and could support their attachment and proliferation. Highest cell growth was observed on the polymer samples produced from corn oil, followed by the polymer produced using coconut oil.

In conclusion, this work established, for the first time, that vegetable oils are a good economical source of carbon for production of MCL-PHA copolymers effectively by *Pseudomonas mendocina.* Moreover, biocompatibility studies suggest that the produced polymers may have potential for dental tissue engineering application.

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LIST OF ABBREVIATIONS

alpha-MEM	**Alpha Minimal Essential Medium**
BDNF	**Brain Derived Neurotrophic Factor**
BF3	**Boron trifluoride**
BMSCs	**Bone marrow stromal cells**
$CHCl_3$	**Chloroform**
CO_2	**Carbon dioxide**
dcw	**dry cell weight**
DMA	**Dynamic Mechanical Analysis**
DMP-1	**Dentin matrix phosphoprotein 1**
DMSO	**Dimethyl sulfoxide**
DPSCs	**Dental pulp stem cells**
DSC	**Differential Scanning Calorimetry**
DSPP	**Dentin sialophosphoprotein**
3D	**3-Dimentional**
ECM	**Extracellular matrix**
EDTA	**Ethylene diamine tetraacetic acid**
FTIR	**Fourier Transform Infrared Spectroscopy**
GAPDH	**Glyceraldehyde 3-phosphate dehydrogenase**
GC	**Gas Chromatography**
GC-MS	**Gas Chromatography Mass Spectroscopy**
GPC	**Gel Permeation Chromatography**
GTR	**Guided tissue regeneration**
HA/TCP	**Hydroxyapatite/tricalcium phosphate**
hMSCs	**human Mesenchymal stem cells**
HMSCs	**Human bone marrow stromal cells**
3HD	**3-hydroxydecanoate**
3HDD	**3-hydroxydodecanoate**
3HHp	**3-hydroxyheptanoate**
3HHx	**3- hydroxyhexanoate**
3HV	**3-hydroxyvalerate**
ICAM-1	**Intercellular adhesion molecule-1**
LPS	**Lipopolysaccharides**

MCL-PHA	**Medium chain length polyhydroxyalkanoates**
M_n	**Number average molecular weight**
MSM	**Mineral salt medium**
MTT	**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide**
M_w	**Weight average molecular weight**
NaOCl	**Sodium hypochlorite**
NIST	**National Institute of Standards and Technology**
NT-3	**Neurotrophin-3**
OD	**Optical Density**
PBS	**Phosphate buffer saline**
PCL	**Polycaprolactone**
PDI	**Polydispersity index**
PDLSCs	**Periodontal ligament derived stem cells**
PECAM-1	**Platelet endothelial cell adhesion molecule 1**
PGA	**Poly(glycolic acid)**
PGA-PLA	**Poly(glycolic acid)-Poly(lactic acid)**
PHAs	**Polyhydroxyalkanoates**
PHO	**Poly(3-hydroxyoctanoate)**
P(3HB)	**Poly(3-hydroxybutyrate)**
P(3HB-3HHx)	**Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)**
P(3HB-co-3HV)	**Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)**
P(3HB-4HB)	**Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)**
P(3HO-co-3HH)	**Poly(3-hydroxyoctanoate-co-3-hydroxyheptanoate)**
P(4HB)	**Poly(4-hydroxybutyrate)**
P(4HB-co-glycolate)	**Poly(4-hydroxybutyrate-co-glycolate)**
PLA/PLGA	**Poly lactic acid/Poly(lactic-*co*-glycolic acid)**
PLG	**Poly(lactide-co-glycolide)**
PLGA	**Poly(L-lactide-co-glycolide)**
PLLA	**Poly(L-lactic acid)**
PTS	**Phosphotransferase system**
rCGNs	**rat Cerebellar Granule Neurons**
SCAP	**Stem cells from the root apical papilla**
SCL-PHA	**Small chain length polyhydroxyalkanoates**
SEM	**Scanning Electron Microscopy**

SHED	**Stem cells from exfoliated deciduous teeth**
TAG	**Triacylglycerides**
T_g	**Glass Transition temperature**
T_m	**Melting temperature**
VCAM1	**Vascular cell adhesion molecule-1**
VE-cadherin	**Vascular endothelial cadherin**
VEGFR2	**Vascular endothelial growth factor receptor-2**
VFAs	**Volatile fatty acids**
v/v	**Volume/Volume**
VVM	**Volume per Volume per Minute**
WAXD	**Wide angle X-ray diffraction**
XRD	**X-Ray Diffraction**

CHAPTER 1

INTRODUCTION

1. Introduction:

1.1. Polyhydroxyalkanoates (PHAs) and its importance

Plastics are synthetic or semi-synthetic organic solids, which are widely used for manufacturing industrial product since the 1940s (Shultz 1979, Panchal *et al.,* 2012). Moreover, the relatively low price, versatile nature and excellent physical and mechanical properties have made them a necessity in our lives to enhance the comfort and quality of life. Plastics are very much advantageous because as synthetic polymers, their structure can be chemically manipulated to have a wide range of strengths and shapes (Reddy *et al.,* 2003). Moreover, they can be easily moulded into almost any desired shape including fibres and thin films. Synthetic polyethylene, polyvinyl chloride and polystyrene are largely used in the manufacture of plastics (Reddy *et al.,* 2003). They have high chemical resistance and are more or less elastic, hence they are popular in many durable, disposal goods and as packaging materials (Panchal *et al.,* 2012). Due to their outstanding features, they have been used for different purposes worldwide such as components in automobiles, home-appliances, computer equipment, packages, construction, sports and leisure equipment and also in medical applications (Abou-Zeid 2001, Panchal *et al.,* 2012). Currently, the worldwide demand for plastics has increased to more than 240 million metric tonnes per year (Zinn *et al.,* 2001, Panchal *et al.,* 2012). However, uncontrolled usage of these plastic materials has led to the accumulation of huge amounts of non-degradable waste materials, which become a major concern in terms of the natural environment. In the UK, only about 3.2 million tonnes of domestic plastic refuse was collected annually, while about 150 million tonnes of plastic waste was disposed of by industry (Philip *et al.,* 2007, Panchal *et al.,* 2012). Moreover, the increased cost of crude oil, depletion of the world's oil reserves and public awareness of the environmental effects of synthetically produced materials has created a lot of interest in the development of biodegradable plastics (Philip *et al.,* 2007, Keshavarz and Roy 2010). Biodegradable plastics synthesised from renewable resources are now being considered as a potential replacement for commercial synthetic plastics due to their biodegradability and non-toxicity. This would help in resolving the problem of plastic waste disposal (Abou-Zeid 2001).

Most synthetic plastics are non-degradable polymers; for instance polyamides, polyfluorocarbons, polyethylene, polypropylene, and polycarbonate. However, some synthetic polymers are semi-degradable by microorganisms such as polyether-polyurethanes. Natural polymers are generally more biodegradable than synthetic polymers; specifically polymers with ester groups like aliphatic polyesters (Shultz 1979). Therefore, several bio-based materials such as polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides, polyisoprenoids and polyphenols are potential candidates for the substitution of synthetic plastics (Steinbuchel 2001) and have been the focus of attention in recent years (Zinn *et al.,* 2001). Amongst these, polyhydroxyalkanoates (PHAs) belong to the polyoxoester group and have received much attention due to their biodegradable thermoplastic properties (Albuquerqueet *et al.,* 2007).

1.2. Discovery of PHAs

Polyhydroxyalkanoates (PHAs) are naturally occurring biodegradable and biocompatible polymers commonly found as storage compounds of carbon, and therefore energy, in various microorganisms. These are produced in the presence of excess carbon and under limiting conditions of other nutrients for example nitrogen, phosphorus, sulphur, magnesium or oxygen (Lee 1996, Steinbuchel 1991, Du 2001). The most simplest PHA is poly(3-hydroxybutyrate) (P(3HB)) which was discovered in 1925 by a French scientist Lemoigne (Doi, 1990). After the first discovery of P(3HB), several bacterial strains (archaebacteria, Gram positive and Gram negative bacteria and photosynthetic bacteria including cyanobacteria) have been known to produce P(3HB) (Doi, 1990, Findlay and White 1983, Williamson and Wilkinson, 1958, Forsyth *et al.,* 1958, Hassan *el al.,* 1998, Hassan *et al.,* 1996, Hassan *et al.,* 1997, Hashimoto *et al.,* 1993, Jau *et al.,* 2005, Jensen and Sicko 1971). It was noted by Dawes and Senior that P(3HB) was similar to starch and glycogen as a storage compound (Dawes and Senior 1973) and was produced by *Bacillus megaterium* under high C/N ratio. In carbon deficient conditions, the organism started to utilise the accumulated P(3HB) (Macrae and Wilkinson 1958, Macrae and Wilkinson 1958). Before 1974, P(3HB) copolymers were the only known members of the PHA family. However, 3-hydroxyvalerate (3HV),

3- hydroxyhexanoate (3HHx) and 3-hydroxyheptanoate (3HHp) monomer units were discovered by Wallen and Rohwedder from activated sewage sludge (Wallen and Rohwedder 1974, Steinbuchel and Valentin 1995, Sudesh *et al.,* 2000). In 1983, two other members of the PHA family such as 3HHp and P(3HO) were also discovered from *B. megaterium* and *Pseudomonas oleovorans* respectively (Findlay and White 1983, De Smet *et al.,* 1983). It was also observed in the above study that production of different PHA monomer units is dependent on the carbon source used for the organisms. Hence, it was possible to produce PHA copolymers of different monomer compositions with straight, branched, saturated, unsaturated and also aromatic structures (Witholt and Kessler 1999). To date, more than 150 different monomer constituents of PHAs have been discovered (Steinbuchel 2001, Steinbuchel and Valentin 1995) and more than 300 different types of PHA producing microorganisms have been identified. The producers include *Ralstonia eutropha* reclassified from *Alcaligenes eutrophus*, *Alcaligenes latus*, *Azotobacter vinelandii*, *Azotobacter chroococcum*, *Methylotrophs*, *Pseudomonads*, *Rhodobacter sphaeroides* and recombinant *Escherichia coli* (Sudesh *et al.,* 2000).

1.3. Properties and different classes of PHAs

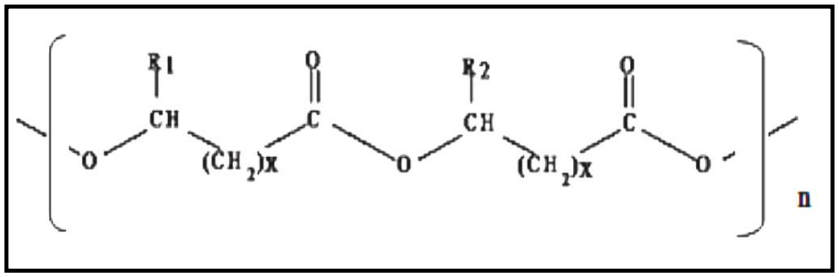


Figure 1.1: General structural formula of PHAs.
(x = 1, 2, 3; n = 100-30000; R_1, R_2 = alkyl groups, C_1-C_{13}) (adapted from Philip *et al*., 2007)

PHAs with different physical and mechanical properties have been identified. Several factors can affect these properties such as the distance between the ester linkages in the polymer backbone, length and type of the side groups and number of the repeating units in the polymer chain (Rai *et al.,* 2011). For instance, flexibility, crystallinity, melting point and glass transition temperature of the polymer produced are dependent on the length and the type of the side groups of the repeating units (Volova 2004, Rai *et al.,* 2011). As the length of

the side chain on the β-carbon of the PHA increases, the physical property of polymer is changed from a glassy state to more soft and sticky material (Hazer *et al.,* 2012). Fermentation conditions, the producing organism and the carbon source used have a direct effect on the PHA monomer composition and thereby polymer properties, as well as PHA yield (Ojumu *et al.,* 2004, Rai *et al.,* 2011).

PHAs are degraded by several microbes in the environment and by enzymes present in the human body (Valappil *et al.,* 2006, Philip *et al.,* 2007). PHAs are thermoplastic and elastomeric materials with different mechanical, physical and thermal properties (Philip *et al.,* 2007, Chen 2010). They are water insoluble biodegradable polymers. Moreover, PHAs are also known to be biocompatible polymers due to their non-toxic degradation products and have ability to support cell adhesion, migration, differentiation and proliferation functions for tissue engineering applications (Saad *et al.,* 1999, Chen and Wu 2005, Zhao *et al.,* 2003, Philip *et al.,* 2007). PHAs have piezoelectric property which makes them suitable for bone regeneration, nerve repair as well as nerve regeneration (Williams *et al.,* 2000).

Basically, PHAs can be broadly subdivided into three groups based on the number of carbon atoms present in its monomer units (Hazer *et al.,* 2012):

(a) Short-chain-length PHAs consisting of 3-5 carbon atoms (PHA_{SCL}).
(b) Medium-chain-length PHAs consisting of 6-14 carbon atoms (PHA_{MCL}).
(c) Long-chain-length PHAs consisting of more than 14 carbon atoms (PHA_{LCL}).

1.3.1. SCL PHAs

The most thoroughly investigated PHA is the poly(3-hydroxybutyrate) (P(3HB)), known as SCL-PHA. The other common example of SCL-PHA is poly(4-hydroxybutyrate) (P(4HB)) (Hazer *et al.,* 2012). *R. eutropha* is widely used for the P(3HB) production (Hazer *et al.,* 2012). It is a highly crystalline, brittle, stiff and piezoelectric material. It has melting temperature in the range of 175-179°C, glass transition temperature of -3-4°C, crystallinity of 60-80%, tensile strength up to 40 MPa and elongation at break of 5-6% (Saad *et al.,* 1999). It has ideal

biocompatibility because the polymer and its degradation product, 3-hydroxybutyric acid is the product of cell metabolism and is present in blood and tissues. Hence, many *in vitro* investigations have shown that P(3HB) is biocompatible to various cell lines, including osteoblasts, epithelial cells and ovine chondrocytes, which triggered commercial interest in the polymer (Zhao *et al.,* 2003). However, P(3HB) has been found to induce some inflammatory responses (Bhubalan *et al.,* 2007). Due to the brittle nature and high stiffness of P(3HB), its application is significantly limited (Bohmert *et al.,* 2002). Due to the poor physical properties of P(3HB), the incorporation of a second monomer unit into P(3HB) can significantly enhance its properties. This has led to an increased interest to produce copolymers with improved qualities. The incorporation of 3-hydroxyvalerate (3HV) into P(3HB) results in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)) which is more flexible and tougher than P(3HB), and is easier to degrade when discarded into the environment (Doi 1990, Kunioka *et al.,* 1989, Saito *et al.,* 1996).

1.3.2. MCL PHAs

MCL-PHAs are polyesters accumulated by fluorescent Pseudomonads. After the discovery of MCL-PHAs in 1983, more than 100 different monomer units have been characterised within MCL-PHAs, in order to achieve different physical as well as mechanical and thermal properties, to be utilized in various applications (Witholt and Kessler, 1999). The melting point (T_m~40-60^oC) and glass transition temperature (T_g~-50 to -25^oC) of MCL-PHAs are much lower than those of P(3HB) (Witholt and Kessler, 1999, Rai *et al.,* 2011). The most common examples of MCL-PHAs are thermoplastic elastomers such as poly(3-hydroxyhexanoate) (P(3HHx)) and poly(3-hydroxyoctanoate) (P(3HO)) (Basnett 2014). These materials have lower crystallinity, are rather flexible and soft. They have elastomeric nature which increases with the length of the side chain. These are also biodegradable, water resistant and biocompatible, which could be utilized in medical implants, such as scaffolding for the regeneration of arteries and nerve axons (Rai *et al.,* 2011, Hazer *et al.,* 2012). The major advantage of the MCL-PHAs is the variability in their biological as well as material properties which can be tailored by altering the culture conditions, carbon source and organism used. Also, they have less acidic end products

compared to synthetic biodegradable elastomeric polymers, PGA (poly-glycolic acid), PLLA (poly-L-Lactic acid) , poly(lactic-*co*-glycolic acid) (PLGA), hence induce lesser inflammatory reaction when used *in vivo* (Williams *et al.,* 1999, Martin and Williams 2003, Rezvan *et al.,* 2006). Moreover, synthetic polymers such as polycaprolactone (PCL) (Rezvan *et al.,* 2006, Prabhakar *et al*., 2005) contain traces of undesirable chemical impurities such as the catalysts used for the synthesis, which are not present in MCL-PHAs. Hence, PHAs are truly considered as "environmentally friendly" biodegradable and highly biocompatible materials, suitable for two promising range of applications: as a potential replacement for synthetic plastics; the other as a biomedical material (Rachana *et al.,* 2008, Ashby and Foglia 1998). However, until recently only a few PHAs such as poly(3-hydroxybutyrate) and poly(-3-hydroxybutyrate-co-3-hydroxyvalerate) were available commercially.

As a result of research developments, PHAs are being considered for use in different industrial applications such as: packaging industry, medicine, pharmacy, agriculture, food industry, paint industry and biomedical applications: medical device development including sutures, stents, nerve repair devices and wound dressing which are discussed in section **1.6.** in detail (Rai *et al.,* 2011, Grage *et al.,* 2009, Keshavarz and Roy 2010, Hazer *et al.,* 2012).

1.4. Biosynthesis of PHAs

As mentioned earlier, PHAs are storage compounds of carbon, and therefore energy, in various microorganisms and accumulated in the form of granules in the organisms. The size and the number of these granules per cell depend on the PHA producing bacterial species (Ojumu *et al.,* 2004). In the previous study carried out by Byrom, it was observed that *Alcaligenes eutrophu*s synthesised 8 to 13 such granules with the diameter of 0.2 to 0.5 µm per cell (Byrom 1994, Ojumu *et al.,* 2004). When there is a short supply of carbon and energy, organisms start to utilise accumulated PHAs to survive difficult conditions (Byrom 1994, Ojumu *et al.,* 2004). Moreover, PHAs can also help bacteria to survive in harsh environmental conditions such as ultraviolet (UV) irradiation, heat and osmotic shock by protecting their DNA from damage (Kadouri *et al.,* 2005, Giin-Yu *et al.,* 2014). PHAs are mainly made up of R(-)-3-hydroxyalkanoic

acid monomer units containing carbon atoms ranging from C_3 to C_{14}. They are saturated or unsaturated polymers with straight or branched chains comprising aliphatic or aromatic side groups (Doi *et al.,* 1992, DeSmet *et al.,* 1983, Ojumu *et al.,* 2004). This is due to the substrate specificity of the PHA biosynthetic enzymes in the particular organism (Anderson and Dawes 1990, Ojumu *et al.,* 2004). For example, only SCL 3-hydroxyalkanoates (3HAs) can be polymerized by the *A. eutrophus,* PHA synthase enzyme. While, in case of *Pseudomonas oleovorans,* the PHA synthase enzyme can only polymerize MCL 3-hydroxyalkanoates (3HAs) (Ojumu *et al.,* 2004). MCL-PHAs can possess different functional groups such as olefins, branched hydrocarbyls, halogens, aromatic and cyano with different physical and mechanical properties. Hence, they are attractive biomaterials for a range of different applications (Anderson and Dawes 1990, Ojumu *et al.,* 2004).

There are three different pathways known for the PHA biosynthesis which are interconnected with the anabolic/catabolic pathways such as glycolysis, Krebs Cycle, β-oxidation, *de novo* fatty acids synthesis, amino acid catabolism, Calvin Cycle, and serine pathway of the producing organism (Lu *et al.,* 2009, Giin-Yu *et al.,* 2014). Acetyl-CoA is the common intermediate between PHA synthesis and metabolic pathways. When there are nutrient rich conditions, acetyl-CoA is transferred into the Krebs Cycle by excessive coenzyme A production which inhibits 3-ketothiolase (PhaA) and blocks PHA synthesis (Ratledge and Kristiansen 2001). However, when there is nutrient limiting conditions, the level of coenzyme A is non-inhibitory which allows acetyl-CoA to be channelled into PHA synthetic pathways (Ratledge and Kristiansen 2001, Jung and Lee 2000).
Biosynthesis of PHAs involves two main steps: 1) Synthesis of hydroxyacyl-CoA, 2) Polymerisation of hydroxyacyl-CoA into PHAs (Rehm and Steinbüchel 2001). As mentioned above, three different pathways are involved in the production of the 3-hydroxyacyl CoA units (**Figure 1.2**) from which, in two pathways, carbohydrates are being utilised as the feedstocks for the organisms in the PHA production. While, in the third pathway, PHA production is carried out using fatty acids as the carbon source for the organisms (Byrom 1994, Doi and Abe 1990, Poirier *et al.,* 1995, Steinbüchel 1991).

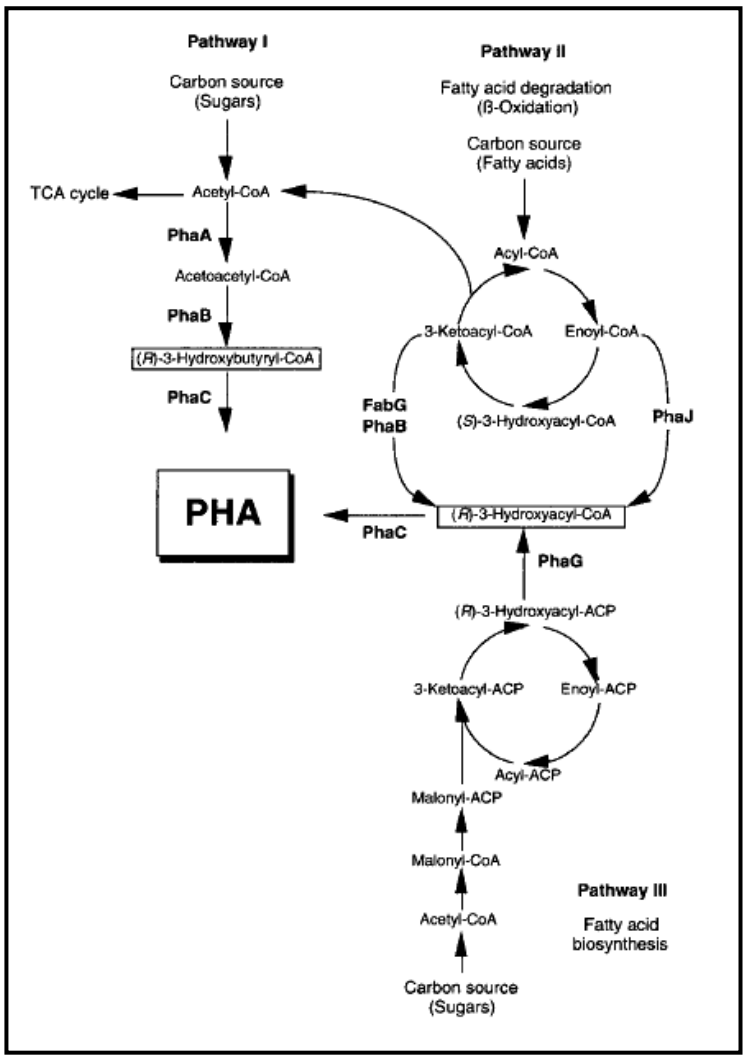


Figure 1.2: Metabolic pathways involved in the biosynthesis of PHAs from related and unrelated carbon sources (adapted from Kazunori *et al*., 2001).

The first pathway involves three key enzymes, β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase and PHA synthase, for SCL-PHAs biosynthesis by using structurally unrelated carbohydrates as the carbon feedstocks for the producer organisms (Kazunori 2001). β-ketothiolase combines two molecules of acetyl-CoA to acetoacetyl-CoA which can then be converted into 3-hydroxybutyryl-CoA by NADPH-dependent acetoacetyl-CoA reductase. The third and the final step is the polymerisation of 3-hydroxybutyryl-CoA units into P(3HB) catalysed by PHB synthase (Philip *et al.,* 2007). P(3HB-co-3HV) can also be synthesised using the same pathway. A second pathway, called β-oxidation pathway, uses structurally related fatty acids as the carbon source for MCL-PHAs production. There are three main enzymes involved in this pathway for PHA biosynthesis: 1) enoyl-CoA hydratase which converts 2-trans-enoyl-CoA into (R)-3-hydroxyacyl CoA , 2) hydroxyacyl-CoA epimerase

which converts (S)-3-hydroxyacyl-CoA into (R)-3-hydroxyacyl CoA and, 3) β-ketoacyl-CoA reductase which converts 3-ketoacyl-CoA to (R)-3-hydroxyacyl CoA (Huisman *et al.,* 1989). Finally, the last step involves polymerization of the produced (R)-3-hydroxyacyl CoA into MCL-PHAs using PHA synthase (Rehm 2007). In this pathway, the resulting polymer is similar in structure to the carbon source used and sometimes shortened by 2, 4 or 6 carbon atoms (Huisman *et al.,* 1989). The last pathway is called the *de novo* fatty acid biosynthesis pathway, which uses structurally unrelated carbohydrates as the feedstocks for the organisms in MCL-PHA production (Kazunori 2001). After the oxidation of the sugars, the generated acetyl-CoA molecules are directed into *de novo* fatty acid biosynthesis pathway which ends up into (R)-3-hydroxyacyl-ACP precursor molecules. Finally, (R)-3-hydroxyacyl-ACP is converted into (R)-3-hydroxyacyl- CoA by (R)-3-hydroxyacyl-ACP-CoA transacylase and gets polymerized into MCL-PHAs by the PHA synthase (Chen 2010).

1.5. PHA production using renewable resources

There has been a great interest in commercialising PHA production due to their various advantages. However, petrochemically derived plastics are still the material of choice due to their comparatively lower production costs. Moreover, utilising the expensive carbon feedstocks for PHA production, high recovery cost and relatively low PHA yield has put up the overall price of PHA production (Jiun-Yee *et al.,* 2010, Chenyu 2012). If expensive raw materials have been used for the PHA production, it contributes about 30-40% of the total fermentation cost (Chenyu 2012). Hence, the major cost in the PHA production is the cost of the substrate (Yamane 1993). Therefore, to make PHA production more economical, many researchers are focusing on the identification of renewable, inexpensive and readily available carbon substrates, which not only reduce the production costs but also increase the polymer yields (Ashby and Foglia 1998). To make the PHA production commercially viable, there are few criteria which could be highlighted. Main criteria involve the utilization of the inexpensive carbon source. Moreover, an effective PHA production strategy such as efficient bacterial strains, fermentation and recovery processes are also very important in commercialisation of PHAs (Grothe *et al.,* 1999, Lee 1996).

In recent years, there has been significant increase in the agricultural and food processing industries. Hence, large amount of waste materials from these industries are being discarded each year. These waste materials could be used as the carbon feedstocks for the PHA producing organisms. Therefore, by using waste effluents of agricultural and food processing industries would not only decrease the PHA production cost but also solve the problem of waste management (Yu 2007). Moreover, various bacterial strains have been identified for PHA production using renewable inexpensive carbon sources such as complex waste effluents, plant oils (Fukui and Doi 1998), fatty acids (Eggink *et al.,* 1992), alkanes (Lageveen *et al.,* 1988) and carbohydrates. In recent years, the use of organic wastes (swine waste liquor, palm oil mill effluents, and vegetable and fruit wastes), many agricultural and dairy by-products (whey, molasses) and vegetable oils have been studied as an alternative substrate for PHA production. Among these, vegetable oils are considered as the most suitable and desirable feedstocks for PHA production due to their high productivity. In contrast to the other carbon sources, the theoretical yield coefficients of PHA production from vegetable oils are as high as over 1.0 g-PHA per g-vegetable oil used, since they have a much higher number of carbon atoms per unit weight. Thus, vegetable oils can reduce production cost and increase polymer productivity when compared with sugar substrates such as glucose or sucrose (Daniel 2006, Chenyu *et al.,* 2012, Chee *et al.,* 2010). Furthermore, there are some studies which show that vegetable oil could significantly improve PHA production compared to sugars, which are normally used for the PHA accumulation by various bacteria. Additionally, previous studies have shown that for each gram of either glucose or plant oils, the P(3HB) yields produced from plant oils were almost twofold higher (0.8 g PHA per 1 g of plant oil) as compared to when glucose (0.3 g PHA per 1 g of glucose) was used as the sole carbon source (Akiyama *et al.,* 2003). Although fats and oils are renewable and inexpensive agricultural co-products, there are a small number of reports published demonstrating the use of fats and oils for PHA production (Fukui and Doi 1998). Below are some examples of the industrial by-products, such as molasses, whey, lignocellulosic raw materials, fats and oils, glycerols and carbon dioxide, used for the production of PHAs.

1.5.1. Fats, vegetable oils and waste cooking oils

In the 1990s, relatively low prices and renewability of triacylglycerides (TAG) (such as fats, vegetable oils and waste cooking oils) and its derived fatty acids attracted interest for the fermentative PHA production (Chenyu *et al.,* 2012). In comparison to carbohydrates, fatty acids deliver more energy per mole when they are converted to PHAs (Solaiman *et al.,* 2006). Shimamura *et al.* (1994) first investigated the PHA production directly from TAGs by *Aeromonas caviae*. Cromwick *et al.* (1996) showed *Pseudomonas resinovorans* accumulated PHA up to 15% of its cell dry weight from tallow. Ashby and Foglia (1998) further investigated PHA production by *Pseudomonas resinovorans* using a whole range of TAGs, such as lard, butter oil, olive oil, coconut oil, and soybean oil for the production of MCL-PHA. Plant oils such as soybean oil, palm oil and corn oil are desirable carbon sources for PHA production as they are cheaper than most sugars. The production of PHAs using sugars has been optimised to achieve high productivity. However, the cost of PHA production using sugars is higher than the 'acceptable' level as it results in low PHA yield (Lee and Choi 1999). Approximately 0.3 to 0.4 g of P(3HB) per g of glucose has been reported to be the highest yield of PHA production. On the contrary, plant oils are predicted to provide higher yield for both cell biomass and PHA production (0.6 to 0.8 g of PHA per g of oil) as they contain higher carbon content per weight compared to sugars (Akiyama *et al.,* 2003). Kahar and coworkers (2004) investigated P(3HB) homopolymer and P(3HB-*co*-5 mol% 3HHx) copolymer production of up to 80 wt% PHA of the dry cell weight by *C. necator* H16 and its recombinant strain (harbouring the PHA synthase gene from *A. caviae*) respectively from soybean oil as the sole carbon source (Fukui Doi 1998, Kahar and coworkers 2004). Recombinant *C. necator* H16 was also able to utilise palm oil as the sole carbon source (Loo 2005). There are also few other bacteria that are known to produce PHA from plant oils, such as *Burkholderia cepacia* (Alias and Tan 2005) and *Comamonas testosteroni* (Thakor *et al.,* 2005). Chee and co-workers have isolated *Burkholderia* sp. USM (JCM15050) from oil polluted wastewater and reported that this bacterium could produce P(3HB) up to 70 wt% of dry cell weight from palm oil (Chee *et al.,* 2010). *C. testosteroni* has been studied for its ability to synthesize MCL-PHA from vegetable oils such as castor seed oil, coconut oil, mustard oil, cotton seed oil,

groundnut oil, olive oil and sesame oil (Thakor *et al.,* 2005). This bacterium was shown to accumulate PHA up to 80 wt% of dry cell weight with major monomer compositions consisting of 3HO and 3HD (Thakor *et al.,* 2005). Owing to the absence of lipase activity in *P. putida*, plant oils in the form of triglycerides could not support both the cell growth and PHA production in *P. putida*. Therefore, an additional saponification step was needed to break down the triglycerides into free fatty acids, which can be assimilated by *P. putida* for growth and PHA production (Tan *et al.,* 1997). Kim and coworkers (Kim *et al.,* 1997) performed a two-stage fed-batch cultivation using *P. putida* by supplying octanoic acid in the first step, which resulted in good growth and could stimulate the biosynthesis of MCL-PHAs efficiently.

1.5.2. Glycerol

Glycerol is the main by-product of the biodiesel production plant and palm oil refining industry. In 2009, the biodiesel production in Europe exceeded 10 billion litres, resulting in about 1 billion litres of coproduced glycerol. Hence, there has been decrease in the world wide market price for glycerol, which makes it a potential carbon feedstock for PHA production (da Silva *et al.,* 2009, Chee *et al.,* 2010, Ashby 2005, Madden *et al.,* 1999). In the early nineties, *Pseudomonas putida* KT2442 was shown to produce MCL-PHA from glycerol which had similar polymer characteristics to the polymer produced by the same strain from glucose or fructose as a carbon source (Solaiman *et al.,* 2006, Huijberts *et al.,* 1992). Bormann and Roth (1999) demonstrated the production of P(3HB) up to 50% and 65% dcw from glycerol using *Methylobacterium rhodesianum* and *C. necator*. Ashby *et al.* 2005 investigated PHA synthesis by *Pseudomonas oleovorans* NRRL B-14682 and *Pseudomonas corrugata* 388. A recombinant *E. coli* strain with the *phaC1* gene from *Pseudomonas sp.* LDC-5 was prepared by Sujatha and Shenbagarathai (2006) which showed 3.4 g/L PHAs on glycerol. As well as pure glycerol, crude glycerol has also been studied for PHA production. Ashby *et al.* (2004) used crude glycerol, derived from a soy-based biodiesel production site, for the microbial production of PHAs. It was shown that *P. oleovorans* NRRL B-14682 and *P. corrugata* 388 could accumulate MCL-PHAs from this carbon source in shaken flasks. Koller *et al.* (2005) was able to produce 5.9 g/L P(3HB-co-3HV) copolymer, without

adding any precursor molecule in the feed by using crude glycerol as the carbon source, combined with meat and bone meals as a nitrogen source. Mothes *et al.* (2007) also investigated the usability of the different crude glycerol streams for P(3HB) production by *C. necator* JMP134 and *Paracoccus denitrificans* and noted that contaminated waste glycerol streams could be a more suitable stream for the PHA production (Cavalheiro *et al.,* 2009).

1.5.3. Whey and whey hydrolysates

Whey is the main by-product produced in large quantities in the manufacturing of cheese. It is estimated that annual whey formation exceeds 40 million tons in the European Union (Koller *et al.,* 2008). Whey is a lactose rich substrate, hence it would be of great interest for PHA production as a good and cheap carbon and energy source. A recombinant *Escherichia coli* strain expressing *Cupriavidus necator phaC2* gene (also known as *Ralstonia eutropha*) was prepared by Lee *et al.* (1997) and showed 81% DCW P(3HB) yield from whey (Lee *et al.,* 1997). Lee and his co-workers also constructed a recombinant *E. coli* GCSC 6576 expressing *Ralstonia eutropha* PHA biosynthesis genes and *E. coli* fts Z gene, which showed 50 g/L P(3HB) from whey powder and 69 g/L P(3HB) from concentrated whey solution containing 210 g/L lactose (Wong and Lee 1998). Ahn *et al.* (2000) used a recombinant *E. coli* strain CGSC 4401 and whey solution containing 280 g/L lactose, which achieved 96.2 g/L P(3HB) in 37.5 hours. Using the system of cell recycle membrane with the same strain and a lactose concentration of 280 g/L, Ahn *et al.* (2001) achieved 168 g/L P(3HB) in 36.5 hours. Some other bacteria such as *Ralstonia eutropha* DSM545, *Pseudomonas hydrogenovora*, *Thermus thermophilus* HB8 and wild strains, such as *Methylobacterium sp*. ZP24, *Hydrogenophaga pseudoflava* DSM1034 have also been explored for their PHA producing abilities using whey which showed comparatively lower PHA accumulation than the recombinant *E. coli* fermentations. (Chenyu *et al.,* 2012).

1.5.4. Molasses

Molasses is a sugar-rich by-product of the sugar manufacturing industries (Albuquerque *et al.,* 2007). Large scale PHA production from molasses has

been exploited due to their abundance production from refining plants (Zhang *et al.,* 1994). The production of P(3HB) by *Azotobacter vinelandii* UWD using sugar beet molasses was first investigated in 1992 (Page 1992). Chen and Page (1997) observed P(3HB) production of 19 to 22 g/L by *Azotobacter vinelandii* UWD. Albuquerque *et al.* (2007) reported PHA production from cane molasses using a three-step fermentation strategy. Apart from normal molasses, fermented molasses and waste water containing volatile fatty acids (VFAs) were also investigated for PHA production using combination of bacterial strains by Pisco *et al.* (2009) and Bengtsson *et al.* (2010) which showed efficient PHA accumulation. Use of sugar cane molasses for PHA production was further studied by Wu *et al.* (2001) using *Bacillus sp.* JMa5 which showed higher cell growth and P(3HB) accumulation of 25-35% dcw. P(3HB) accumulation of up to 43% dcw was observed in *B. megaterium* ATCC 6748 when sugar cane molasses was used as the carbon feed for this organism (Chaijamrus & Udpuay 2008). Kulpreecha *et al.* (2009) observed 42% dcw P(3HB) accumulation in *B. megaterium* BA-019 using cane molasses. Soy molasses is another attractive carbon source for PHA production because it is very rich in sucrose. MCL-PHA production by *Pseudomonas corrugate* using soy molasses was first observed by Solaiman *et al.* (2006a). In this study, PHA accumulation of 5-7% dcw was observed giving higher concentrations of 3-hydroxydodecanoate, 3-hydroxyoctanoate and 3-hydroxytetradecenoate monomer units. Lower PHA yield in this study suggested that efficient PHA production using soy molasses was obtained from Gram-positive bacteria, such as *Bacillus. Sp* CL1 which exhibited up to 90% dcw PHA accumulation. *Bacillus* strains, HF-1 and HF-2, were also able to accumulate P(3HB) from disintegrated soy and malt wastes (Law *et al.,* 2001).

1.5.5. Lignocellulosic raw materials

Increasing food prices has reinforced scientist's interest in utilizing lignocellulosic materials for the production of biofuel and biochemical compounds (Lin *et al.,* 2012). Due to their high abundance and cellulose rich nature, they have been an attractive carbon feedstock for PHA production. Approximately 80 billion tons of woody biomass is generated annually worldwide, contributing to a total annual production of 180 billion tons plant

matter (Perlack *et al.,* 2005). *Saccharophagus degradans* ATCC 43961 is known to produce PHA from cellulose (Munoz and Riley 2008). Lignocellulosic materials need hydrolysis to prepare free sugars due to their recalcitrant nature. *Pseudomonas pseudoava* exhibited P(3HB) production of 22% dcw using the hemicellulosic fraction of wood (Bertrand *et al.,* 1990). *Burkholderia cepacia* ATCC 17759, *Burkholderia cepacia* IPT 048 and *B. sacchari* IPT 101 were found to produce P(3HB) yield of 1.6-3.7 g/L and 60% dcw from xylose (Ramsay *et al.,* 1995, Young *et al.,* 1994, Silva *et al.,* 2004). Ramsay *et al.* (1989) and Ramsay *et al.* (1990) also observed the production of P(3HBV) from fructose and glucose using propionic acid as a co-substrate. *Burkholderia cepacia* ATCC 17759 was further investigated by Keenan *et al.* (2006a and 2006b) for the production of 1.3-4.2 g/L P(3HBV) using xylose and laevulinic acid. Moreover, 2.0 g/L P(3HBV) accumulation was observed from the hemicellulosic content of the aspen and maple. Li *et al.* (2007) constructed an *E. coli* phosphotransferase system (PTS) mutant using *Ralstronia eutropha phaCRe* and *phaABRe* genes which produced SCL-PHA using a substrate containing a mixture of glucose and xylose. When the same mutant was prepared using *Pseudomonas aeruginosa phaC1* gene, it produced MCL-PHAs using this carbon source. Recently, Van-Thuoc *et al.* (2008) demonstrated that *Halomonas boliviensis* LC1 could produce P(3HB) on enzymatically hydrolysed wheat bran. Huang *et al.* (2006) investigated *Haloferax mediterranei* bacteria for PHA biosynthesis ability from extruded rice bran and corn starch.

1.5.6. Carbon dioxide

Transgenic plants are other producers of PHAs which utilise carbon dioxide (CO_2) (abundantly available on earth) as the carbon source for PHA accumulation (Braunegg *et al.,* 1998). Poirier and coworkers (Poirier *et al.,* 1992) were the first to produce PHAs using genetically modified plants (*Arabidopsis thaliana*) which encode the PHA synthesis genes from *C. necator*. Another example of a PHA producer, utilizing carbon dioxide as the feedstock is cyanobacteria. They naturally possess the key enzyme (PHA synthase) for the production of PHAs (Sudesh *et al.,* 2002). *Spirulina platensis* UMACC 161 (Jau *et al.,* 2005) and *Synechocystis* sp. PCC6803 (Sudesh *et al.,* 2001) are some of the cyanobacteria that can produce up to 10% dcw P(3HB) homopolymer in

nitrogen limiting conditions with added acetate (Jau *et al.,* 2005, Sudesh *et al.,* 2001).

Table 1.1: Bacteria used for production of PHAs from plant oils and wastes (adapted from Jiun-Yee *et al.,* 2010).

Strains	PHA type	Substrates	PHA content (wt%)
***Alcaligenes latus* DSM 1124**	P(3HB)	Soya waste, malt waste	33, 71
Bacillus megaterium	P(3HB)	Beet molasses, date syrup	~50
***Burkholderia sp.* USM (JCM 15050)**	P(3HB)	Palm oil derivatives, fatty acids, glycerol	22-70
Comamonas testosteroni	MCL-PHA	Castor oil, coconut oil, mustard oil, cottonseed oil, groundnut oil, olive oil, sesame oil	79-88
Cupriavidus necator	P(3HB)	Bagasse hydrolysates	54
***Cupriavidus necator* H16**	P(3HB-*co*-3HV)	Crude palm kernel oil, olive oil, sunflower oil, palm kernel oil, cooking oil, palm olein, crude palm oil, coconut oil + sodium propionate	65-90
***Cupriavidus necator* DSM 545**	P(3HB)	Waste glycerol	50
Recombinant *Cupriavidus necator*	P(3HB-*co*-3HHx)	Palm kernel oil, palm olein, crude palm oil, palm acid oil	40-90
Recombinant	P(3HB-*co*-	Soybean oil	6

Escherichia coli	3HHx-*co*-3HO)		
***Pseudomonas aeruginosa* IFO3924**	MCL-PHA	Palm oil	39
***Pseudomonas aeruginosa* NCIMB 40045**	MCL-PHA	Waste frying oil	29
Pseudomonas guezennei* biovar. *tikehau	MCL-PHA	Coprah oil	63
***Thermus thermophilus* HB8**	P(3HV-*co*-3HHp-*co*-3HN*co*-3HU)	Whey	36

1.6. Applications of PHAs

PHAs are “environmentally friendly” biodegradable and highly biocompatible materials; hence there has been a great amount of interest in the commercialisation of these biopolymers. They have shown their applicability as the potential replacement for synthetic plastics in bulk applications (packaging industry), medicine, pharmacy, agriculture, food industry and paint industry. Moreover, due to research developments, they have been considered as the efficient biomaterials for medical device development including sutures, stents, nerve repair devices and wound dressing. Some of the applications of PHAs are discussed here as follows.

1.6.1. Bulk Applications of PHAs

The PHAs have various properties which make them suitable replacement material for petro chemically derived plastics. Hence, they can be used for several bulk applications. The first consumer product made out of PHAs was biodegradable shampoo bottles made of Biopol (ICI, UK), launched in April 1990 by Wella AG. Initially, PHAs were used in packaging films mainly in bags,

containers and paper coatings. Similar applications of PHAs as conventional commodity plastics include disposable items, such as razors, utensils, diapers, feminine hygiene products, foils, films and diaphragms, combs, pens, bullets, cosmetic containers-shampoo bottles and cups (Akaraonye *et al.,* 2010, Chen 2005, Reddy *et al.,* 2003, Rehm 2006, Panchal *et al.,* 2012). PHA latex can be used to cover paper or cardboard to make water-resistant surfaces. A P(3HB) and P(3HO) blend was commercialised by Metabolix, a US-based company, for the use as a food additive approved by the FDA (Philip *et al.,* 2007, Clarinval and Halleux 2005, Panchal *et al.,* 2012). One other example of a commercial PHA was poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(3HB-3HHx)) copolymer used to make flushables (wipes), nonwoven fabrics, binders, flexible packaging, thermoformed articles, synthetic paper and medical devices. P(3HB-3HHx) can also be used to prepare pressure sensors for keyboards, stretch and acceleration measuring instruments, material testing, shock wave sensors, lighters, gas lighters; acoustics: microphone, ultrasonic detectors, sound pressure measuring instruments; oscillators: headphones, loudspeakers, for ultrasonic therapy and atomization of liquids due to their unique piezoelectric property (Panchal *et al.,* 2012). Moreover, food packages, paper milk cartons and plastic beverage bottles could also be prepared due to the gas barrier properties of P(3HB-co-3HV) (Panchal *et al.,* 2012). P(3HB), SCL-MCL copolymers (Nodax^{TM}) and P(3HB-co-3HV) have also been used for controlled release of fertilisers, herbicides and insecticides (Galego *et al.,* 2000, Ren *et al.,* 2005, Scholz 2000, Steinbuchel 2001, Panchal *et al.,* 2012). Another use of PHAs in agriculture is as bacterial inoculants used to enhance nitrogen fixation in plants. This was confirmed by field experiments in Mexico with maize and wheat. Increase in the crop yield was observed when PHA-rich nitrogen fixing bacteria (*Azospirillum brasilense*) was used. This PHA accumulating bacteria was able to survive the harsh conditions and help in nitrogen fixation resulting in higher crop yield (Philip *et al.,* 2007).

1.6.2. Biomedical Applications of PHAs

PHAs are natural polymers with biocompatibility, biodegradability and lack of cytotoxic properties (Valappil *et al.,* 2006). Hence, PHAs are attractive materials for biomedical applications. They have been broadly utilised to prepare some

medical devices such as sutures, stents, nerve repair devices and wound dressing (Rai *et al.,* 2011, Hazer *et al.,* 2012). Moreover, PHAs support cell attachment, migration, differentiation and proliferation functions which make them the material of choice for biomedical applications (Zhao *et al.,* 2003). Some of the biomedical applications of PHAs are discussed here as follows.

1.6.2.1. PHAs as the drug-delivery systems

The traditional way of administering drugs is an either intravenous or extravascular route including oral administration. These methods have a major drawback of uncontrollable drug release at the target site. Hence, more advanced methods should be developed to overcome this problem. One such approach is to utilize biodegradable polymers as the drug delivery vehicles. Homo and copolymers of lactate and glycolate are commercially available and have shown sustained drug release over a 30 day period (Pouton and Akhtar 1996, Valapil *et al.,* 2007). However, they do not show controlled release which reinforced scientists' interst in developing an alternative material for controlled drug delivery systems. In early 1990s, researchers noted PHAs with biodegradability and biocompatibility properties which made them potential biomaterials for drug delivery. P(3HB) was analyzed for controlled release of the 7- hydroxyethyltheophylline which showed that polymer composition and its porosity combined with molecular weight of this drug affected the controlled release of the drug (Korsatko *et al.,* 1983, Gould *et al.,* 1987). Moreover, when metoclopramide (a drug used in the treatment of cattle disease) was encapsulated in P(3HB) and implanted subdermally in cattle, long term controlled release of this drug showed a positive response for this particular application (Jones *et al.,* 1994). P(3HB) was also investigated for the release of an anti-cancer agent lomustine (CCNU), which showed rapid release of the drug in 24 hours from P(3HB) microspheres compared to the 7 day drug release from PLA microspheres (Bissery *et al.,* 1984, Bissery *et al.,* 1985). To study the treatment of chronic osteomyelitis by sulbactam-cefoperazone antibiotic release, poly(3HB-co-22mol%-3-HV) rods were prepared with this antibiotic encapsulated and implanted into a rabit tibia. These studies showed a decrease in the infection within 15 days of implantation and complete healing within 30 days (Yagmurlu *et al.,* 1999). In a study by Francis *et al.* (2010) tetracycline,

was encapsulated in P(3HB) microspheres, which changed polymer film surface morphology and roughness. Observations revealed that drug loaded P(3HB) microspheres were not only efficient substrates for drug delivery but also enhanced human keratinocyte cell line (HaCaT) attachment (Francis *et al.,* 2010). In another study by Francis *et al.* (2011) gentamicin loaded poly(3-hydroxybutyrate), P(3HB) microspheres were prepared and investigated for drug delivery applications. The results showed burst release of the drug in the initial stage followed by its sustained release (Francis *et al.,* 2011). P(3HB) microspheres with encapsulated rifampicin (chemoembolising agent) were studied for the controlled release. However, it showed almost 90% of the drug released in 24 h which could be prevented by regulating the drug loading and the particle size (Zinn *et al.,* 2001).

1.6.2.2. PHAs as the scaffold materials in wound management

Efficient wound treatment sutures must meet certain criteria such as high tensile strength, lack of inflammatory reactions, easy handling and good absorbability. Mainly two kinds of wound sutures are available; absorbable and non-absorbable. Among these, absorbable natural (catgut), as well as synthetic materials (polygalactin-910 (Vicryl®), polydioxanone (PDS), polyglyconate, polyglecaprone-25 (Monocryl), and polygalactin-910 rapide (Vicryl Rapide®)), are available as the wound suture applications (Valappil *et al.,* 2006). The use of catgut has been prohibited due to the chance of getting Creutzfeldt-Jakob Disease (CJD) (Singh and Maxwell 2006). P(3HB) was first suggested as an absorbable suture in mid 1960s. P(3HB) in the form of nonwoven fiber was then investigated for wound dressing materials, such as swabs, gauze, lint or fleece, by Steel and Norton-Berry in 1986 (Baptist and Ziegler 1965). In earlier investigations, it was observed that P(3HB) and P(3HB-co-3HV) sutures had the required strength for myofacial (skeletal muscle) wound healing (Volova *et al.,* 2003, Shishatskaya *et al.,* 2004). These sutures were also compared with natural absorbable (catgut) and nonabsorbable (silk) sutures. In these investigations, P(3HB) and P(3HB-co-3HV) sutures were implanted in female wistar rats in which a prominent macrophagal stage was observed throughout the post-surgery monitoring period (Shishatskaya *et al.,* 2004). Moreover, a prolonged 1 year *in vivo* investigation by Shishatskaya and colleagues also

showed a positive response, including less inflammatory reactions, necrosis and carcinogenesis, when P(3HB) and P(3HB-co-3HV) sutures were used. No change was observed in test animals in their weights, internal organs, blood morphology, biochemistry and lymphoid tissue reactions after suture implantation. Therefore, these investigations have revealed that PHAs can be further explored for the development of future natural absorbable wound sutures. P(3HB) and P(3HB-co-3HV) were also assessed by Webb and Adsetts in 1986 for their use as wound plaster in case of emergency to avoid any contamination through airborne bacteria (Williams and Martin 2005). P(3HB-co-4HB) film was studied for its applicability as a wound healing suture. In this study, the prepared film was implanted in the abdominal cavity of the rat, between the cuts of skin and intestine to prevent adhesions. After 1 month of implantation of the polymer film, a complete healing of the wound was achieved without adhesions. However, the film showed poor degradation properties which suggested further development of the polymer properties (Ishikawa 1996).

1.6.2.3. PHAs as the nerve repair devices

Increasing amount of interest has been noted to develop efficient strategies for the treatment of the peripheral nerve and spinal cord injuries in which nerve regeneration has got a lot of attention. Four major components such as scaffolds, Schwann cells (supportive cells), growth factors and extracellular matrix are needed for the complex nerve regenerative engineering (Yang *et al.,* 2005, Yang *et al.,* 2005). Continuous growth of axonal nerves is disrupted in case of spinal cord injury that is why it is very hard to repair and sometimes life threatening compared to peripheral nervous damage. Small distance nerve injuries are regenerated on their own in case of peripheral nerve damage. However, nerve grafts from elsewhere in the body are needed to cure larger nerve damage (Schmidt and Leach 2003). Mostly there are two medical devices prepared, conduits and carrier scaffolds for peripheral and spinal cord nerve injuries (Valappil *et al.,* 2006). Conduits are commonly known as guidance channels and bridges which are being produced using some natural (e.g. collagen, chitosan, alginate, laminin, fibronectin) and synthetic (e.g. silicone, ethylene vinyl coacetate, ethyl vinyl acetate co-polymer, poly(lactide-co-

glycolide) (PLGA)) polymers. However, these polymers can only be used for short nerve gaps (Yang *et al.,* 2005, Yang *et al.,* 2005).

Earlier investigations showed the use of nonwoven P(3HB) sheets to repair 2-3 mm nerve gaps in cat models. Normal tissue response was observed in the investigation (Hazari *et al.,* 1999, Hazari *et al.,* 1999). In the subsequent investigation, it was observed that the P(3HB) conduit was able to regenerate 10 mm rat sciatic nerve gap with less inflammatory reactions (Hazari *et al.,* 1999, Hazari *et al.,* 1999). In another report, P(3HB) filled with alginate hydrogel and Schwann cells (SC) showed 10 mm rat sciatic nerve gap bridging with no inflammatory responses (Valappil *et al.,* 2006). Preparation of P(4HB) nerve guide conduits demonstrated better axonal regeneration and improved sensory functions compared to previously studied P(3HB) conduits. *In vivo* investigations on 30 male Sprague-Dawley rats showed 10 mm sciatic nerve gap restoration with 0.8 mm per day regeneration rate (Opitz *et al.,* 2004, Valappil *et al.,* 2006). Moreover, P(4HB) showed better mechanical properties and less inflammatory reactions to be used as the carrier scaffolds for spinal cord injuries. To repair spinal cord injuries, P(3HB) polymer was also investigated as the carrier scaffold for extracellular matrix components and cell lines to support neuronal survival. *In vivo* investigations in rat models showed that when P(3HB) fibers coated with alginate hydrogel and fibronectin were implanted in adult rats, cell loss was reduced to 50% which was comparable to the animals treated with neurotrophic factors such as brain derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3) (Novikov *et al.,* 2002). Moreover, full axonal growth along the entire length was observed when neonatal Schwann cells were incorporated into the graft material.

From the above investigations, it was demonstrated that PHAs would make efficient nerve tissue engineered devices for peripheral nerve and spinal cord injuries. Moreover, it was revealed that highly porous materials can allow the inflow of the growth factors and nutrients to the host cells which would enhance nerve regeneration by increasing cell migration, proliferation and differentiation (Maquet *et al.,* 2000, Ding *et al.,* 2010, Queen 2006, Vondran *et al.,* 2006). Several electrospun fibers of 100nm - 1µm were prepared from chitosan, poly(β-caprolactone), poly(L-lactide-co-glycolide) (PLGA), PHAs and have been

studied for nerve tissue regeneration applications. P(3HB) electrospun fibers and solvent cast films were investigated to analyze rat cerebellar granule neurons (rCGNs) behavior on them. This study revealed that electrospun P(3HB) fibers showed higher cell growth than 2D films (Bo-Yi *et al.,* 2009).

1.6.2.4. PHAs as materials for development of cardiovascular devices

In recent years there has been an increase in cardiovascular disease worldwide. Hence, researchers are focusing on the development of new efficient treatment procedures for heart diseases. Commonly synthetic polymers are used for this treatment. However, these polymers are mostly non-degradable and contain high risk of immune response. Therefore, development of new alternative biomaterials would help to overcome this challenge. The desired properties for cardiovascular devices include lack of inflammatory reactions, resistance to microbial infection, long endurance, supporting repair and regeneration by cell attachment and proliferation (Kofidis *et al.,* 2002, Morosco 2002, Smaill *et al.,* 2000).

There are several examples of PHAs being utilised for cardiovascular diseases within which pericardial patches made of P(3HB) are the most advanced amongst cardiovascular devices. Such patches have been utilised to prevent postsurgical adhesions between the heart and the sternum (Valappil *et al.,* 2006, Williams and Martin 1996). To study its applicability as a pericardial patch, *in vivo* investigations with 18 sheep showed less inflammation, no infection and lack of adhesion formation in the test animals receiving P(3HB) patches compared to control animals in which the pericardium was left open (Malm *et al.,* 1992a). Moreover, human studies were also carried out to investigate post surgical adhesions after heart bypass or vascular replacement. In this study, 19 patients were implanted with a P(3HB) patch and 20 patients were left without the P(3HB) patch (Duvernoy *et al.,* 1995). The final observations of this study revealed that the group of patients receiving the P(3HB) patches showed lower incidence of postsurgical adhesions than patients without the P(3HB) patches.

The other examples of PHA applications in cardiovascular diseases are artery augmentation, atrial septal defect repair, vascular grafts and heart valves. Patches made of P(3HB) in a non-woven form were also investigated for their use as artery augmentation devices for arterial regeneration (Malm *et al.,* 1994). These patches were compared with patches made of Dacron materials. The patches made of P(3HB) were found covered with regenerated endothelial layers on both sides of the patch. Moreover, the disordered host arterial tissue showed complete regeneration with smooth muscle cells, collagen and elastic fibres on it. A non-woven P(3HB) patch has also been used to repair atrial septal defects giving complete endothelial regeneration on both sides of the atrium with sub-endothelial collagen layer and smooth muscle cells (Malm *et al.,* 1992c). In artery augmentation device development, porous P(4HB) patches were also investigated as the scaffold material in autologous cardiovascular tissue preparations. In sheep models, scaffolds seeded with endothelial, smooth muscle and fibroblast cells were implanted in the pulmonary artery. This experiment showed tissue regeneration and lack of adverse host tissue reactions such as thrombus, dilation or stenosis in the sheep models containing P(4HB) patches. In contrast, polytetrafluoroethylene (PTFE) patches showed swelling at the site of implantation, less tissue regeneration and blood leakage in the sheep models (Stock *et al.,* 2000a, Valappil *et al.,* 2006, Williams and Martin 1996). In another study, it was found that P(4HB) scaffolds were able to reconstruct viable ovine blood vessels which were functionally similar to native aorta. Moreover, tissue engineered blood vessels were also successfully prepared in a bioreactor using P(4HB) scaffolds seeded with autologous cells (Opitz *et al.,* 2004).

Metallic materials were the material of choice for cardiovascular stent preparations until now. However, the major drawback of this material is the restenosis due to excessive growth of the blood vessel wall. Moreover, they are non-degradable materials hence, require a second operation to remove them after tissue repair. Therefore, PHAs have received a lot of attention for the development of the biodegradable stents which could prevent reocclusion of the vessel wall and also the subsequent operation to remove them. In earlier investigations, it was observed that drug eluting biodegradable stents made of P(3HB) and P(3HB-co-3HV) showed prompt degradation within four weeks of

implantation *in vivo* with temporary atrial cell proliferation (Van dar Giessen *et al.,* 1996). Recently in a study carried out by Basnett *et al.* (2013), it was observed that the drug eluting stent prepared from Poly(3-hydroxyoctanoate)/poly(3-hydroxybutyrate), P(3HO)/P(3HB), had controlled release of aspirin without any burst release over the period of 25 days (Basnett *et al.,* 2013).

Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)(P(3HB-co-4HB)) scaffold aterials were studied as vascular grafts for the repair of damaged smaller diameter blood vessels in dog models for 10 weeks, which showed graft degradation after 2 weeks of implantation (Noisshiki and Komatsuzaki 1995). Poly(3-hydroxyoctanoate-co-3-hydroxyheptanoate) (P(3HO-co-3HH)) was also investigated for the same application, which showed slower degradation with mild tissue inflammation (Marois *et al.,* 1999c, 2000). In another study, P(3HO-co-3HH) conduits were prepared with non-woven PGA, Poly(glycolic acid) mesh coating inside and incubated for 7 days with autologous endothelial, smooth muscle and fibroblasts cells. After incubation, grafts were implanted in the lambs' abdominal aortic segments which showed less tissue irritations with higher cell growth. Moreover, native aorta like tissue regeneration was observed with collagen formation (Shum-Tim *et al.,* 1999).

In recent years, PHAs have been considered most promising biomaterials for heart valve developments. The materials used for the heart valve replacement surgery have undesirable properties which result in subsequent surgeries. In the case of adults, improved valve stability is required which stimulated scientists to develop more reliable materials for heart valve surgeries. In earlier investigations, synthetic absorbable materials such as PGA (Poly(glycolic acid)) and PLA (Poly(lactic acid)) were studied *in vivo* in lambs (Williams and Martin 2005). This study revealed that the scaffolds prepared using these materials were very stiff and did not have enough elasticity to function as the valve leaflets in a trileaflet valve. Finally, the scaffold prepared using a porous P(3HHx-co-3HO)-PGA mesh showed significantly improved results in *in vivo* investigations in the lamb models (Opitz *et al.,* 2004). However, until now the most prominent results were observed with trileaflet heart valve scaffold preparations using P(4HB) coated on a nonwoven PGA mesh. *In vivo*

implantation of the engineered valve in juvenile sheep showed a fully functional trileaflet heart valve regeneration in 20 weeks (Hoerstrup *et al.,* 2000).

1.6.2.5. PHAs as the dental materials

PHAs have also been shown to be useful in the treatment of the periodontal ligament and jaw bone defects. Periodontosis is a disease caused by bacterial infection which halts the ligament development. Galgut *et al.* (1991) demonstrated the use of P(3HB-co-3HV) membranes for guided tissue regeneration (GTR) or guided bone regeneration in which they functioned not only as a barrier to make space for tissue regeneration, but also as a permeable membrane for contiguous nutrient supply (Williams and Martin 2005, Zinn *et al.,* 2001). Comparison of P(3HB-co-3HV) membranes with the membrane prepared from polytetraflouroethylene, polylactic acid and polycaprolactone showed that P(3HB-co-3HV) membranes were mechanically more stable and gained better tissue regeneration (Galgut *et al.,* 1991). Studies on P(3HB-co-3HV) membranes to treat jaw bone defects also showed complete bone regeneration within 6 months of implantation in rats (Kostopoulos and Karring 1994a).

1.7. Dental pulp regeneration

Dental pulp is made up of loose vascular connective tissue with fibroblasts, blood vessels, nerves, and a population of stem cells, which provides nutritional and sensory properties to dentine, vitality and sensitivity to the tooth (Liu *et al*., 2006, Syed-Picard *et al.,* 2014). Moreover, it also has its own reparative capability and hence plays an important role in maintaining the long life of teeth (Schmalz and Galler 2011, Ravindran *et al.,* 2013, Huangqin and Mingwen, 2007).

The most common diseases of dental pulp tissue are dental caries and irreversible pulpitis which affectes both children and adults (Petersen *et al.,* 2005, Ravindran *et al.,* 2013, Suzuki *et al.,* 2013). Root canal therapy is the common practice to treat these diseases, in which the whole pulp is removed and the empty space created is filled with inert material such as gutta percha,

resilon or silicone. (Ingle and Bakland 2002, Suzuki *et al.,* 2013, Huang 2008, 2009b, Hai-Hua *et al*., 2011, Orstavik 2005). This procedure makes the tooth insensitive, devitalised, brittle and susceptible to reinfection (Ingle and Bakland, 2002, Kim *et al*., 2010a). This would increase the chances of needing a second operation. This conventional root canal treatment is getting success for most necrotic permanent teeth. However, there is less chance of complete treatment of immature teeth due to some difficulties such as poor apical sealing and frequent cervical root fracture (Cvek 1992, Sakai *et al.,* 2011). This could halt the root formation of the child's immature teeth (Cvek 1992). The common root canal treatment can prevent reinfection but cannot support root development. (Lentzari and Kozirakis 1989, McTigue *et al.,* 2013, Rosa *et al.,* 2013). Moreover, the loss of pulp vitality in young permanent teeth terminates dentine formation and subsequent tooth maturation. Thus, researchers are getting involved in the development of alternative methods to replace the conventional root canal treatment and to overcome common difficulties associated with this treatment of dental caries and irreversible pulpitis. Moreover, due to the high treatment cost of root canal therapy and increasing demand for maintaining pulp vitality, development of pulp regenerative methods has been the focus of attention in dental tissue engineering (Rania *et al.,* 2008).

Further reasons for approaches to the regeneration of a functional dental pulp include the wetting of dentin, new dentin formation after caries attack, transmission of pain as an indicator of tissue damage and active tissue defense mechanisms against invading micro-organisms (Schmalz and Galler 2011). Therefore, tissue-engineering-based approaches have been considered an attractive strategy for dental pulp regeneration (Rosa *et al.,* 2013). Using dental pulp tissue engineering strategies, it would be possible to replace the damaged and necrotic pulp with healthy pulp tissues which would help in completion of the root development in immature teeth (Nör, 2006, Sakai *et al.,* 2010). The development of this strategy could possibly substitute the commonly used endodontic treatments and would have a positive impact on the long-term outcome (Syed-Picard *et al.,* 2014, Sakai *et al.,* 2011). Therefore, many scientists and dentists are working together to develop strategies to regenerate lost or diseased dental tissue (Hai-Hua *et al.,* 2011).

1.7.1. Use of stem cells for pulp tissue regeneration

There are two ways to achieve this goal: 1) In this approach host cells are used to regenerate the pulp tissues by inducing them to move towards the inside of the root canal space from the apical end and differentiate into a vascularised pulp tissue (Sakai *et al.,* 2011); 2) This approach involves exogenous stem cell based strategies where, stem cells are delivered to the root canal space from outside and allowed to differentiate into a new dental pulp (Sakai *et al.,* 2011). In both the approaches, stem cells play an important role for dental pulp regeneration due to their multipotency. They are capable of differentiating into odontoblasts, vascular endothelial cells, and neural cells which are the key cells of the pulp tissues (Sakai *et al.,* 2011, Rania *et al.,* 2008). Hence, using the multipotency of stem cells for dental pulp tissue engineering would stop the requirement of multiple cell types (*i.e.,* odontoblasts, endothelial cells, neural cells, fibroblasts) transplantation which is technically a very challenging approach (Sakai *et al.,* 2011).

It was observed that dental pulp stem cells (DPSCs) (Gronthos *et al.,* 2000), periodontal ligament derived stem cells (PDLSCs) (Gould *et al.,* 1977, Gronthos *et al.,* 2006), stem cells from the root apical papilla (SCAP) (Sonoyama *et al.,* 2006) and stem cells from exfoliated deciduous teeth (SHED) (Miura *et al.,* 2003) are multipotent and can differentiate into different cell types. However, it is very difficult to get host dental stem cells specially DPSCs for regenerative purposes from a clinical perspective (Demarco *et al.,* 2011, Ravindran *et al.,* 2013). Hence, it would be advantageous for pulp regenerative engineering if other stem cell sources or if a combination of the multipotent mesenchymal cells could be used (Ravindran *et al.,* 2013). Ravindran *et al.* (2013) investigated human periodontal ligament stem cells (PDLSCs) and human bone marrow stromal cells (HMSCs) for their possible differentiation into odontoblasts by using biomimetic dental pulp extracellular matrix (ECM) incorporated scaffold. This study revealed that these somatic mesenchymal stem cells were able to form vascularised pulp like tissue. These findings also demonstrated that the dental pulp stem derived ECM scaffold stimulated odontogenic differentiation of PDLSCs and HMSCs without the need for exogenous addition of growth and differentiation factors. This study represents a translational perspective toward

possible therapeutic application of using a combination of somatic stem cells and extracellular matrix for pulp regeneration (Ravindran *et al.,* 2013). Bone marrow stromal cells (BMSCs) also have multipotency characteristics with high proliferation rate and are easy to get compared to other stem cell types. They can differentiate into a variety of cell types such as osteoblasts, chondrocytes, adipocytes, myelosupportive fibrousstroma and also muscle and neural tissues. Hence, they are attractive candidates for the pulp regenerative strategies (Gronthos *et al.,* 2000).

1.7.2. Scaffold materials for pulp tissue regeneration

Together with stem cells, scaffold and growth factors are also important components for the success of the regenerative strategies. During the last decade, several studies have been carried out using different dental cell types, scaffolds made up of natural and synthetic materials and growth factors for pulp regenerative investigations which showed a positive response towards the pulp repair strategy (**Table 1.2**) (Hai-Hua *et al.,* 2011, Ravindran *et al.,* 2013).

Table 1.2: Candidate stem cells, active factors and biomaterials for dental pulp tissue engineering (adapted from Hai-Hua *et al.,* 2011).

Components	Suggested candidates	Selected references
Stem cells	DPSCs	Batouli *et al*., 2003; Gronthos *et al*., 2000, 2002; Prescott *et al*., 2008; Miura *et al*., 2003; Cordeiro *et al*., 2008
	SHED	Sonoyama *et al*., 2006; Huang *et al*., 2008; Sonoyama *et al*., 2008
	SCAP	Morsczeck *et al*., 2005; Wu *et al*., 2008; Guo *et al*., 2009; Tsuchiya *et al*., 2010
	DFPCs	Iohara *et al*., 2009
	SPCs	Hu *et al*., 2006; Sloan and Smith, 2007; Morsczeck *et al*., 2008

	BMSCs	Ravindran *et al.,* 2013
Active factors	BMPs	Iohara *et al*., 2004; Nakashima and Reddi, 2003; Nakashima, 2005
	TGF*β*	Chan *et al*., 2005; Liu *et al*., 2007
	FGF-2	Ishimatsu *et al*., 2009
	DSP	Suzuki *et al*., 2009
	DPP	Suzuki *et al*., 2009
	BSP	Decup *et al*., 2000
	DMP-1	Srinivasan *et al*., 1999; Smith *et al*., 1995; He *et al*., 2003; Narayanan *et al*., 2003; He and George, 2004; Almushayt *et al*., 2006; Prescott *et al*., 2008
	MEPE	Six *et al*., 2007; Wang *et al*., 2010
	A + 4 and A −4	Six *et al*., 2004
Biomaterials	Dentine chips	Cordeiro *et al*., 2008; Guo *et al*., 2009
	MTA	Bogen *et al*., 2008; Kuratate *et al*., 2008
	Fibrin	Bashutski and Wang, 2008; Danilovi´c *et al*., 2008
	Collagen	Prescott *et al*., 2008; Iohara *et al*., 2009; Kim *et al*., 2010a
	Gelatin	Ishimatsu *et al*., 2009
	PGA	Mooney *et al*., 1996; Bohl *et al*., 1998; Buurma *et al*., 1999
	PLG	Huang *et al*., 2010
	PLGA	El-Backly *et al*., 2008
	Composites	Shi *et al*., 2005; Mao *et al*., 2010

Successful pulp regeneration is mainly dependent on the scaffold material used which is required ultimately to support cell attachment, migration, proliferation, differentiation and 3D spatial organisation of the cell population required for structural and functional replacement of the target tissue. Hence, choosing the right scaffold material is a very crucial step in pulp tissue engineering. To make pulp regeneration more efficient and reliable, some of the 3D cell culture models such as porous sponges, mesh, fibers, and hydrogels that can mimic the natural environment have been used, as discussed earlier. (Schmalz *et al.,* 1999, Camps *et al.,* 2002, Huangqin and Mingwen 2007). Most of the time organic materials are being used for the regenerative strategies, due to their biocompatibility property which is the most impostant criteria to prevent adverse tissue reactions since the host cells will, in any case, interact with the scaffold. Secondly, biodegradability of the scaffold materials is also very important since it must degrade completely to be replaced by the appropriate tissues at the target site (Galler *et al.* 2011).

Various investigations have been carried out recently using collagen, polyesters, chitosan, or hydroxyapatite as the scaffold material for pulp regeneration showing soft connective tissue formation and newly generated dentin (Galler *et al.,* 2011). Smart materials such as self-assembling peptide hydrogels together with cell adhesion motifs, enzyme cleavable sites, and suitable growth factors would be of great interest for the purpose of dental tissue regeneration. Early studies carried out by Mooney *et al*. (1996) and Bohl *et al*. (1998) showed pulp like tissue formation on PGA scaffolds after 45 to 60 days of *in vitro* cell culture using human dental pulp cells. Gronthos *et al*. (2000) and Miura *et al*. (2003) also demonstrated the formation of dentin, bone, and dentin-pulp complexes, when dental pulp stem cells were cultured on to hydroxyapatite/tricalcium phosphate (HA/TCP). When collagen I and III, alginate, and chitosan were compared for their cell supporting abilities and mineralisation activity, type I collagen showed the highest results.

The most promising investigation was carried out by Nor's group (Cordeiro *et al*., 2008). They showed that when SHED cells were cultured onto PLA, formation of vascularised pulp-like tissue, odontoblast-like cells, and newly generated dentin were observed. Use of PGLA as the scaffold material was

carried out by Huang *et al*. (2010). They observed soft tissue and new dentin formation by culturing the stem cells from apical papilla in an empty root canal space (Huang *et al*., 2010). These studies have concluded that out of all the polymers used for pulp regeneration applications, collagen I and the synthetic polymers (PLA and PLGA) are materials of choice (Galler *et al.* 2011). The above studies carried out to find an ideal scaffold material for dental pulp regeneration had shown that above materials are relatively good materials that exhibit adequate amount of biodegradability and biocompatibility. However, neither of the studies demonstrated an ideal scaffold preparation for pulp regeneration application.

Synthetic polymers (PLA, PGA and copolymers) are commonly used for the dental tissue engineering applications due to their cytocompatibility, degradability by hydrolysis, lower price and easy to prepare properties. Moreover, they have been approved by FDA for various applications (Chan and Mooney 2008). However, they do not have signal molecules commonly present in the natural extracellular matrix (ECM) which control the surrounding cell behavior. Natural polymers such as collagen, alginate and chitosan are also gaining much of the interests for this particular application. However, poor processing methods of collagen and chitosan, uncontrollable degradation of alginate have reinforced the interests of many scientists to develop more efficient and tailor made smart materials for pulp regenerative engineering (Boontheekul *et al*., 2005, Jiang *et al*., 2008).

An ideal scaffold should combine the best properties of each of these groups of biomaterials. These would be structurally similar to ECM at the nanoscale, be able to present complex molecular information to the cells, and be easy to modify for specific applications. To address these deficiencies, novel synthetic matrices are being developed for tissue engineering. Among these, peptide-based nanofibers are particularly promising because of their ease of synthesis, chemical diversity, and high control over various aspects of material behavior (Hartgerink *et al*., 2002, Zhang 2003, Silva *et al*., 2004).

Regarding dentin-pulp-complex engineering, the scaffold should allow us to address the particular challenges of this approach, including contamination

control in the root canal, vascularization and innervation of a long and narrow space, the incorporation of growth and differentiation factors relevant to odontoblast differentiation, the support of mineral formation, and the possibility for creation of acellular matrices capable of recruiting resident stem cells in the respective tissues (Galler *et al.,* 2011).

In this study we have investigated, for the first time, the PHA production by *Pseudomonas mendocina* using different vegetable oils as feedstocks for the culture. The ultimate goal was to obtain novel PHAs with properties suitable for a range of applications, with a special focus on dental tissue engineering.

Aims of the study:

The aim of this work is the production and characterisation of a range of MCL-PHAs with different structural, thermal, mechanical, physical and chemical properties which can be used for different applications. The organism used in this study was *Pseudomonas mendocina*. Various carbon sources used for the production of PHAs for the project were sodium octanoate, coconut oil, groundnut oil, corn oil and olive oil. The polymers produced were characterised in depth with respect to their chemical, physical, mechanical properties and biocompatibility in order for them to be assessed for biomedical applications in particular as the scaffold material for dental pulp tissue engineering in future.

The Specific Aims of the project leading to the above overall aim were:

- Production of MCL-PHAs using *Pseudomonas mendocina* and a range of vegetable oils at shaken flask level: The main objective of this section was to produce MCL-PHAs in shaken flasks by *Pseudomonas mendocina* using cheap carbon sources such as vegetable oils (coconut oil, groundnut oil, olive oil and corn oil) as the replacement of the more expensive carbon feedstocks for this organism. The PHA yields obtained using vegetable oils were then compared with that obtained during the production of PHAs using sodium octanoate as the sole carbon source. This is because sodium octanoate is predominantly being used for MCL-PHA production from *P. mendocina* in our laboratory.

- Growth and PHA production profiles at shaken flask level: To investigate the temporal *P. mendocina* growth and PHA production profiles, flask level fermentations were carried out. Different parameters such as cell growth, pH, nitrogen concentration, dry cell weight and % PHA yield were measured and compared. Samples were withdrawn at regular intervals of time to get continuous growth and production profile.
- Scaling up the production of polymers from shaken flask level to 2 L bioreactor level: PHA production from *P. mendocina* using five different sole carbon sources was also carried out in 2 L bioreactors. In order to produce PHAs in a more controlled manner, scaled up production in 2 L bioreactors were investigated using continuous air supply and agitation.
- Growth and PHA production profiles at fermenter level: Similar to flask level profiling, samples were withdrawn at regular interval of time to get continuous growth and production profile. Overall good carbon feedstock was identified by comparing different parameters as mentioned in flask level fermentations. Finally flask level and 2 L bioreactor level fermentations were compared to identify the higher PHA producing fermentation condition.
- Characterisation: The produced PHAs were analysed and characterised for their chemical nature using Gas Chromatography-Mass Spectroscopy (GC-MS), and Fourier Transform Infrared Spectroscopy (FTIR), mechanical properties using tensile testing Dynamic Mechanical Analysis (DMA), thermal properties using Differential Scanning Calorimetry (DSC), microstructural properties using X-Ray Diffraction (XRD), Scanning Electron Microscopy (SEM) and Static Contact Angle Analysis, molecular weight analysis using Gel Permeation Chromatography (GPC) and finally cytocompatibility of the polymers was assessed using *in vitro* cell analysis towards human Mesenchymal stem cells (hMSCs).

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials:

2.1.1. Bacterial strain and cell line

Pseudomonas mendocina (NCIMB 10542), used in this study to produce a range of MCL-PHAs was obtained from the culture collection of Dr. Roy's laboratory, University of Westminster, London, UK. Biocompatibility studies of the produced polymers were investigated using human Mesenchymal Stromal Cells (hMSCs) which was obtained from Eastman Dental Institute of University College London's cell line collection, London, UK.

2.1.2. Chemicals and Reagents

All the chemicals used in this study were obtained from Sigma-Aldrich or VWR (Leicestershire, UK). Bacterial media preparations were done using general purpose reagents. Analytical studies were carried out using analytical grade reagents. Chromatography grade reagents were used to investigate Gas chromatography mass spectroscopy (GC-MS) of the produced polymers. Distilled and HPLC grade water were used for the estimating experiments. Cell culture studies were carried out using cell culture grade media and reagents purchased from Sigma-Aldrich, UK, and VWR, UK.

1. Reagents required for nitrogen estimations were prepared as follows:

a) Phenol nitroprusside buffer: 3 g of sodium phosphate tribasic, 3 g of sodium citrate and 0.3 g ethylene diamine tetraacetic acid (EDTA) were dissolved in 100 ml HPLC water. pH of the solution was adjusted to 12. Finally, 6 g of phenol and 20 mg of sodium nitroprusside (disodium pentacyano(nitroso)irondiuide) were dissolved in this solution.

b) Alkaline hypochlorite reagent: 2.5 ml of sodium hypochlorite (NaOCl) solution containing 4% chlorine was added to 40 ml of 1M NaOH solution. Finally, the volume was made up to 100 ml by adding HPLC water.

Note: The prepared reagents were kept in dark glass bottles and stored in a fridge for further use. These reagents were prepared fresh every 3 weeks.

2. Trace element solution: **Table 2.5** is the list of the chemicals required for the preparation of trace element solution. All the listed chemicals in **Table 2.5** were well dissolved in 0.1 N HCl and then filter sterilised under aseptic conditions for further use. 1 ml/L filter sterilised trace element solution was used for preparing seed and production media at all time.

2.1.3. Media

In this study, PHA production was carried out using sodium octanoate (purchased from Sigma-Aldrich) and 4 different vegetable oils (coconut oil, groundnut oil, olive oil, and corn oil purchased from Sainsbury supermarket) as sole carbon sources.

2.1.3.1. Inoculum growth medium

Nutrient broth media was used for the growth and the inoculum preparation of *P. mendocina* according to the manufacturer's specifications. The chemical composition of this medium is as followed:

Table 2.1: Chemical composition of inoculum growth medium.

Inoculum growth medium	
Chemicals	**Composition (g/L)**
'Lab- Lemco' Powder	1.00
Yeast extract	2.00
Peptone	5.00
Sodium Chloride	5.00

2.1.3.2. MCL-PHAs production media

In this study, production of MCL-PHAs was carried out from *P. mendocina* using five different sole carbon sources namely sodium octanoate and 4 different vegetable oils (coconut oil, groundnut oil, olive oil, and corn oil). *P. mendocina* was first grown in the inoculum media and then transferred to the production mineral salt medium (MSM) which was divided into two stages, seed and production stages.

Table 2.2: Composition of the second stage MSM (Rai *et al*., 2011).

Seed Mineral Salt Medium (MSM)	
Chemicals	**Composition (g/L)**
$(NH_4)_2SO_4$	0.45
Na_2HPO_4	3.42
KH_2PO_4	2.38
$MgSO_4$	0.4

Table 2.3: Composition of the production stage MSM (Rai *et al*., 2011).

Production Mineral Salt Medium (MSM)	
Chemicals	**Composition (g/L)**
$(NH_4)_2SO_4$	0.5
Na_2HPO_4	3.8
KH_2PO_4	2.65
$MgSO_4$	0.4

Table 2.4: List of sole carbon sources used in this study for PHA production.

Sole Carbon Sources	Seed Medium	Production Medium
Sodium Octanoate	20mM concentration 3.24 g/L	20mM concentration 3.24 g/L
Vegetable Oils	**Seed Medium**	**Production Medium**
Coconut oil	1% (v/v)	1% (v/v)
Groundnut oil	1% (v/v)	1% (v/v)
Olive oil	1% (v/v)	1% (v/v)
Corn oil	1% (v/v)	1% (v/v)

Table 2.5: Composition of the trace element solution (Basnett *et al*., 2014).

Trace Element Solution	
Chemicals	**Composition (g/L)**
$CoCl_2$	0.22
$FeCl_3$	9.70
$CaCl_2$	7.80
$NiCl_3$	0.12
$CrCl_6.H_2O$	0.11
$CuSO_4.6H_2O$	0.16

2.2. Methods:

2.2.1. Production of PHAs

The production of PHAs includes four main steps **(Figure 2.1)**, described as bellow:

1. Culturing of the organisms in suitable growth and PHA production medium.
2. Harvesting of the cell biomass at specific time points and then lyophilisation.
3. Extraction of PHAs from the lyophilised bacterial biomass.
4. Purification of the produced PHAs.

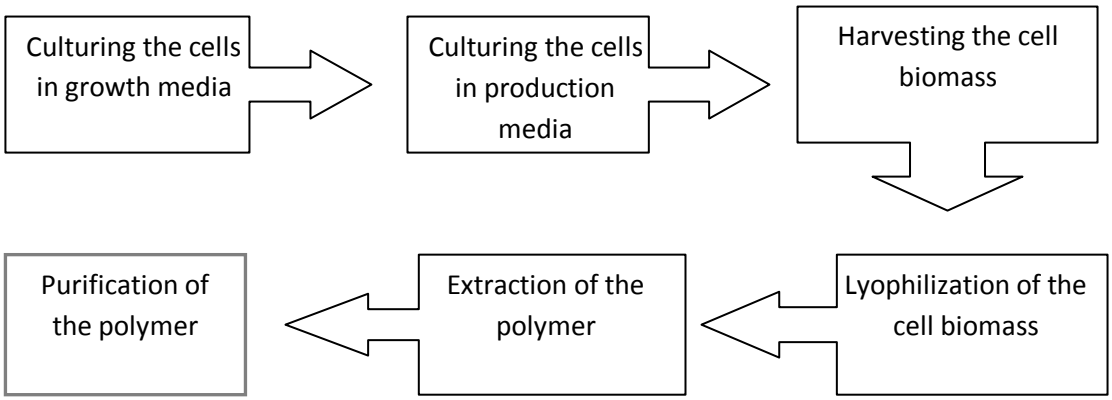


Figure 2.1: Schematic diagram of the steps involved in PHA production.

2.2.1.1. Production of PHAs at shaken flask level

Firstly, to grow the culture, nutrient broth (inoculum growth medium) was prepared by inoculating an isolated single colony of *P. mendocina* and growing it for 24 hours in an orbital shaker at 30°C at 150 rpm. This grown culture was then used to inoculate sterile second stage seed culture medium and incubated under the same culture conditions of 30°C at 150 rpm. The growth of the organism was monitored by measuring optical density (OD) readings at 450nm. For OD values above 0.8, a tenfold diluted culture was used to inoculate sterile third stage production medium and grown for 48 hours at 30°C at 150 rpm.

Throughout the study, while inoculating the production medium, the inoculum volume used was 10% of the final working volume of the production medium.

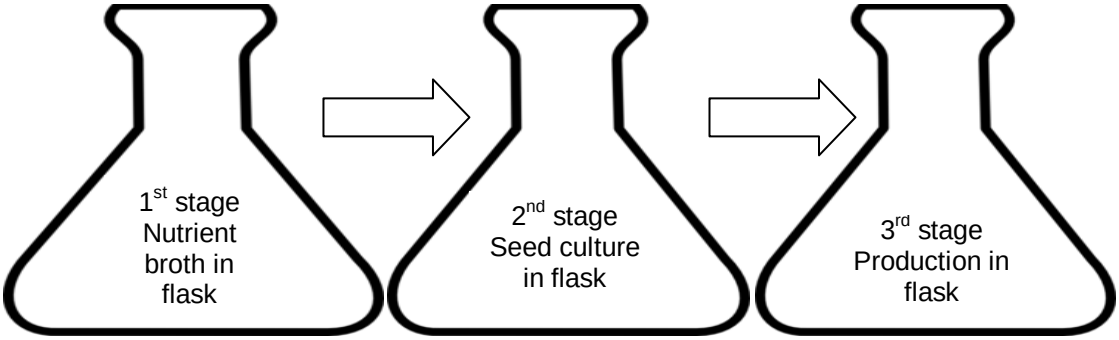


Figure 2.2: Schematic diagram of the three stage PHA production at flask level fermentations.

Note: All the PHA seed and production media (MSM, carbon sources, and magnesium sulphate), was set at a final pH of 7 using 1 M NaOH and 1 M HCl. The sodium octanoate sole carbon source and magnesium sulphate were prepared and sterilised separately. The remaining inorganic salt components of MSM of the media were sterilised together. Vegetable oil for seed and production media were added directly into MSM components while preparing and then sterilised together. All these components (MSM, carbon sources, and magnesium sulphate) were sterilised at 121°C for 15 minutes. At the time of inoculation, all these prepared components were mixed together aseptically first and then inoculated with the 10% grown inoculum and/or seed cultures for seed and production stage respectively. 1 ml/L filter sterilised trace element solution was used for preparing seed and production media at all time. (Rai 2010)

Table 2.6: List of the components for seed/production media at flask and fermenter level productions.

Components of seed/production media
MSM
$MgSO_4$
Carbon source
Inoculum
Trace elements

2.2.1.2. Growth and production profiles at shaken flask level

To investigate growth and production profiles, 5000 ml Erlenmeyer flasks containing 2000 ml of MSM and five different sole carbon sources such as sodium octanoate 20 mM and four 1% vegetable oils (coconut oil, groundnut oil, olive oil, corn oil) were prepared and inoculated with *P. mendocina* when OD reached 1.6 (450nm). These production flasks were then incubated on rotary shaker at 150 rpm at 30°C temperature from which samples were withdrawn at regular interval of time to get continuous growth and production profile. OD was measured at 450nm for each time point. Samples were then centrifuged at 8700 g for 10 minutes to separate biomass and supernatant. pH and nitrogen concentration were measured from the supernatant. From lyophilised cell pellet (biomass), dry cell weights of the harvested samples were determined followed by its PHA extraction. The exeperiments were carried out in triplicates where, three different flasks were used for experimental replications. Moreover, for continuous growth and production curve, day and night flasks were used for each experimental replication.

2.2.1.3. Production of PHAs in bioreactors

PHA production from *P. mendocina* using five different sole carbon sources was also carried out in 2 L bioreactors. Inoculum and seed cultures were prepared in the flasks, similar to shaken flask PHA production. The fermenters were sterilised at 121°C for 30 minutes containing MSM salts. The sodium octanoate sole carbon source and magnesium sulphate were sterilised separately at 121°C for 15 minutes. Whereas, vegetable oil sole carbon sources were added directly to the fermenters and sterilised together with MSM. At the time of production stage, seed cultures, sodium octanoate, magnesium sulphate, and trace element solution were mixed in the fermenters aseptically. The fermenters were also inoculated with 10% of second stage seed cultures in all cases. pH was set at 7.0 at the beginning of the fermentations. Prepared fermenters were then incubated for 48 hours at 30°C with continuous stirring at 200 rpm and 1 volume per volume per minute (VVM) air.

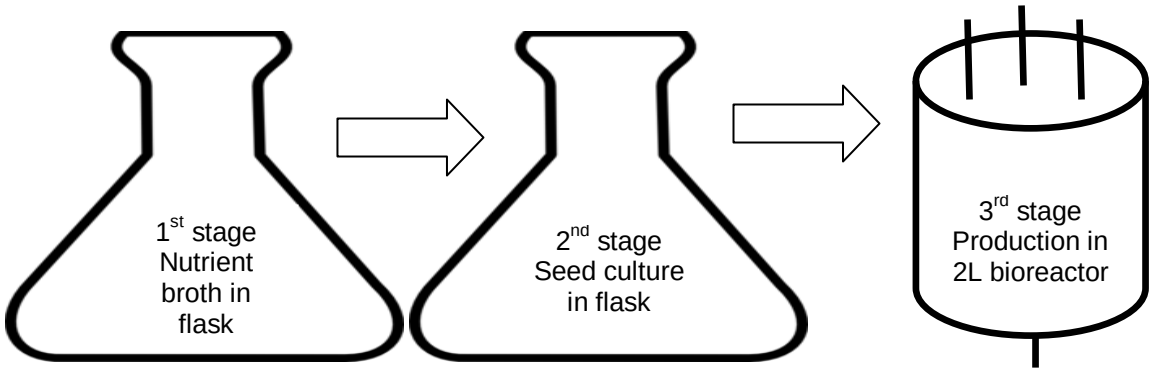


Figure 2.3: Schematic diagram of the three stage PHA production at 2 L bioreactor level fermentations.

2.2.1.4. Growth and production profiles in bioreactors

P. mendocina growth and PHA production were also investigated in 2 L bioreactor level fermentations. The working volume used in the 2 L fermenter was 1.5 L. Fermenters were prepared as stated in **2.2.1.3.** Under aseptic conditions, samples were withdrawn at regular interval of time to analyse continuous growth and production profile as described earlier in **2.2.1.2.** All these profiling studies were carried out in duplicates where, two different bioreactors were used for experimental replications. Moreover, for continuous growth and production curve, day and night bioreactors were used for each experimental replication.

2.2.2. Extraction of the PHAs

From production media, cells were harvested at appropriate time periods by centrifugation at 12,000 g for 30 minutes. Supernatant was discarded and the cell pellets were dried and lyophilised for 48 hours. PHAs were extracted from the lyophilized cells using dispersion of chloroform ($CHCl_3$) and sodium hypochlorite (NaOCl) method. In this method, dried cells were incubated in 80% sodium hypochlorite and chloroform in 1:4.5 ratio for 2 hours at 30^{o}C and 150 rpm. After incubation, the suspension was centrifuged at 12,000 g for 20 minutes. As a result of centrifugation 3 distinct layers formed in the centrifuge tubes. The top layer was an aqueous phase of sodium hypochlorite, the middle layer contained cell debris, and the bottom layer was the chloroform containing

dissolved PHAs. The lower most layer of the chloroform containing polymer was then collected and filtered. The extract was concentrated by evaporating chloroform using a rotary vacuum evaporator. PHAs were finally precipitated by adding concentrated chloroform solution dropwise into ten volumes of chilled methanol with continuous stirring.

2.2.3. Analytical methods used for profiling

At regular time interval, samples were withdrawn to get continuous growth and production profiles. Optical density was measured at 450nm wavelength for each time point. Samples were then centrifuged to separate biomass and supernatant. pH and nitrogen concentration were measured from the supernatant. From lyophilised cell pellet (biomass), dry cell weights (dcw) and PHA estimations were carried out.

2.2.3.1. Biomass estimation

For each time intervals, 1 ml samples were taken out aseptically from the production medium to get biomass estimations. Optical density was measured using a spectrophotometer at 450nm wavelength for each time point. The samples were then centrifuged at 8700 g for 10 minutes and cell pellets were weighed after freeze drying to get dry cell weight measurements.

2.2.3.2. Nitrogen estimation

For each time point, the available nitrogen molecules' estimations were carried out using the phenol hypochlorite reaction method in order to know the utilisation of nitrogen for the cell growth. As mentioned above, 1 ml samples were taken out aseptically from production media which were centrifuged at 8700 g for 10 minutes. From supernatants, nitrogen concentrations were analysed by making dilutions of the samples. 2.5 ml of these diluted samples were then gently mixed with 1 ml of phenol nitroprusside buffer by swirling. The next step was to add 1.5 ml of the hypochlorite reagent quickly and gently mix by inversion. It was followed by 45 minutes of incubation at room temperature in

the dark. Finally, spectrophotometric readings were taken at 635nm wavelength.

2.2.3.3. PHA estimation

In case of profiling, at specific time intervals, samples were taken out aseptically from production media to get PHA estimations. Culture samples were then centrifuged at 12,000 g for 30 minutes. The collected cell mass was then freeze dried to follow PHA extraction method as described earlier in section **2.2.2.** The precipitated PHAs were then weighed after drying to get PHA production profiles for specific time intervals.

2.2.4. Purification of the produced PHAs

The polymer extracted using the dispersion of hypochlorite and chloroform was subjected to sequential repeated steps of precipitation to reduce or remove contaminants. Further purification of the produced polymers was carried out using the following method stepwise. This procedure was repeated several times to obtain purified polymers. **Note:** In each step, polymer solution and solvent were used in 1:10 ratio for precipitation.

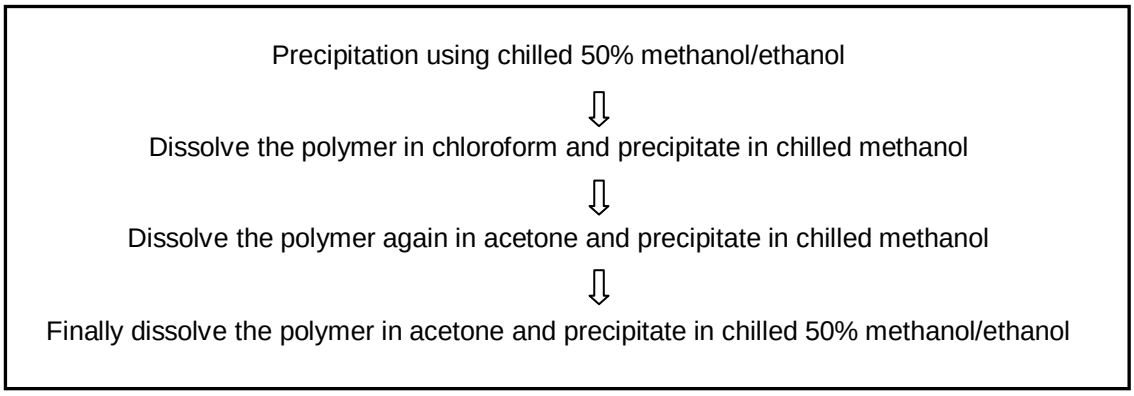


Figure 2.4: Flowchart of the polymer purification procedure.

2.2.5. Solvent Cast film preparation

Of the five PHAs extracted in this study, polymers produced from *P. mendocina* using sodium octanoate and coconut oil were fabricated into 2D films. Neat films were fabricated by dissolving 0.5 g of the polymer in 10 ml of $CHCl_3$. The

polymers were well dissolved in chloroform after which the polymer solutions were filtered. The films were casted by pouring the filtered polymer solutions into 60 mm glass Petri dishes. The solutions were then left to air dry at room temperature for 1 week followed by freeze drying for 10 days.

It was very difficult to prepare solvent cast films of the PHAs produced from *P. mendocina* using groundnut oil, olive oil and corn oil as sole carbon sources due to their very sticky nature. Therefore, thin films were made on glass cover slips using 100µl solution of 5 wt% polymers in chloroform followed by air drying at room temperature for 1 week and freeze drying for 10 days.

2.2.6. Characterisation of the produced PHAs

Chemical, physical, mechanical and biocompatibility properties of the produced polymers have been characterised by performing the following analysis.

2.2.6.1. Fourier Transform Infrared Spectroscopy (FTIR)

To identify the kind of the PHAs (SCL/MCL) produced, preliminary analysis of the polymer was performed using FTIR. Approximately 5 mg of the polymer was used for the study. The analysis was performed under the following conditions: Spectral range 4000 to 400 cm^{-1}; window material, CsI; 10 scans and resolution 4 cm^{-1}. The analysis was carried out at the Department of Biomaterials and Tissue engineering, Eastman Dental Institute, University College London, UK.

2.2.6.2. Gas Chromatography-Mass Spectroscopy (GC-MS)

For identification of the produced PHAs compositions, GC-MS analysis was carried out on the methanolysed products of these PHAs. Methanolysed samples were prepared as described by Furrer *et al*. (2007). 10 mg of polymer were added into 1 ml of methylene chloride containing 10 mg/ml of 2-ethyl-2-hydroxybutyric acid. This reaction mixture was then covered and incubated at room temperature for 1 hour to dissolve the polymer. Following incubation, 1 ml of 0.65M boron trifluoride (BF3) solution in methanol was added, after which the tube was tightly sealed and vigorously shaken. This reaction mixture was then

refluxed for 16 hours. After the reaction, the tubes were cooled on dried ice for 5 minutes, 2 ml HPLC water was added and the tubes were vortexed for 1 minute. After phase separation, the bottom organic phase was collected, dried over 10 mg anhydrous sodium sulphate and sodium carbonate. It was then filtered and used for carrying out the GC-MS study (Furrer *et al.,* 2007), which was conducted at the School of Chemistry, University of Southampton, UK.

2.2.6.3. Differential Scanning Calorimetry (DSC)

The thermal properties (glass transition temperature (T_g) and melting temperature (T_m)) of the polymers produced were analysed by differential scanning calorimetry (DSC) using a Perkin Elmer Pyris Diamond DSC (Perkin Elmer Instruments). The sample mass used for these measurements was in the range of 5-13 mg. Samples were encapsulated in standard aluminium pans and tests were performed under inert nitrogen environment. The samples were heated/cooled/heated at a heating rate of 20^{o}C min^{-1} between -50 and 200^{o}C. The measurements were carried out in triplicates. The analysis was done at the Department of Biomaterials and Tissue engineering, Eastman Dental Institute, University College London, UK.

2.2.6.4. Tensile testing

Tensile strength tests were conducted on flat specimens (width: 1.15 mm, length: 3 mm) cut out from the solvent cast polymer films, using a Perkin Elmer Dynamic Mechanical Analyser (DMA 7e, Perkin Elmer Instruments, USA) at room temperature. The initial load was set to 1 mN and then increased to 6000 mN at the rate of 200 mN min^{-1}. The tests were carried out in triplicate. Young's modulus, tensile strength and elongation at break were recorded during the test. The analysis was done at the Department of Biomaterials and Tissue engineering, Eastman Dental Institute, University College London, UK.

2.2.6.5. Gel Permeation Chromatography (GPC)

The molecular mass data of the polymers produced i.e. number average molecular weight, (M_n) and weight average molecular weight, (M_w) were

determined by carrying out gel permeation chromatography analysis. Chloroform was used as an eluent at a flow rate of 1.0 ml min^{-1}. Polystyrene standards having a low polydispersity rate were used to produce the calibration curve. The samples were sent for the analysis at the Department of Chemistry, University of Nottingham, UK.

2.2.6.6. X-ray Diffraction (XRD)

Thin films of the polymers produced were made on the cover slips using 100 µl of 5 wt% polymer solution in chloroform as described earlier in section **2.2.5.** These prepared films were then used to analyse the crystallinity of the produced polymers for X-ray diffraction (XRD) using a Bruker D8 Advance. Cu Kα radiation (l = 1.54 A˚) operating at 40 kV and 40 mA was used for this investigations. Scans were performed with a detector step size of 0.02^{o} over an angular range 2θ = $10\text{-}100^{o}$ and counting for 1 second per step. The analysis was carried out at the Department of Biomaterials and Tissue engineering, Eastman Dental Institute, University College London, UK.

2.2.6.7. Scanning Electron Microscopy (SEM)

SEM images of the produced polymers for their surface topography were carried out using INCAx-Sight scanning electron microscope (OXFORD INSTRUMENTS). The samples were placed on 8 mm diameter aluminium stubs and then coated with gold using the gold spluttering device (EMITECH-K550) for 2 minutes. The operating pressure of 7 x 10^{-2} bar and deposition current of 20 mA was used. The SEM images were taken at various magnifications with an acceleration voltage of 5 kV (maximum) to avoid incineration of the polymer due to the beam heat. Analysis was carried out at the Department of Biomaterials and Tissue engineering, Eastman Dental Institute, University College London, UK.

2.2.6.8. Contact angle analysis

To characterise the wettability i.e. hydrophilicity/hydrophobicity of the fabricated films of PHA produced, the surface contact angle analysis was carried out at the

Department of Biomaterials and Tissue engineering, Eastman Dental Institute, University College London, UK. Through the profile of a liquid drop placed on a polymer film surface, wettability was measured using a KSV Cam 200 optical contact angle meter (KSV Instruments Ltd). By using a gas tight micro-syringe an equal volume of the liquid on each sample was placed forming a drop and photos (frame interval of 1 second, number of frames = 10) were taken to record the shape of the drops. The contact angles of the water droplets on the specimens were measured by analysing the recorded drop images using the Windows based KSVCam software.

2.2.7. Cell culture studies

The *in vitro* cell culture studies were carried out on the PHA films prepared on glass cover slips using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay and SEM.

2.2.7.1. Cell culture preparation

Human Mesenchymal Stem Cells (hMSCs) were cultured into standard tissue culture flasks using alpha-MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were incubated at 37^{o}C in a 5% CO_2 humidified atmosphere until 90% confluence, and the culture medium was changed every 2 days. 2-3 cell passages were carried out before seeding onto the test samples. For each passage, cells were detached from the flask by trypsinisation using 5% trypsin at 37^{o}C for 2 minutes. The reaction was stopped by adding equal volume of supplemented alpha-MEM. Samples were then centrifuged at 400 g for 10 minutes and the resulting pellet was resuspended in fresh supplemented alpha-MEM. Cell suspension was then distributed into two sterile 75 cm^2 tissue culture flasks and incubated for further growth. Media, PBS and trypsin used in this study were pre-warmed at 37^{o}C and filter sterilised prior to use.

2.2.7.2. Test sample preparation

PHA films were prepared on glass cover slips as described earlier in section **2.2.5.** After drying, the cover slip polymer films were placed in 24 well tissue culture plates and sterilised under UV light for 1 hour. These prepared test samples were then passivated for 12 hours with supplemented alpha-MEM culture media prior to hMSCs seeding.

2.2.7.3. hMSCs seeding onto test samples

Following three passages and 90% confluence growth, cells were harvested using trypsinisation and centrifugation as described earlier. The cells were counted using haemocytometer. Passivated test samples were seeded with 2 x 10^4 cells/well and maintained in supplemented alpha-MEM. Cells seeded in the wells without test samples were used as the positive controls. While, test samples incubated in culture media without cells were used as the negative controls. Plates were then incubated at 37^{o}C with 5% CO_2 for further analysis. The medium was changed every 2 days. The cells were allowed to proliferate for a period of 14 days. Cell culture studies were carried out in triplicate samples per experiment.

2.2.7.4. MTT colorimetric assay

Human Mesenchymal Stem Cells (hMSCs) were allowed to proliferate for 1, 4, 7, and 14 days, and % cell viability was determined by the MTT assay. First of all, 100 µl (5 mg/ml) MTT assay solution was added to each sample and incubated at 37^{o}C with 5% CO_2 for 4 hours. Followed by incubation, films were transferred to a new 24 well plate and 500 µl of DMSO was added. After 10 minutes incubation at room temperature, 100 µl of solution was transferred to a 96 well plate and the absorbance was measured at 570nm using a microtitre plate reader. Negative control readings were deducted from the test samples readings to avoid any background absorbance. By using the following equation, % cell viability was measured. The positive control was normalised to 100%.

$$\% \text{ cell viability} = \frac{\text{Mean absorbance of samples X 100}}{\text{Mean absorbance of control}}$$

2.2.7.5. Cell proliferation SEM

For each time point, constructs containing the cells were visualised using SEM to observe the human Mesenchymal Stem Cells spreading and attachment to the surface of the test samples. Immediately after incubation is over, the specimens were fixed in 0.1 M phosphate buffer containing 3% glutaraldehyde for 12 hours at 4^{o}C. These fixed samples were then dehydrated using a series of graded ethyl alcohols such as 20%, 50%, 70%, 90% and 100% for 10 minutes incubation in each solution. In the final step, samples were dried using hexamethyldisilazane for 2-5 minutes. The samples were then left to air dry for one hour in the fume cupboard. Finally, the dried samples were attached to aluminium stubs, gold coated and examined using INCAx-Sight scanning electron microscope (OXFORD INSTRUMENTS).

2.2.8. Statistical analysis

Data are reported as mean ± STDEV. Statistical significance was assessed using ANOVA single factor. Differences were considered statistically significant when $*p<0.05$, very significant $**p<0.01$ and highly significant when $***p<0.001$.

CHAPTER 3

Production of PHAs at shaken flask and fermenter level

3.1. Introduction:

In 1983, MCL-PHAs were first discovered in *P. oleovorans* using octane as the sole carbon source (Rai *et al.,* 2011). *Pseudomonas* sp. belonging to the rRNA homology group I, are particularly known to produce MCL-PHAs. MCL-PHAs contain 6-14 carbon atoms, for example poly(3-hydroxyhexanoate), P(3HHx), poly(3-hydroxyoctanoate), P(3HO), poly(3-hydroxydecanoate), P(3HD), poly(3-hydroxydodecanoate), P(3HDD), poly(3-hydroxytetradecanoate), P(3HTD) and poly(3-hydroxyhexadecanoate), P(3HHD) (Hazer *et al.,* 2012). Until now, more than 100 different monomer units have been characterised within the MCL-PHAs from *Pseudomonas* strains using different carbon sources. Several *Pseudomonas* strains such as *P. oleovorans*, *Pseudomonas sp.* DSY-82, *Pseudomonas stutzeri*, *Pseudomonas sp.* 61-3, *Pseudomonas sp.* A33, *Aeromonas* sp. e.g. *Aeromonas hydrophila*, and *Cuprivadus necator* are known to produce MCL-PHAs using broad range of structurally related and unrelated carbon feedstocks (Rai *et al.,* 2011). These organisms are not only able to synthesise MCL-PHAs but also accumulate SCL-MCL copolymers. *Pseudomonas* sp. predominantly produces MCL-PHA copolymers. When the substrate with even carbon atoms (C_6, C_8, C_{10}, C_{12}, C_{14}) are used as the feedstock for the organisms, the produced polymer contains 3-hydroxyoctanoate as the major monomer unit. Substrate with odd number carbon atoms (C_7, C_9, C_{11}), it was observed that the organism produced polymer with 3-hydroxynonanoate as the predominant monomer unit (Rai *et al.,* 2011).

SCL-PHAs such as P(3HB) are highly rigid and brittle in nature. Hence, their applicability is limited to hard tissue engineering (Rai *et al.,* 2011, Hazer *et al.,* 2012). MCL-PHAs are more flexible and have elastomeric nature. Moreover, copolymerisation of the MCL-PHAs makes them structurally more diverse than SCL-PHAs. This allows efficient tailoring of the physical and mechanical properties of MCL-PHAs for the required applications (Hazer *et al.,* 2012). Although MCL-PHAs have outstanding physical and mechanical properties to be utilised in various applications, their development as the potential industrial material has been limited due to their high price (approximately US $ 16/kg) (Fukui and Doi 1998). Substrate costs, downstream processing costs and

relatively low PHA yields contribute to the high price of PHAs. Petroleum-based plastics are commercially viable because of their high abundance and low production cost. Whereas, in case of the MCL-PHA production, expensive carbon substrates for the producing organisms increase the overall cost of the polymer production. In recent years, scientists are focusing on using cheap renewable substrates for the organism growth and PHA accumulation in order to reduce the overall PHA production cost. Several inexpensive carbon feedstocks such as sugars, organic wastes, many agricultural and dairy by-products and vegetable oils have been identified to be utilised as the renewable substrates for the organisms (Daniel *et al.,* 2006, Chenyu *et al.,* 2012, Chee *et al.,* 2010). Amongst these, vegetable oils showed higher PHA productivity with the ability to reduce polymer production cost. There are few reports on using plant oils as the carbon feedstocks of the organisms for PHA production. *Aeromonas caviae, Pseudomonas aeruginosa, Pseudomonas resinovorans, Ralstonia eutropha, Pseudomonas putida, Comamonas testosteroni, Pseudomonas saccharophila, Pseudomonas stutzeri* have been shown to produce both SCL and MCL PHAs using plant oils (soybean oil, castor oil, sunflower oil, and palm oil) (Daniel *et al.,* 2006, Chenyu *et al.,* 2012, Chee *et al.,* 2010). Among these, Pseudomonads were able to produce MCL PHAs using vegetable oils (Ashby and Foglia 1998). However, there has not been a single study carried out on *Pseudomonas mendocina* for PHA production using vegetable oils. Hence, it remains a relatively unexplored organism for PHA production using vegetable oils as the carbon source. In this study we have investigated, for the first time, PHA production by *Pseudomonas mendocina* using different vegetable oils as the feedstocks for the culture.

The main objective of this chapter was to produce MCL-PHAs using *Pseudomonas mendocina* and cheap carbon sources such as vegetable oils (coconut oil, groundnut oil, olive oil and corn oil) as the replacement of the more expensive carbon feedstock (i.e. sodium octanoate) for this organism. To investigate temporal *P. mendocina* growth and PHA production profiles using vegetable oil carbon feeds, different parameters such as cell growth, pH, nitrogen concentration, dry cell weight and % PHA yield were measured at regular intervals of the fermentation time. The temporal fermentation profile of *P. mendocina* using sodium octanoate sole carbon source was also assessed

at shaken flask and fermenter levels in this study. Finally, the results obtained for *P. mendocina* growth and production profiles using sodium octanoate and vegetable oils as the sole carbon sources were compared at shaken flask and 2 L fermenter levels.

3.2. PHA production at shaken flask level:

Investigations of PHA productions from *P. mendocina* using five different sole carbon sources (sodium octanoate, coconut oil, groundnut oil, corn oil and olive oil) were first carried out at the shaken flask level. This was necessary to gain more information for the scaling up of the production process. To investigate growth and production profiles, 5000 ml Erlenmeyer flasks containing 2000 ml of MSM and five different sole carbon sources, sodium octanoate 20 mM and four 1% vegetable oils (coconut oil, groundnut oil, olive oil, corn oil), were prepared and inoculated using cells of *P. mendocina,* as described earlier. At the shaken flask level, the agitation speed was kept constant at 150 rpm throughout the duration of the fermentation and the fermentation was continued until the optical density of the broth culture began to decrease. At this time, the maximal PHA accumulation was expected to have completed. The cultivation time was extended for a further six hours after the death phase was attained. This was necessary to understand the effect of the harvesting time on PHA yield. Samples were withdrawn at regular intervals of time, i.e., every 3 hours to monitor the growth profile of *Pseudomonas mendocina* throughout the fermentation process. Optical density of the culture was measured at 450nm. Samples were then centrifuged to separate biomass and supernatant. pH and nitrogen concentration were measured using the supernatant. From lyophilised cell pellet (biomass), dry cell weight of the harvested samples was determined and dry cells were used for PHA extraction (discussed in section **2.2.1.2.** in detail).

Figure 3.1: Shaken flask level PHA production.

3.2.1. PHA production using sodium octanoate as the sole carbon source

Using 5 L flasks, batch production of MCL-PHA was carried out from *P. mendocina* using sodium octanoate as the sole carbon source. The organism was grown for 54 hours and samples were withdrawn every 3 hours to investigate the growth and production of PHAs. **Figure 3.2** shows the fermentation profile obtained for *P. mendocina* using sodium octanoate as the sole carbon source.

The fermentation profile of PHA production shows the temporal variation in optical density (OD) at 450nm, dry cell weight (dcw) g/L, pH, nitrogen concentration (g/L), and PHA yield (% dcw), when *P. mendocina* was grown in sodium octanoate as the sole carbon source.

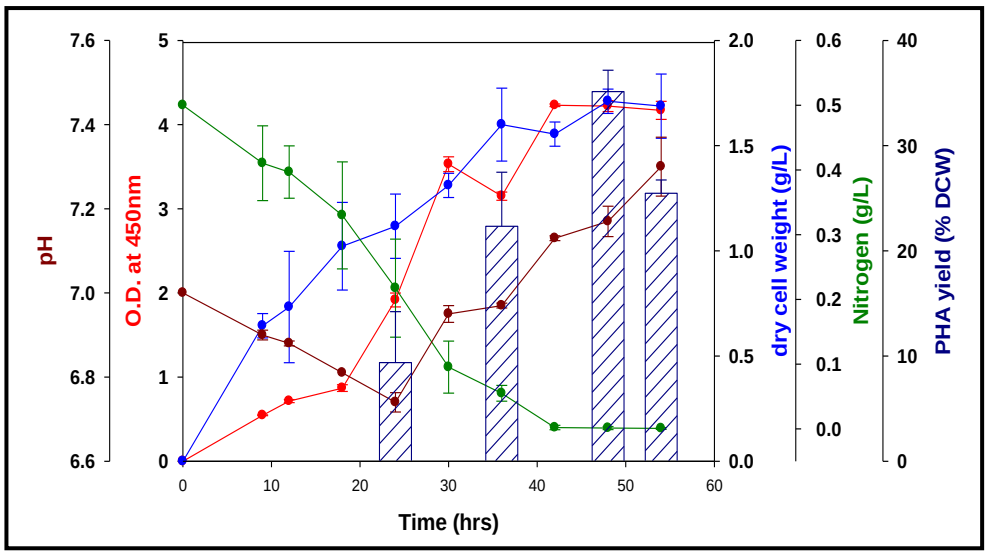


Figure 3.2: Fermentation profile for MCL-PHA production by *P. mendocina* using sodium octanoate as the sole carbon source in shaken flasks.

From this profile, it can be seen that OD and dry cell weight (dcw) increased simultaneously as the fermentation progressed. The dcw increased from 0.6 g/L at the 9th hour to 1.7 g/L at the 48th hour. After the 54th hour, both OD and dcw became constant. PHA accumulation at 24, 36, 48, and 54 hours were measured and the results obtained showed that the highest amount of PHA accumulation observed was 35.1% dcw at 48 hour. The PHA accumulation had however decreased to 25.5% dcw by the 54th hour. In the beginning of the fermentation, nitrogen concentration was 500 mg/L. As the fermentation progressed, it was observed that the amount of nitrogen decreased steadily in the media and dropped from 500 mg/L to 0.6 mg/L. As the fermentation progressed, the pH of the culture medium, which was initially set to 7.00 had decreased and dropped to 6.74 by 24 hours. However, increase in the pH was observed after 24 hours reaching 7.30 at the end of the fermentation.

3.2.2. PHA production using coconut oil as the sole carbon source

In order to evaluate the potential of vegetable oils as the replacement of the more expensive carbon sources in PHA production, *P. mendocina* was grown on MSM and supplemented with 1% v/v vegetable oils as the sole carbon sources in 5 L shaken flasks. The first vegetable oil used for PHA production from *P. mendocina* was coconut oil as the sole carbon source and the results

obtained are shown in **Figure 3.3.** The production was carried out for 60 hours and samples were withdrawn every 3 hours to investigate growth as well as production profile.

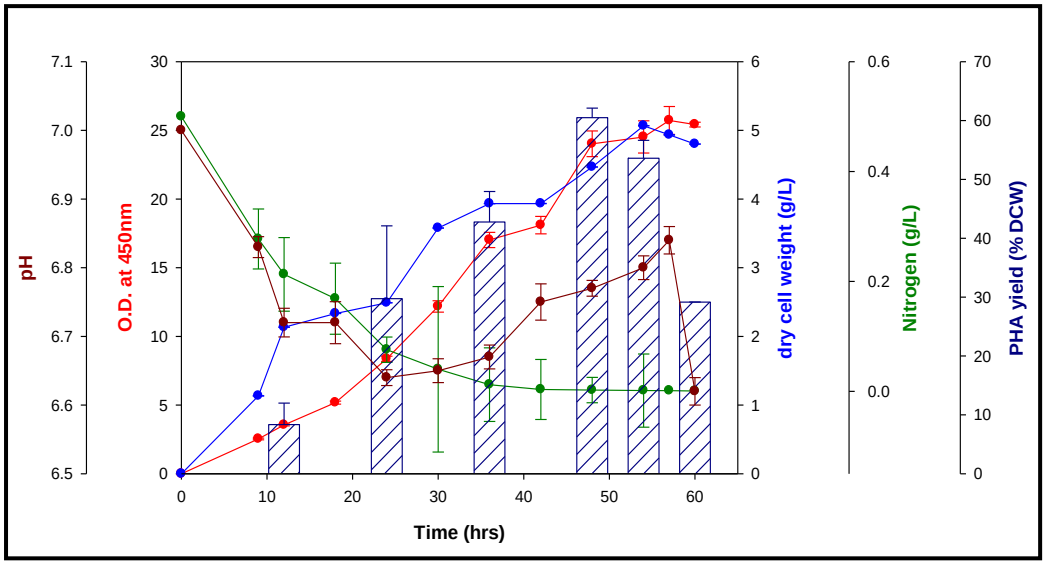


Figure 3.3: Fermentation profile for MCL-PHA production by *P. mendocina* using coconut oil as the sole carbon source in shaken flasks.

This fermentation profile showed simultaneous increase in both, the cell growth and dried biomass until 54 hours of the fermentation time. The dcw observed for the organism ranged between 1.1 to 5.1 g/L. Measurement of PHA accumulation at 12, 24, 36, 48, 54 and 60 hours were carried out and results obtained showed that the highest amount of PHA accumulated was 60.5% dcw at the 48th hour, after which a decrease was observed at the 54th hour. The amount of nitrogen decreased steadily in the media as the fermentation progressed and dropped from the initial value of 500 mg/L to 0.05 mg/L. At the beginning of the fermentation the pH was set at 7.00 and as the fermentation progressed the pH dropped to 6.64 at the 24th hour. This was followed by an increase in pH and the pH reached a value of 6.84 at the 57th hour. At the end of the fermentation, pH of the media dropped to 6.62.

3.2.3. PHA production using groundnut oil as the sole carbon source

The shaken flask PHA production was also carried out using groundnut oil as the sole carbon source in order to get preliminary data of cell growth and PHA production by *P. mendocina.* Fermentation was carried out for 54 hours.

Different parameters (OD, pH, nitrogen concentration, dcw, PHA yield) were measured at regular intervals of time in order to get temporal fermentation profile of *P. mendocina* using groundnut oil as the sole carbon source.

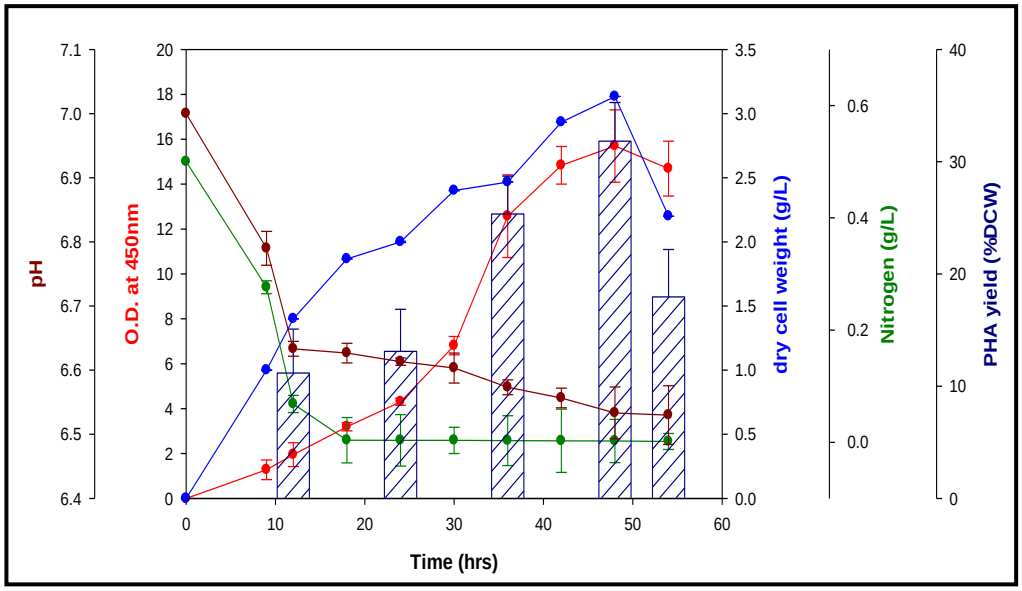


Figure 3.4: Fermentation profile for MCL-PHA production by *P. mendocina* using groundnut oil as the sole carbon source in shaken flasks.

Groundnut oil was the next chosen carbon source for growth and polymer production using *P. mendocina*. The results obtained are shown in **Figure 3.4.** The profiles also showed steady increase in both, OD and dcw as the fermentation progressed. The dcw was achieved in the range of 1.0 g/L at the 9th hour to 3.1 g/L at the 48th hour. After 48 hours, both OD and dcw seem to have decreased. Polymer was already starting to accumulate in the organism after 12 hours of the fermentation. The polymer yield ranged between 11.2% dcw to 31.8% dcw. The highest PHA yield of 31.8% dcw was achieved at the 48th hour, after which the yield decreased to 18.0% dcw at the 54th hour. At this point, the amount of nitrogen dropped to 1.5 mg/L from the initial value of 500 mg/L. During the fermentation, the pH of the culture medium decreased from 7.00 to 6.53.

3.2.4. PHA production using olive oil as the sole carbon source

The next shaken flask PHA production was carried out using olive oil as the sole carbon source in order monitor cell growth and PHA accumulation in *P. mendocina.* In this fermentation, cells were incubated for 60 hours and samples were assessed for different parameters similar to earlier fermentations.

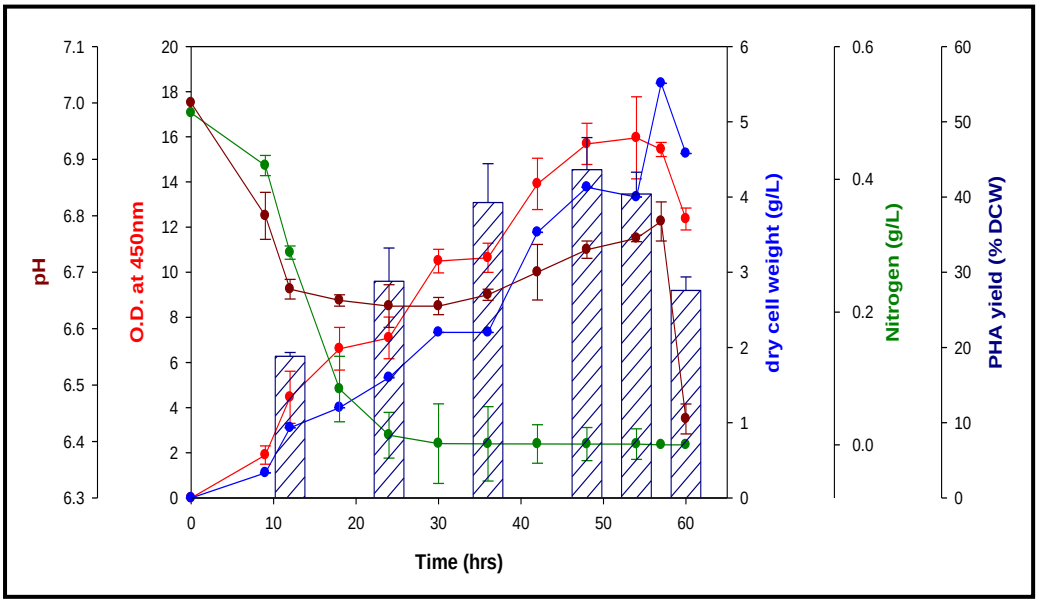


Figure 3.5: Fermentation profile for MCL-PHA production by *P. mendocina* using olive oil as the sole carbon source in shaken flasks.

The results obtained are shown in **Figure 3.5.** Simultaneous increase in OD and dcw was observed during this fermentation. The dcw of the organism ranged between 1.3 to 5.5 g/L. The highest dcw (5.5 g/L) was observed after 54 hours of fermentation and by 57 hours, the dcw started to decrease reaching a value of 4.6 g/L at the end of the fermentation. Here too the polymer started to accumulate at very early stage of the fermentation. PHA produced by the organism ranged between 18.8 to 43.6% dcw. The polymer yield increased up to 48 hours with the highest PHA accumulation of 43.6% dcw, after which the yield decreased to 40.4% dcw by 54 hours. The level of nitrogen decreased from the initial value of 500 mg/L to that of 1.0 mg/L at the end of the fermentation. As the fermentation progressed, the pH of the culture medium, which was initially set to 7.00, decreased and dropped to 6.64 at 30 hour, after which it started to increase again and reached 6.79 at the 54th hour. At the end of the fermentation, pH again dropped to 6.44.

3.2.5. PHA production using corn oil as the sole carbon source

The final vegetable oil used as the sole carbon source was corn oil. The shaken flask level PHA production was carried out for 72 hours using corn oil carbon feed and at regular interval of time different parameters were measured in order to get preliminary data of *P. mendocina* growth and PHA production.

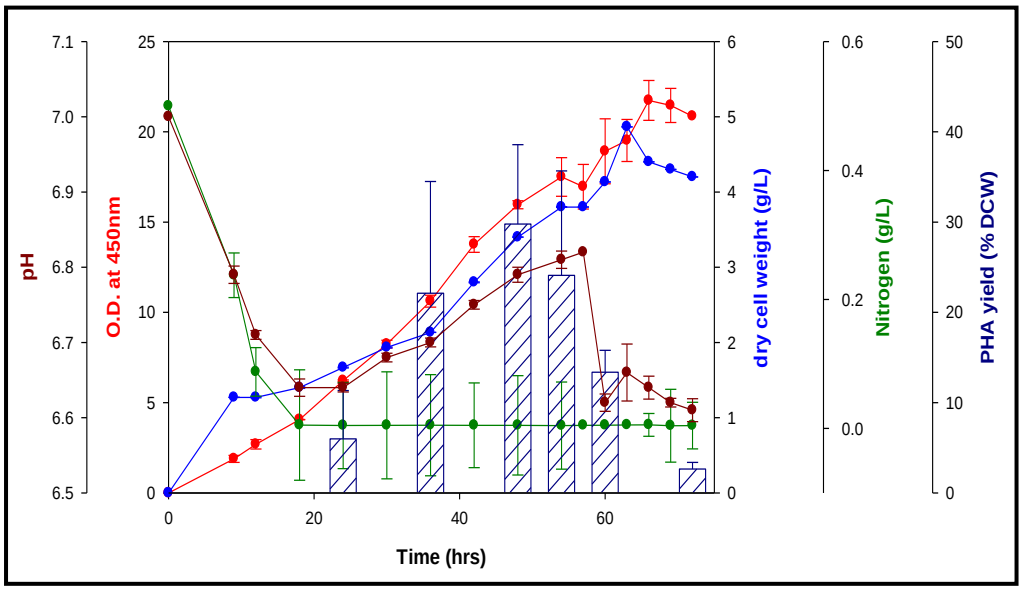


Figure 3.6: Fermentation profile for MCL-PHA production by *P. mendocina* using corn oil as the sole carbon source in shaken flasks.

Figure 3.6 shows the temporal variation of the various relevant parameters during the fermentation. Growth profile of *P. mendocina* using corn oil as the sole carbon source showed longer cell growth phase compared to the other *P. mendocina* growth profiles at shaken flask level. The organism entered stationary phase within 66 hours of the fermentation, after which it started to decline. The dcw of the organism ranged between 1.3 to 4.9 g/L. Here polymer accumulation was only observed after 24 hours of the fermentation. The organism exhibited maximum accumulation of the polymer at the 48th hour which was 29.8% dcw. By 72 hours of the fermentation, amount of nitrogen in the medium had dropped to 4.2 mg/L. During the fermentation, the pH of the medium which was set at 7.00 decreased and reached to a value of 6.62 by the end of the fermentation.

3.3. PHA production at fermenter level:

Optimisation of the PHA production by *P. mendocina* using four vegetable oils (coconut oil, groundnut oil, olive oil and corn oil) as the sole carbon sources was also investigated in 2 L bioreactors, for the first time, in this study. The PHA yield obtained in the bioreactors using vegetable oils was compared to the PHA yield obtained using sodium octanoate as the sole carbon source in the 2 L bioreactors. The working volume used in the 2 L fermenter was 1.5 L. Fermenters were prepared as stated in **2.2.1.3.** Temperature of the fermenter was set to 30°C followed by continuous stirring at 200 rpm and 1 vvm air flow. At the beginning of the fermentation pH was set at 7.0. Under aseptic conditions, samples were withdrawn at regular intervals of time to analyse continuous growth and production profiles as described earlier in **2.2.1.2.** All profiling experiments were carried out in duplicates.

Figure 3.7: 2 L bioreactor level PHA production.

3.3.1. PHA production using sodium octanoate as the sole carbon source

MCL-PHA production study using sodium octanoate as the main carbon source was further extended to the fermenter level (2 L). The organism was grown for 54 hours and the samples were withdrawn at regular intervals to investigate growth as well as production profiles. **Figure 3.8** shows the profile of the parameters (optical density (OD) at 450nm, dry cell weight (dcw) g/L, pH, nitrogen concentration (g/L), and PHA yield (%dcw)) measured during the study.

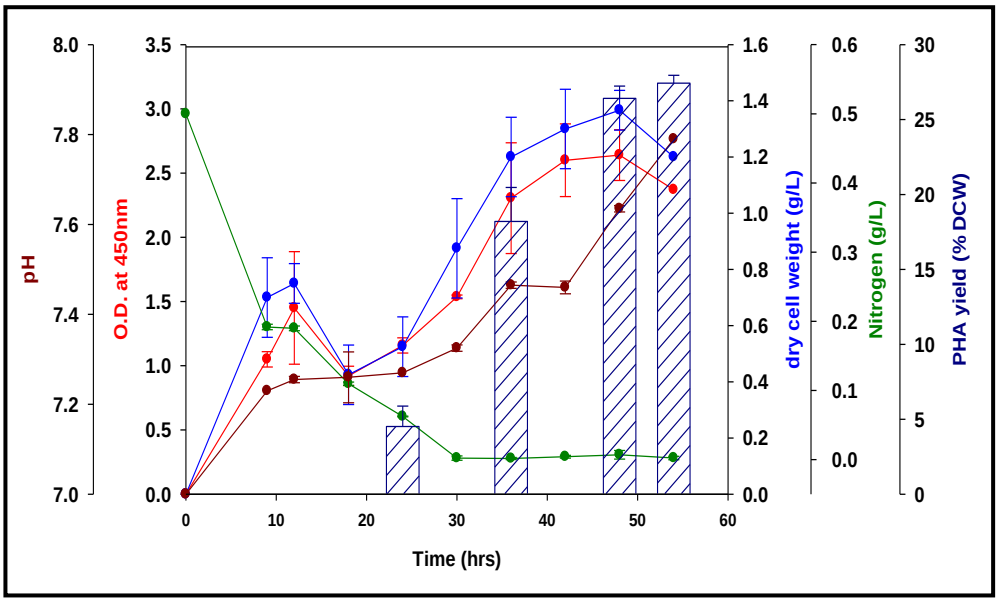


Figure 3.8: Fermentation profile for MCL-PHA production by *P. mendocina* using sodium octanoate as the sole carbon source at 2 L bioreactor level fermentation.

From this profile, it can be seen that, *P. mendocina* cell growth and dry cell weight (dcw) increased simultaneously until 48 hours of the fermentation. At the 54th hour, cells entered stationary phase. The maximum dcw/L (1.4 g/L) was achieved during the end of the log phase at the 48th hour. PHA accumulation at 24, 36, 48, and 54 hours were measured and the results obtained showed that the highest amount of PHA accumulation observed was 27.4% dcw at the 54th hour. As the fermentation progressed, it was observed that the amount of nitrogen decreased steadily in the medium and dropped from 500 mg/L to 0.2 mg/L. pH of the medium increased steadily from 7.00 at 0 hour to 7.79 at the end of the fermentation time.

3.3.2. PHA production using coconut oil as the sole carbon source

To further understand the potential of *P. mendocina* in utilizing coconut oil as an alternative relatively cheaper carbon source for large scale PHA production, studies were extended to a 2 L bioreactor level. Fermentation was carried out for 60 hours. At regular intervals of time, samples were withdrawn and analysed for *P. mendocina* growth and PHA accumulation using coconut oil as the sole carbon source.

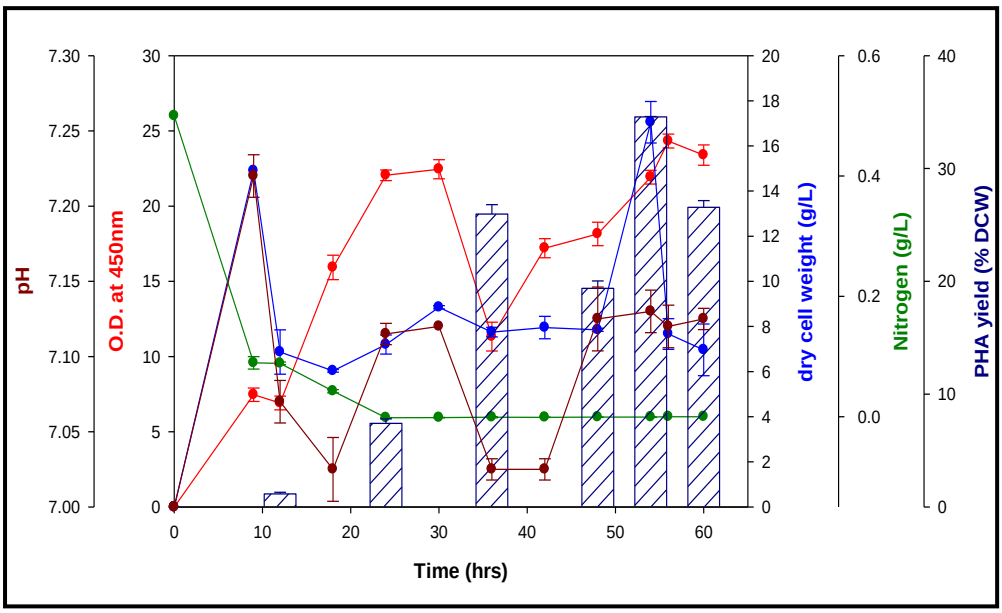


Figure 3.9: Fermentation profile for MCL-PHA production by *P. mendocina* using coconut oil as the sole carbon source at 2 L bioreactor level fermentation.

The result (**Figure 3.9**) showed increase in the OD until 57 hours of the fermentation time after which, cells entered stationary phase. Dcw showed gradual increase after 12 hours of the fermentation until 54 hours of the fermentation. Decline in the cell mass was observed after 54 hours in the fermentation. The maximal dcw value of 17.1 g/L was observed at the 54th hour. Measurement of PHA accumulation at 12, 24, 36, 48, 54 and 60 hours were carried out. The polymer yield ranged between 1.2% dcw to 34.6% dcw. The highest amount of PHA accumulated was 34.6% dcw at the 54th hour, after which a decrease was observed at the end of the fermentation time at the 60th hour. The level of nitrogen started to decrease at very early stage of the fermentation and reached to zero at 24 hours of the fermentation time. At the beginning of the fermentation the pH was set at 7.00 and as the fermentation progressed the pH increased until 9 hours and started to decrease until 18 hours of the fermentation. After 18 hours of the fermentation period, pH followed the same trend as the cell growth and reached a value of 7.13 at the end of the fermentation.

3.3.3. PHA production using groundnut oil as the sole carbon source

An optimisation study of the PHA production using groundnut oil as the sole carbon source was also investigated further using a 2 L bioreactor. The organism was grown for 54 hours and different parameters such as OD, pH, nitrogen concentration, dcw and % PHA yield were measured at regular intervals of time.

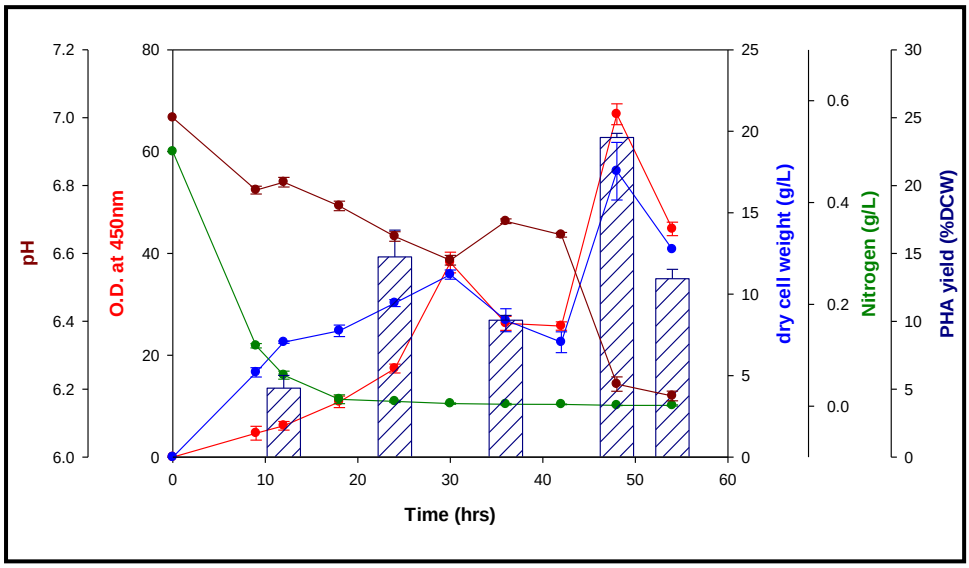


Figure 3.10: Fermentation profile for MCL-PHA production by *P. mendocina* using groundnut oil as the sole carbon source at 2 L bioreactor level fermentation.

The profile of the parameters measured during the investigations is shown in **Figure 3.10**. Gradual increase in the OD and dcw was observed simultaneously until 48 hours of the fermentation. At the 54th hour of fermentation, the rate of cell growth decreased. The highest value of dcw achieved at the 48th hour was 17.6 g/L. Further extension in fermentation time resulted in a decline in the accumulated dry cell weight to 12.8 g/L. The polymer accumulation of the organism ranged between 5.1% dcw to 23.5% dcw. As the fermentation progressed, the amount of nitrogen started to decrease in the medium. By the 54th hour, the amount of nitrogen dropped to 1.4 mg/L from the initial value of 500 mg/L. During fermentation, the pH of the culture medium decreased from 7.00 to 6.58 until 30 hours. A slight increase in the pH was observed until 42 hours of the fermentation, followed by a sudden decrease of up to 6.18 until the end of the fermentation.

3.3.4. PHA production using olive oil as the sole carbon source

Based on previous work at shaken flask level PHA production using olive oil as the sole carbon source, production in 2 L bioreactor was chosen for further investigations. To understand in detail, the role of the olive oil carbon source in the production medium towards cell growth and PHA accumulation, several parameters were analysed for 60 hours of fermentation.

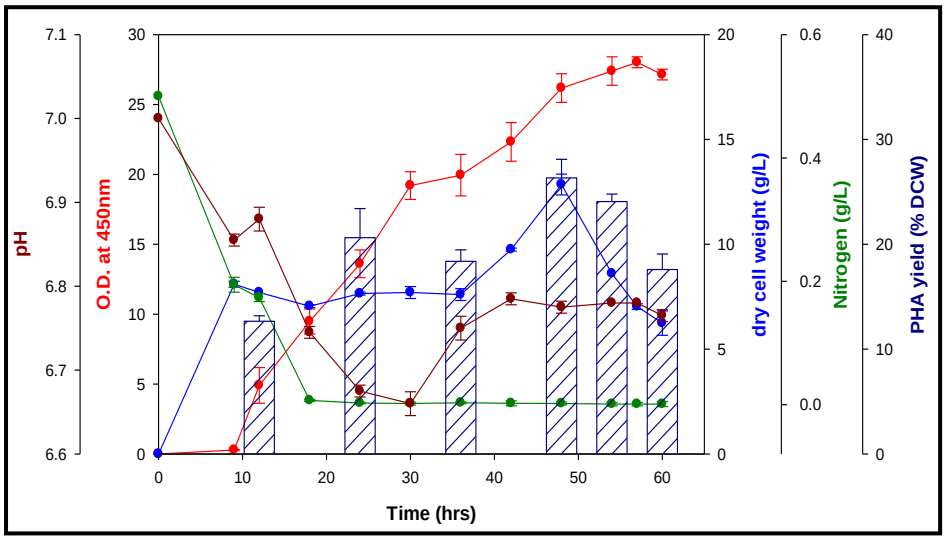


Figure 3.11: Fermentation profile for MCL-PHA production by *P. mendocina* using olive oil as the sole carbon source at 2 L bioreactor level fermentation.

Figure 3.11 shows the profile of different parameters' (optical density (OD) at 450nm, dry cell weight (dcw) g/L, pH, nitrogen concentration (g/L), and PHA yield (%dcw)) measured during the study. Lag phase was noticed at the beginning of the fermentation until 9 hours into the cell growth after inoculation with 10% of the seed culture followed by gradual cell growth increase until 57 hours of the fermentation. Slow progress in the dcw was observed until 48 hours of the fermentation. A decline in the dry cell mass was noticed at the 54th hour of the fermentation. The maximum dry cell weight and PHA yield achieved were 12.9 g/L and 26.3% dcw respectively. The level of nitrogen decreased from the initial value of 500 mg/L to that of 0.8 mg/L at the end of the fermentation. Decrease in the pH of the medium was observed until 30 hours after which it started to increase again and reached a value of 6.77 at the end of the fermentation.

3.3.5. PHA production using corn oil as the sole carbon source

MCL-PHA production study using corn oil as the sole carbon source was also further extended to the fermenter scale (2 L). To analyse the effect of the use of corn oil as the carbon feed on *P. mendocina* growth and PHA production, different parameters were investigated for 54 hours in a bioreactor at regular intervals of time.

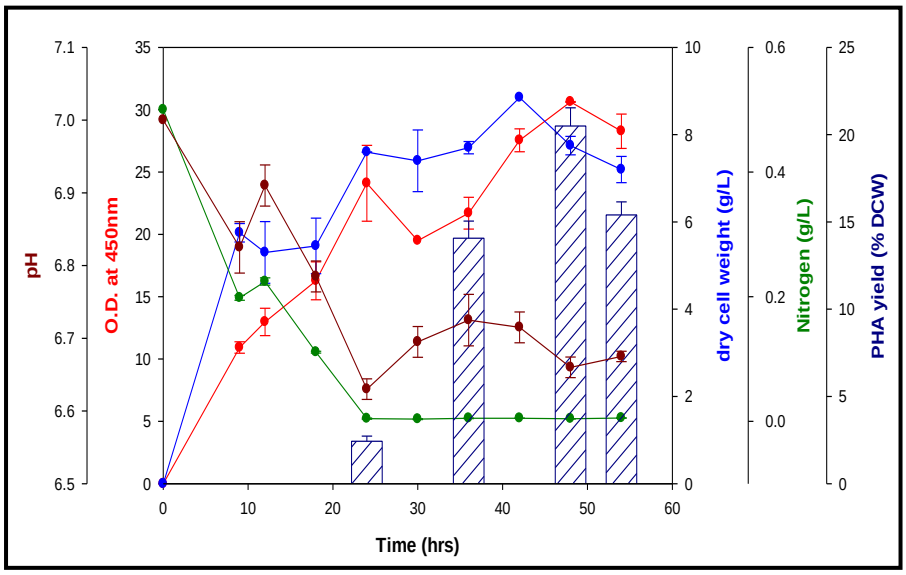


Figure 3.12: Fermentation profile for MCL-PHA production by *P. mendocina* using corn oil as the sole carbon source at 2 L bioreactor level fermentation.

Figure 3.12 shows the profile of the results obtained from the study. Gradual increase in the cell growth was observed until 48 hours after which the cells entered the stationary phase. Dcw also followed the same trend of cell growth until 42 hours of fermentation. The maximum dcw achieved at the end of log phase was 8.9 g/L. PHA accumulation was only observed after 24 hours of the fermentation. The maximum PHA yield obtained using corn oil was 20.5% dcw at the 48th hour. The nitrogen amount in the media decreased as the fermentation progressed and dropped from 500 mg/L to 5.4 mg/L. pH started to decrease until 24 hours followed by an increase at the end of the fermentation, reaching to a value of 6.68.

3.4. Comparison of growth and production profiles in shaken flask and 2 L fermenter level:

In this study, *P. mendocina* growth and production profiles were investigated in shaken flask and 2 L bioreactor level fermentations. Overall comparison of the values obtained for the parameters measured during the growth of *Pseudomonas mendocina* in mineral salt medium, containing five different sole carbon sources showed that when vegetable oils were used as the sole carbon sources, more cell growth was observed in the fermenter (2 L) than in the shaken flask (5 L) (**Figure 3.13 and 3.14**). However, higher PHA yields were obtained in shaken flask level fermentations for all five carbon sources (**Figure 3.14**). When sodium octanoate was used as the sole carbon source, more cell growth and PHA yield was observed in the shaken flask (5 L) than in the fermenter (2 L) study. Also, these results obtained showed that coconut oil as the sole carbon source supported comparatively higher cell growth and PHA accumulation for *Pseudomonas mendocina* in both shaken flask and fermenter.

3.4.1. Comparison of PHA production at shaken flask level

Table 3.1: Summary of the shaken flask level PHA production studies using different carbon sources by *P. mendocina*.

Carbon Source	% PHA Yield (%dcw)	Time (hours)	PHA (g/L)	Time (hours)
Sodium octanoate	35.1	48	0.3	48
Coconut oil	60.5	48	1.8	48
Groundnut oil	31.8	48	0.4	48
Olive oil	43.6	48	1.0	48
Corn oil	29.8	48	0.4	48

3.4.2. Comparison of PHA production at 2 L fermenter level

Table 3.2: Summary of the 2 L bioreactor level PHA production using different carbon sources by *P. mendocina*.

Carbon Source	% PHA Yield (%dcw)	Time (hours)	PHA (g/L)	Time (hours)
Sodium octanoate	27.4	54	0.3	54
Coconut oil	34.6	54	1.0	54
Groundnut oil	23.6	48	0.3	48
Olive oil	26.3	48	1.2	48
Corn oil	20.5	48	0.7	48

3.4.3. Comparison of cell growth in shaken flask and 2 L fermenter level productions

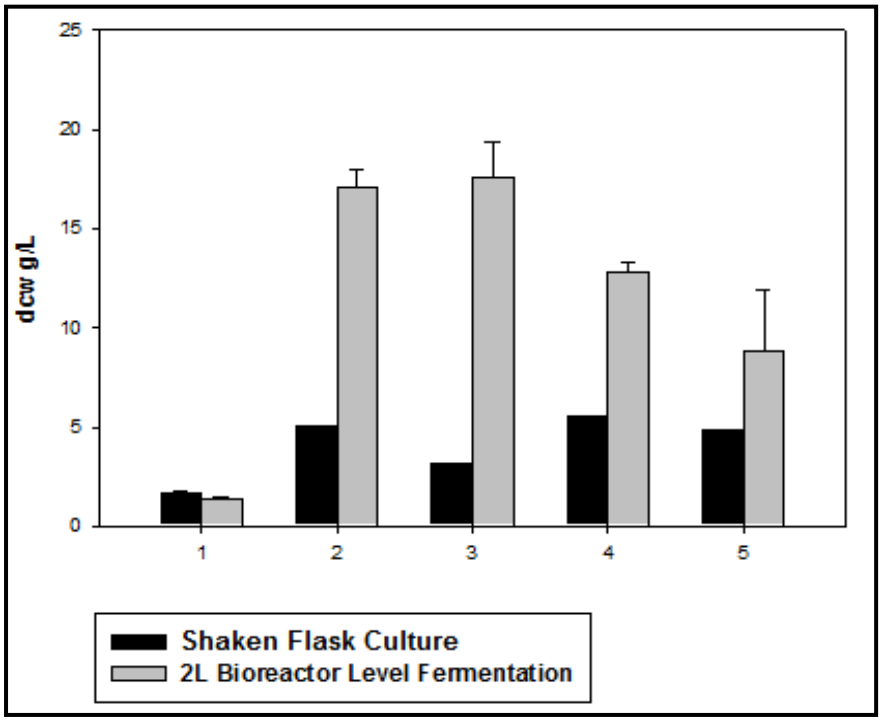


Figure 3.13: Comparison of the maximum *P. mendocina* growth (g/L) obtained using 1) sodium octanoate, 2) coconut oil, 3) groundnut oil, 4) olive oil and 5) corn oil as the sole carbon source in shaken flask and 2 L bioreactor level fermentations (n=2-3, Error bars = ±SD).

3.4.4. Comparison of PHA yields in shaken flask and 2 L fermenter level productions

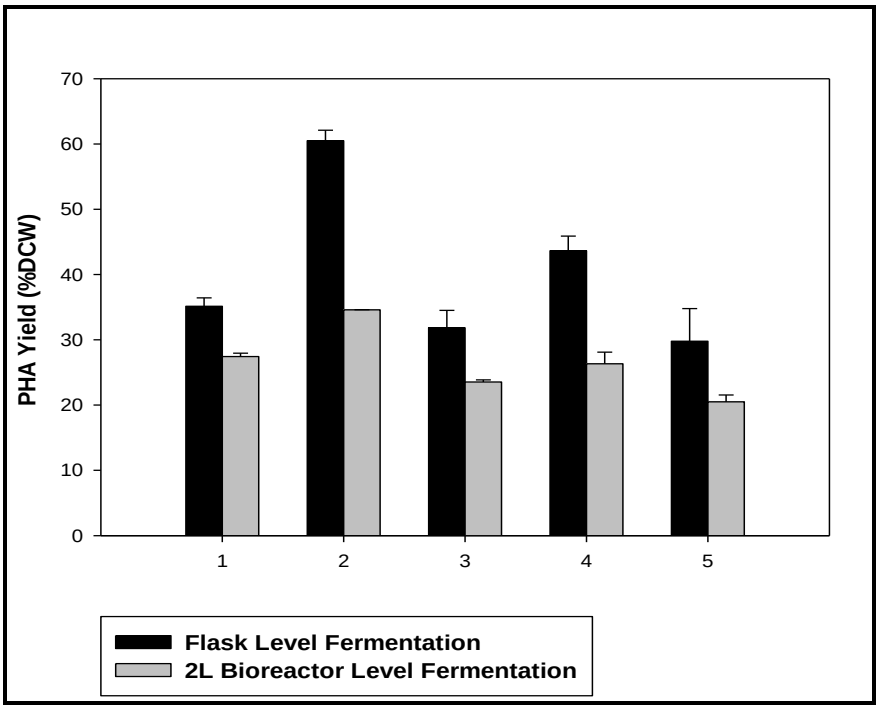


Figure 3.14: Comparison of the maximum PHA yield (%dcw) accumulated by *P. mendocina* using 1) sodium octanoate, 2) coconut oil, 3) groundnut oil, 4) olive oil and 5) corn oil as the sole carbon source in shaken flask and 2 L bioreactor level fermentations (n=2-3, Error bars = ±SD).

3.5. Discussion:

Investigations of PHA production by the Gram negative bacteria, *P. mendocina* have been recently published showing P(3HO) production in the range of 27 - 45% dcw, when grown on sodium octanoate as the sole carbon source (Rai 2010, Bagdadi 2013, Basnett 2014). However, production of PHAs by *P. mendocina* using vegetable oils has not been reported in literature previously. Hence, PHA production using *P. mendocina* and vegetable oils as the sole carbon source was the main focus for the production of PHAs during this study. One of the aims was to make the production of PHAs using *P. mendocina* more economical through the utilisation of cheap and readily available carbon sources such as vegetable oils (coconut oil, groundnut oil, olive oil and corn oil). In this work, the use of vegetable oils as the sole carbon source was investigated at shaken flask (5 L) level. To further optimise PHA yield, fermenter (2 L) level PHA production was also investigated in order to study in detail the

effect of fermentation conditions (air supply and agitation) on cell growth and PHA accumulation using these sole carbon sources. Throughout this study, *P. mendocina* was grown using five different carbon sources to see which carbon source resulted in higher cell growth and accumulation of PHAs. Detailed results of these studies are discussed below.

It was found that, when *P. mendocina* was grown in the MSM media with different sole carbon sources such as sodium octanoate, coconut oil, groundnut oil, olive oil and corn oil, the organism was able to grow and accumulate a range of different PHAs with varying yields. In the previous reports by Ashby (1998) and Daniel *et al.* (1999), it was observed that *Pseudomonas resinovorans* and *Pseudomonas saccharophila* were able to produce MCL-PHAs from vegetable oil substrates. In both of these organisms, *P. resinovorans* and *P. saccharophila,* extracellular esterase (lipase) activity was reported by Cromwick *et al.* (1996) and Hou and Johnson (1992). The inability of other PHA producing Pseudomonads without lipase activity to grow and produce PHA from triglycerides suggests that the esterase (lipase) activity is necessary for PHA biosynthesis by the organism (Ashby and Foglia 1998). Therefore, the cell growth and PHA accumulation results obtained in this study also suggest that *P. mendocina* should have esterase activity in order to utilise vegetable oils for PHA biosynthesis. The results obtained in the production studies revealed that coconut oil as the sole carbon source was able to increase cell growth and PHA accumulation in the organism giving higher PHA productivity (60.5% at shaken flask level and 34.6% dcw at fermenter level) as compared to the other sole carbon sources used in this study. Throughout the study for all five sole carbon sources, the parameters such as fermentation conditions and the producing organism were kept identical. Previous studies have demonstrated that there are three important fermentation criteria i.e. the culture conditions, type of the carbon feedstock and finally the producer organism that have a direct effect on the polymer accumulation or PHA yield (Basnett 2014). It could therefore be suggested that the higher productivity in coconut oil fermentation was largely due the carbon feedstock used for the PHA accumulation in *P. mendocina*.

In order to investigate the temporal variation of *P. mendocina* cell growth and PHA production profiles, different parameters as shown in **Figures 3.2 – 3.13** were measured and compared. When sodium octanoate, groundnut oil, and olive oil were used as the sole carbon sources, both OD as well as dcw seemed to have increased up to 48 hours after which they started to decrease at 54 hours. The decrease could be due to the level of carbon source reaching to insufficient and could no longer support the growth of the organism. However, when coconut oil and corn oil were used as the sole carbon sources, both OD and dcw still increased simultaneously as fermentation progressed during the final stage of the fermentation. Vegetable oils contain a much higher number of carbon atoms per unit weight as per their chemical compositions, compared to the other carbon sources used as the feedstocks. The available carbon fractions for *P. mendocina* growth in case of coconut and corn oil fermentations could be higher compared to the other carbon sources in this study. Hence, the level of the carbon source left in the media at the end of the fermentation could still support the growth of the organism. In the second section, growth and production profiling were carried out in 2 L bioreactors which showed gradual increase in the cell growth up to 54 hours when sodium octanote, groundnut oil and corn were used as the sole carbon sources. In case of coconut and olive oil feedstocks; organism grew until 60 hours of fermentation period. Thus, bioreactor level fermentation results showed much longer cell growth phase compared to the shaken flask cultures. This difference could be due to the variation of the agitation speed (200 rpm) and continuous air supply (1 vvm) in bioreactor level fermentations compared to shaken flask fermentations (agitation 150 rpm).

As can be seen from the temporal profiles observed in **Figure 3.2 – 3.6** at shaken flask level, the maximum dcw (5.5 g/L) accumulated at 57 hours when olive oil was used as the sole carbon source (**Figure 3.5**). The lowest dcw (1.7 g/L) at 48 hour was achieved when sodium octanoate was used as the sole carbon source. Coconut oil, groundnut oil, and corn oil showed significantly higher cell mass (5.1, 3.1 and 4.9 g/L respectively) compared to sodium octanoate. At fermenter level profiling, maximum dcw (17.6 g/L) accumulation was obtained at 48 hour using groundnut oil, followed by 17.1 g/L dcw at 54 hour from coconut oil feedstock. Olive oil, corn oil and sodium octanoate gave

maximum of 12.9, 8.6 and 1.4 g/L dcws respectively. The nature of the carbon source thus plays a very important role for the growth of the organism. Moreover, the organism has selective preference for the carbon source used supporting maximal cell growth due to its genetic makeup which leads to the metabolism of the specific carbon feed (Ashby and Foglia 1998). Study carried out by Daniel *et al.* (1999) showed that *P. saccharophila* grew well in plant oils that contained saturated fatty acyl components. For example, coconut oil, which contains lauric acid (C_{12}) as its major (47%) fatty acid component (Canapi *et al.,* 1996), supported *P. saccharophila* growth to a high density within 48 hours of incubation. On the other hand, soybean and sunflower oils (Davidson *et al.,* 1996, Sipos and Szuhaj 1996), with their high contents (50-75%) of unsaturated linoleoyl ($C_{18:2}$) and oleoyl ($C_{18:1}$) groups, gave lower density cell growth after 72-hour incubation (Daniel *et al.,* 1999). Vegetable oils used for PHA production in this study apart from coconut oil such as groundnut oil, olive oil and corn oil have some degree of unsaturation in their fatty acid composition. For instance, groundnut oil has the high content of oleic acid (52-60%) and linoleic acid (13-27%). Olive oil has an unsaturated fatty acid content of oleic acid (65-80%) and linoleic acid (4-10%). Corn oil also has unsaturated fatty acid components such as oleic acid (19-49%) and linoleic acid (34-62) (Chempro-Edible-Oil-Refining-ISO-TUV-Austria). However, in the study carried out by Chaudhry *et al.* (2011) excellent results were observed of *Pseudomonas* cell growth and biomass production of 12.53 g/L dcw after 24 hours of fermentation using corn oil as the sole carbon source. PHA accumulation seemed to have decreased after this point while biomass remains relatively high until 72 hours. These results showed that this strain has ability to utilise medium-chain-length fatty acids and give good results. Moreover, Shang *et al.* (2008) also observed much higher biomass (109 g/L dcw) production in *P. putida* KT2442 using corn oil hydrolysate (Chaudhry *et al.,* 2011). In the study carried out by Ashby and Foglia (1998), six triglycerides (lard, butter oil, olive oil, high-oleic-acid sunflower oil, coconut oil, and soybean oil) were used as substrates for PHA production in *Pseudomonas resinovorans*. Each of these triglyceride supported relatively high average cell growth (3.3 g/L) after 48 hours of fermentation compare to the values previously reported for tallow (Cromwick *et al.,* 1996). In Cromwick study, cell density of 3.4 g/L and 3.8 g/L were obtained using olive oil and coconut oil as sole carbon sources respectively. These results revealed that

even though corn oil contains highly unsaturated fatty acid composition, some *Pseudomonas sp.* are able to utilise corn oil more efficiently. This could be depending on the organism' lipase specificity. In the present study, the lowest *P. mendocina* growth was observed from corn oil sole carbon source compared to the other vegetable oils used as the sole carbon sources. This demonstrates *P. mendocina's* lipase specificity for saturated fatty acids.

In the study carried out by Thakor *et al.* (2005), different vegetable oils such as castor seed oil, coconut oil, mustard oil, cotton seed oil, olive oil, groundnut oil and sesame oil were used as sole source of carbon/energy and also for PHA accumulation in *C. testosteroni*. It was observed that *C. testosteroni* degrades Long Chain Fatty Acids of vegetable oils by β-oxidation and synthesises (R)-3-hydroxyacyl CoA from the intermediates of this pathway, which can act as a substrate for PHA synthase. Under physiological conditions permissive for synthesis and accumulation of MCL-PHAs, the fatty acids are not completely degraded to acetyl-CoA, and intermediates of the β-oxidation pathway are partially or completely withdrawn and converted to (R)-3-hydroxyacyl-CoA by enoyl CoA hydratases for PHA biosynthesis (Thakor *et al.,* 2005). Previous investigations have revealed that *Pseudomonas* sp. uses the structurally related carbon sources for PHA accumulation by fatty acid β-oxidation pathway (Rai 2010). While, structurally unrelated carbon sources are being utilised by the *de novo* fatty acid synthesis pathway (Rai 2010). Steinbuchel and Fuchtenbusch (1998) observed that when *Pseudomonas putida* and *Pseudomonas aerugenosa* synthesise MCL-PHA from glucose or gluconic acid, they synthesise fatty acid by *de novo* biosynthesis pathway. In the present study, *P. mendocina* was able to grow and produce PHAs using sodium salts of fatty acids like sodium octanoate and vegetable oils which suggests that this organism is able to utilise both the fatty acid β-oxidation pathway and the *de novo* biosynthetic pathway for MCL-PHA production.

At the shaken flask level of PHA production, the maximum PHA yield of 60.5% dry cell weight (dcw) was achieved using coconut oil as the sole carbon source as compared to PHA yield of 35.1% dcw obtained using sodium octanoate as the sole carbon source. Olive oil, groundnut oil and corn oil showed PHA yield of 43.6%, 31.8% and 29.8% dcw respectively at shaken flask cultures. PHA

accumulation using all five carbon sources were also investigated at 2 L bioreactor level which also observed the highest PHA accumulation (34.6% dcw) with coconut oil as the sole carbon source. Sodium octanoate, groundnut oil, olive oil and corn oil showed PHA yield of 27.4%, 23.6%, 26.3% and 20.5% dcw respectively at fermenter level PHA production. When groundnut oil and corn oil were used as the sole carbon sources, the maximum dry cell weights obtained were 3.1 g/L and 4.9 g/L respectively, which were higher in comparison to the dry cell weight (1.7 g/L) obtained using sodium octanoate as the carbon source. However, PHA yields obtained using groundnut oil and corn oil (31.8% dcw and 29.8% dcw) were lower in comparison to the 35.1% dcw PHA yield obtained with sodium octanoate. Similar observations were also obtained in case of fermenter level profiling, where higher cell mass and lower PHA yields were obtained compared to flask level production. This suggested that under the specific conditions used, maximum energy was utilised in growth instead of PHA biosynthesis when groundnut oil and corn oil were used as the sole carbon sources. Previously, it was reported by Rai (2010) and Durner *et al.* (2000) that when hexanoate was used as the carbon feed for *P. mendocina* and *P. oleovorans*, organisms grew well but no polymer was produced. This indicates that hexanoate possibly led to the induction of the fatty acid β-oxidation related enzymes, but not the PHA biosynthesis related enzymes. Therefore, in the present study comparatively high cell growth and low PHA yield using groundnut and corn oils demonstrate that these carbon feeds induced β-oxidation related enzymes more compared to PHA synthesis related enzymes in *P. mendocina*. The above observation indicates that the PHA yield obtained using coconut oil as the sole carbon source in this study was one of the highest yields of polymer obtained from *Pseudomonas mendocina*. One study carried out by Chaudhry *et al.* (2011) demonstrated that corn oil is also a good carbon source for PHA accumulation in other producing *Pseudomonas sp.* since having the high carbon number fatty acids. Results showed that the maximum PHA content of 35.6% dcw was observed in the first 24 hours of fermentation. In another study by Shang *et al.* (2008), PHA content of 28.5% dcw from *P. putida* KT2442 was observed when grown using corn oil hydrolysate. Ashby and Foglia (1998) obtained 43.1% and 51% dcw PHA yields from *P. resinovorans* using olive oil and coconut oil as the sole carbon sources

respectively. While Cromwick *et al.* (1996) showed that *P. resinovorans* accumulated PHA to 15% of its cell dry weights from tallow.

The rate of accumulation of PHA at the early stage of fermentation is slightly slower with sodium octanoate as the sole carbon source compared to vegetable oils used as the carbon source. *Pseudomonas sp* is able to produce MCL PHAs using linear and branched alkanes, 1-alkenes, and alkanoate substrates. Previously, it was reported that when these substrates are used as the carbon feedstock for the organism, they are either immiscible or toxic to the bacteria even at low concentrations (Yao *et al.*, 1999, Tian *et al.,* 2000, Durner *et al.,* 2000). Thus, the lower PHA yield from sodium octanoate as the sole carbon source at early fermentation stage suggests that the higher concentration of this carbon source in the beginning of the fermentation hindered the organism's growth due to its toxic nature and prevented PHA accumulation.

Nitrogen limitation triggers the accumulation of PHAs (Lageveen *et al*., 1988). In this study, nitrogen limitation was maintained during all the fermentation experiments. It is also known that the depletion of the nitrogen concentration in the media guides the cells into the stationary phase and causes the accumulation of PHAs (Lageveen *et al*., 1988, Kim *et al.,* 1994, Wang and Lee, 1997, Choi and Lee 1999). The concentration of the nitrogen in sodium octanoate fermentation reduced gradually compared to the other vegetable oil fermentations. Hence, PHA accumulation in the early phase of the fermentation was not obtained in the organism when sodium octanoate was used as the sole carbon source. While for vegetable oil fermentations, nitrogen concentration decreased during the early hours of fermentation maintaining a nitrogen limiting condition throughout the course of the fermentation. This could be the major reason for the higher yield of PHA accumulation in *P. mendocina* when vegetable oils were used as the sole carbon sources. Hence, it could be concluded that nitrogen limitation was a major factor for the triggering of the polymer production.

Decline in the cell growth and simultaneous increase in the polymer accumulation by the organism was observed during the stationary phase, where the cells were unable to grow further due to the depletion of the nutrients in the

culture media. Hence, the amount of residual biomass decreased due to decline in the cell growth. However, increase in polymer production was noted at this stage without any simultaneous growth of the organism. This is due to the amount and the size of the PHA granules, which increases during the early stationary phase and occupies a large proportion of the intracellular space. Hence, maximal PHA production was achieved at the early stationary growth phase without a simultaneous increase in the dry cell weight. In a previous study carried out by Daniel *et al.* (1999) it was observed that approximately 80% of the *P. saccharophila* cells contained PHA inclusion bodies. These results also revealed that *P. saccharophila* growing on triacylglycerol substrates continued to produce PHA granules even after an extended growth period in contrast to the observation by Young *et al.* (1972) with cells grown on sucrose.

When *P. mendocina* was grown using sodium octanoate as the sole carbon source in the present study, the pH of the production medium which was set at 7.00 at the beginning of fermentation, increased and reached 7.3 (flask) and 7.8 (fermenter). In the study carried out by Rai (2010), similar observations were made which showed that when *P. mendocina* was grown using octanoate, heptanoate and nonanoate, pH of the media increased from 7.00 to 7.82, 7.72 and 7.35 respectively. However, when vegetable oils were used as the sole carbon sources (coconut oil, groundnut oil, olive oil and corn oil), the pH of the medium, which was set at 7.00, dropped gradually and decreased. Similar observations were also reported by Basnett (2014) that when *P. medocina* was grown using sugarcane molasses, biodiesel waste and glycerol, the pH of the media dropped from 7.00 to 6.5. In the present study, it was observed that at shaken flask level PHA productions using coconut and olive oil as sole carbon sources when pH of the media decreased, comparatively higher PHA yields were obtained. Previously, it was revealed that low pH conditions inhibit PHA degradation (Valappil *et al.,* 2006, Valappil *et al.,* 2007). However, relatively lower PHA yields were obtained with groundnut oil and corn oil fermentations, which point out that along with pH of the media, there must be some other fermentation conditions which also play an important role in PHA accumulation. Kim (2002) showed that for *Pseudomonas sp*, pH stat fermentations at 7.00, led to an increase in the yield of MCL-PHA production when compared to non pH stat study. This could be looked into in future in order to better understand the

effect of the pH on cell growth and PHA accumulation using vegetable oils as feedstock for *P. mendocina*.

Pseudomonas sp requires more oxygen for cell growth (Sun *et al.,* 2007). However, PHA accumulation could be observed in oxygen limiting conditions. In this study, continuous air supply of 1 vvm was set with 200 rpm agitation at 2 L bioreactor level fermentations. This suggests that there was never oxygen limiting conditions obtained in 2 L bioreactors throughout the study. Hence, this could be the main reason of getting higher cell growth and lower PHA yield at 2 L bioreactor level PHA productions compared to shaken flask level productions. However in general, it is expected that polymer yields in fermenters should be greater than those achieved in shaken flask fermentation since the physical parameters can be well controlled in order to improve the performance of the organism. Nevertheless, the results obtained in this study showed maximum polymer yield in the shaken flask level fermentation (**Figure 3.14**). Hence, the conditions for polymer production using *Pseudomonas mendocina* in the fermenter needs optimisation to further enhance the polymer yield.

At the end of the fermentation, both the levels of carbon and nitrogen present in the media seemed to have decreased. After 48 hours of fermentation the polymer yield dropped for all the carbon sources possibly due to the utilisation of PHA for growth under carbon deficient conditions. As PHAs are accumulated as energy resources, therefore it is seen that under carbon limiting conditions the organism starts to utilise the accumulated PHAs to sustain its growth. Since all the fermentation studies in this project were carried out in batch production, the media was not replenished. Therefore, the organism has higher possibility of utilizing the accumulated PHA granules when faced with nutrient limiting conditions. (Anderson and Dawes 1990, Huijberts and Eggink 1996). This finding suggested that the mode of fermentation plays a very important role in PHA accumulation (Kim *et al*., 2007). Therefore, to achieve a high yield of PHA production this further utilisation of the accumulated PHAs by the organism needs to be eliminated. One such approach is to carry out a two step fermentation, whereby a high cell concentration is 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 a two

step fed batch cultivation of *P. putida* by combining the use of glucose and octanoate. Continuous cultivation has also been proposed which allows growth of an organism under a defined limitation under prolong periods of time (Durner *et al.*, 2000). High PHA productivity (0.68 g L^{-1} h^{-1}) was achieved when corn oil hydrolysate was used as carbon source in fed batch cultures of *Pseudomonas putida* KT2442, giving cell densities of up to 109 g/L (Shang *et al.,* 2008). In another study by Lee *et al.* (2000), it was observed that when oleic acid was used as the carbon feed, high density *P. putida* cells were obtained in fed batch cultures showing 141 g/L dcw in 38 hours of fermentation time (Lee *et al.,* 2000).

In this report, the results have clearly demonstrated that *P. mendocina* is capable of producing PHAs effectively from vegetable oils and are excellent carbon sources for both cell growth and PHA biosynthesis. The application of inexpensive vegetable oils as carbon sources for PHA biosynthesis is predicted to reduce the production cost of bacterial polyhydroxyalkanoates. Hence in conclusion, as observed at the shaken flask and fermenter levels, the coconut oil sole carbon source, used in the study, was by far the best carbon source in terms of both the cellular growth (5.1 g/L) and PHA accumulation (60.5% dcw) in *P. mendocina*. Overall, reasonably high level of polymer accumulation and cell growth was achieved in this study; a maximal PHA content of 60.5% dcw was achieved from shaken flask investigations with coconut oil as the sole carbon source. These results therefore confirm that *P.mendocina* was able to accumulate PHAs with a yield ranging from 30-61% dcw at shaken flask and 20-35% dcw at fermenter levels, when grown on the structurally related carbon sources, such as sodium octanoate, coconut oil, groundnut oil, olive oil and corn oil. In order to know, wether *P. mendocina* is able to utilise vegetable oils for growth and PHA accumulation, initial investigations were carried out in this study. To enhance PHA yield and further reduce the production cost, optimisation of the MCL-PHA production using vegetable oils as the sole carbon source would be an area of interest in the future.

CHAPTER 4

Characterisations of the produced PHAs

4.1. Introduction:

Biomedical applications of SCL-PHA such as P(3HB) have been significantly limited to hard tissue engineering due to its brittle nature. In contrast, for soft tissue engineering such as heart valves, cardiac patches and other vascular applications, skin tissue engineering, wound healing and controlled drug delivery, more flexible and elastomeric polymer materials are required (Rai *et al.,* 2011, Hazer *et al.,* 2012). MCL-PHAs such as poly(3-hydroxyhexanoate) P(3HHx) and poly(3-hydroxyoctanoate) P(3HO) are elastomers (Rai *et al.,* 2011, Basnett 2014). MCL-PHAs have lower crystallinity with higher flexibility and softness than SCL-PHAs, which make them materials of choice for biomedical applications. Moreover, different physical, mechanical and thermal properties have been achieved due to compositional variations allowing MCL-PHA utilisation in various applications.

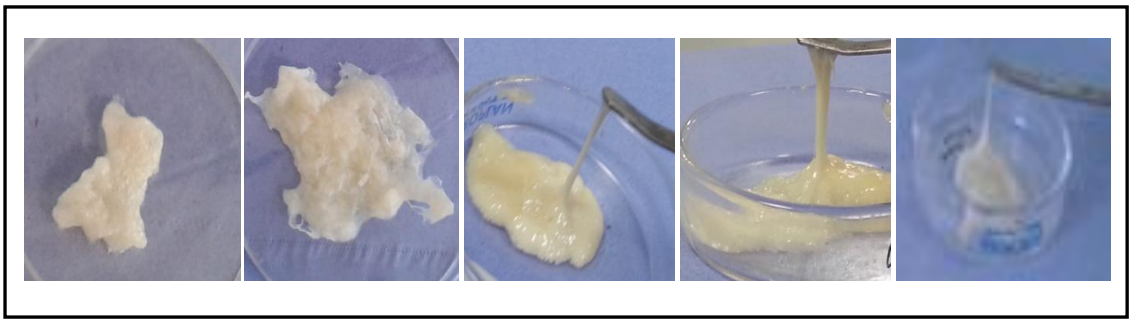
Root canal treatment is the only choice of treatment until now for dental caries and pulpitis. In this treatment the whole pulp is removed and filled with inert sealing materials such as gutta percha, rasilon (Ingle and Bakland 2002). After this surgery, the tooth becomes dead and if the infection spreads again, subsequent operation is needed. Therefore, there has been great amount of interests in the regenerative endodontics of dental pulp and dentin. Combinations of stem cells, scaffolds and growth factors have been investigated previously for the dental pulp tissue engineering applications (Hai-Hua *et al.,* 2011, Ravindran *et al.,* 2013). Scaffold materials used for the dental tissue engineering will have a direct effect on the success of the pulp regeneration. Therefore, choosing a right material for regenerative endodontics is a very crucial step. An ideal material should be biodegradable, biocompatible, permeable and facilitate cell support. Natural as well as synthetic polymers have been investigated as the potential scaffold materials in the pulp tissue engineering applications. Natural polymers (collagen, gelatin, dextran and fibronectin) show good cytocompatibility and bioactivity, whereas synthetic polymers (PGA) show better degradation rate and appropriate physical and mechanical properties for pulp tissue regeneration (Vasita and Katti 2006). However, none of the above scaffold materials have proved to be an ideal scaffold preparation for pulp regeneration application.

PHAs have been extensively studied for their biomedical applications in the development of medical devices such as cardiac patches, sutures, stents, nerve repair devices, drug delivery tablets and wound dressings (Rai *et al.,* 2011, Hazer *et al.,* 2012). Previously, PHAs have been investigated as the scaffold materials for the treatment of periodontal ligament and jaw bone defects (Williams and Martin 2005, Zinn *et al.,* 2001). However, their use as the scaffold materials for dental tissue engineering remains relatively unexplored.

The main objective of this chapter was to characterise PHAs, produced by *Pseudomonas mendocina* using five different sole carbon sources, sodium octanoate, coconut oil, groundnut oil, olive oil and corn oil. These polymers were characterised with respect to their chemical and material properties and finally assessed for their cytocompatibily using human Mesenchymal Stem Cells (hMSCs) in order to investigate their possible application as the scaffold materials for dental tissue engineering, with a particular interest in the dental pulp regeneration application.

4.2. Characterisations:

The polymers produced were extracted and analysed for their physical, chemical, mechanical and cytocompatibility properties as described below. The resulting polymers are shown in **Figure 4.1**.



(a) (b) (c) (d) (e)

Figure 4.1: Polymers produced by *P. mendocina* using different sole carbon sources; (a) sodium octanoate, (b) coconut oil, (c) groundnut oil, (d) olive oil, (e) corn oil.

4.2.1. FTIR

Preliminary confirmation of the chemical structure of the polymers produced from *P. mendocina* using different sole carbon sources was carried out using FTIR. **Figure 4.2** shows the combined FTIR spectra of these polymers. FTIR analyses of the polymers produced from different carbon sources confirmed the presence of the characteristic marker ester carbonyl bond and C-O stretching bond for MCL-PHAs which is observed in the range between 1728-1736 cm^{-1} and 1159-1162 cm^{-1} respectively (Randriamahefa *et al.,* 2003), as shown in **Figure 4.2**. The bands at 2954-2956cm^{-1}, 2922-2928 cm^{-1} and 2853-2858 cm^{-1} correspond to the aliphatic C-H group of the polymer backbone (Sánchez *et al.,* 2003). Hence, the results indicated that the polymers produced contain structural elements characteristic of MCL PHAs.

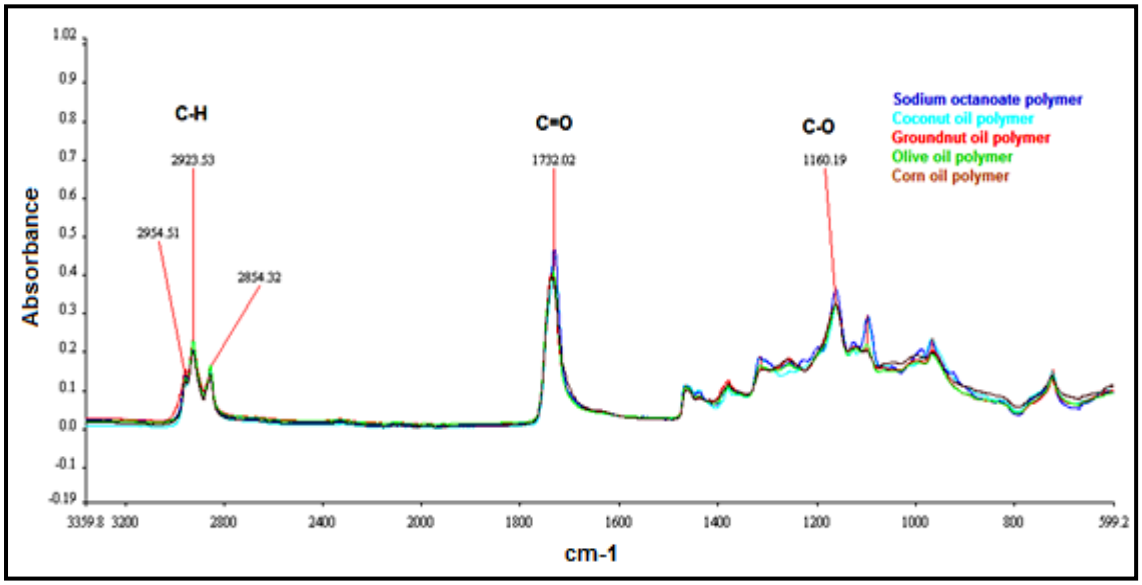


Figure 4.2: Combined FTIR spectra of the PHAs produced by *P. mendocina* using different sole carbon sources: sodium octanoate, coconut oil, groundnut oil, olive oil and corn oil.

4.2.2 GC-MS

Further detailed structural characterisation of the monomers present in the extracted PHAs was carried out using GC-MS. The methanolysis of the PHA obtained using sodium octanoate resulted in a GC peak, the MS spectrum of which was identical to that of the methyl ester of 3-hydroxyoctanoate in the NIST (National Institute of Standards and Technology) library. The total ion

current chromatogram for the methanolysed product of the polymer produced from sodium octanoate can be seen in **Figure 4.3 (a)**. The mass spectrum of the molecular ion related mass fragment peak was observed at an R_t of 6.67 minutes. The peaks obtained due to the fragmentation of the molecular ion is the same as that for the methyl ester of 3HO. The MS fragmentation pattern of 3HO in **Figure 4.3 (b)** showed the following main peaks at m/z 40.9, 70.8, 74.9 and 102.8. The peak at m/z 40.9 corresponded to the alkyl end of the molecule formed due to the cleavage between the C_5 and C_6 carbon atoms**.** The peak at m/z 70.8 represented the alkyl end of the molecule occurring due to the cleavage between C_3 and C_4 carbon atoms. The peak at m/z 74.9 represented the carbonyl end of the molecule occurring due to the cleavage between C_3 and C_4 carbon atoms. The peak at 102.8 corresponded to the hydroxyl end of the molecule occurring due to the cleavage between C_3 and C_4 atoms. Thus, it was confirmed that polymer produced from *P. mendocina,* using sodium octanoate as the sole carbon source, was indeed a homopolymer of 3-hydroxyoctanoic acid.

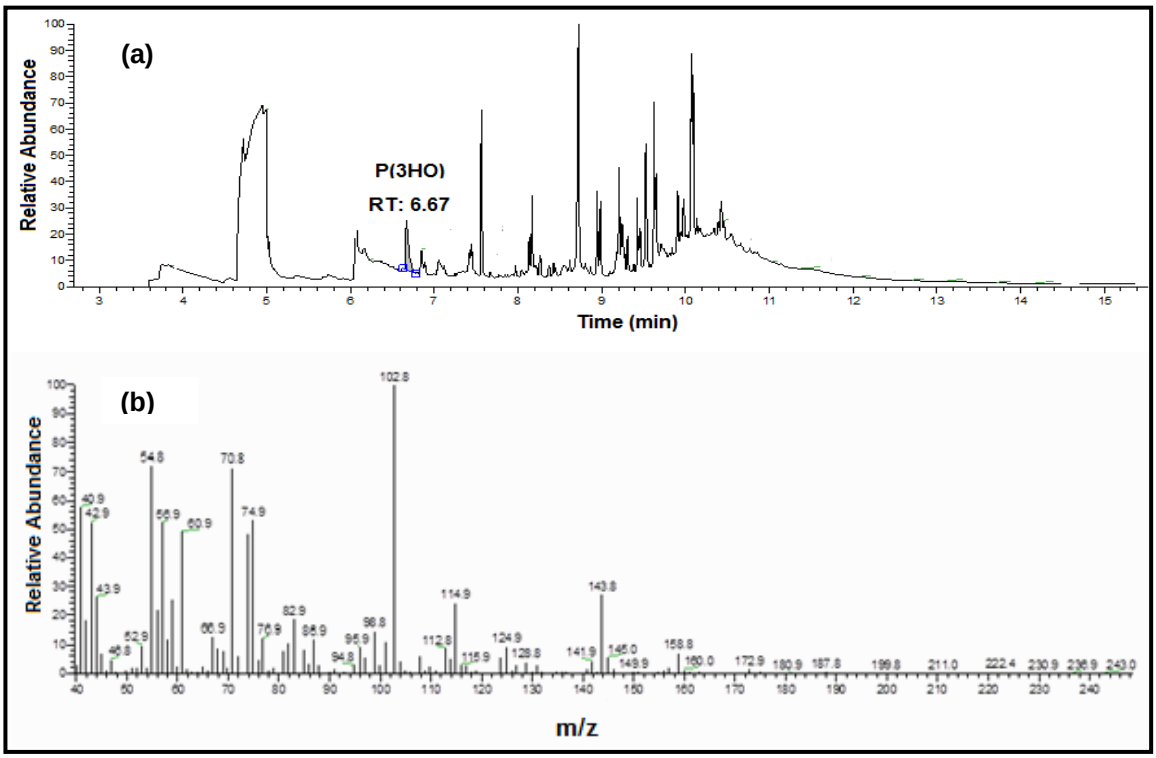
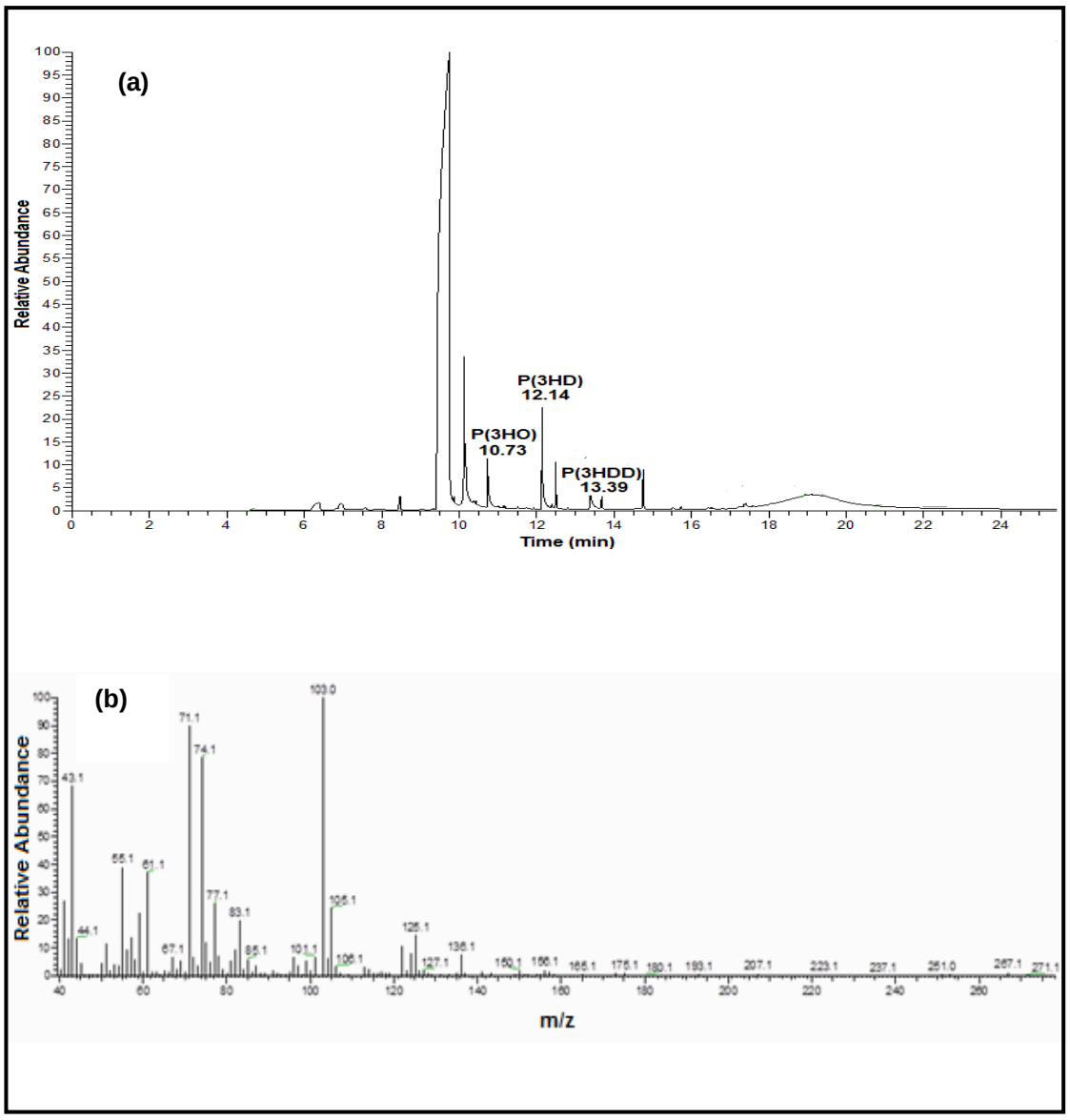


Figure 4.3: GC-MS analysis of the polymer produced when *P. mendocina* was grown on sodium octanoate: (a) Total ion chromatogram of the methanolysis product of PHA, (b) Mass spectrum of the methylester of 3-hydroxyoctanoic acid with a Rt of 6.67 minutes.

The polymers extracted from coconut oil, groundnut oil, olive oil and corn oil were also analysed using GC-MS. The analysis confirmed the presence of three different monomers in the methanolysed product of these polymers. The total ion current chromatogram for these methanolysis products is shown in **Figures 4.4 and 4.5**. The molecular ion related mass fragment peak at R_t of 6.64 minutes, due to the methyl ester of 3HO was observed again in polymers produced from groundnut, olive and corn oil. Whereas, in case of the polymer produced from coconut oil, it was observed at 10.73 minutes. The peaks obtained due to the fragmentation of the molecular ion were the same as the methyl ester of 3HO produced and discussed previously from *P. mendocina* using sodium octanoate.



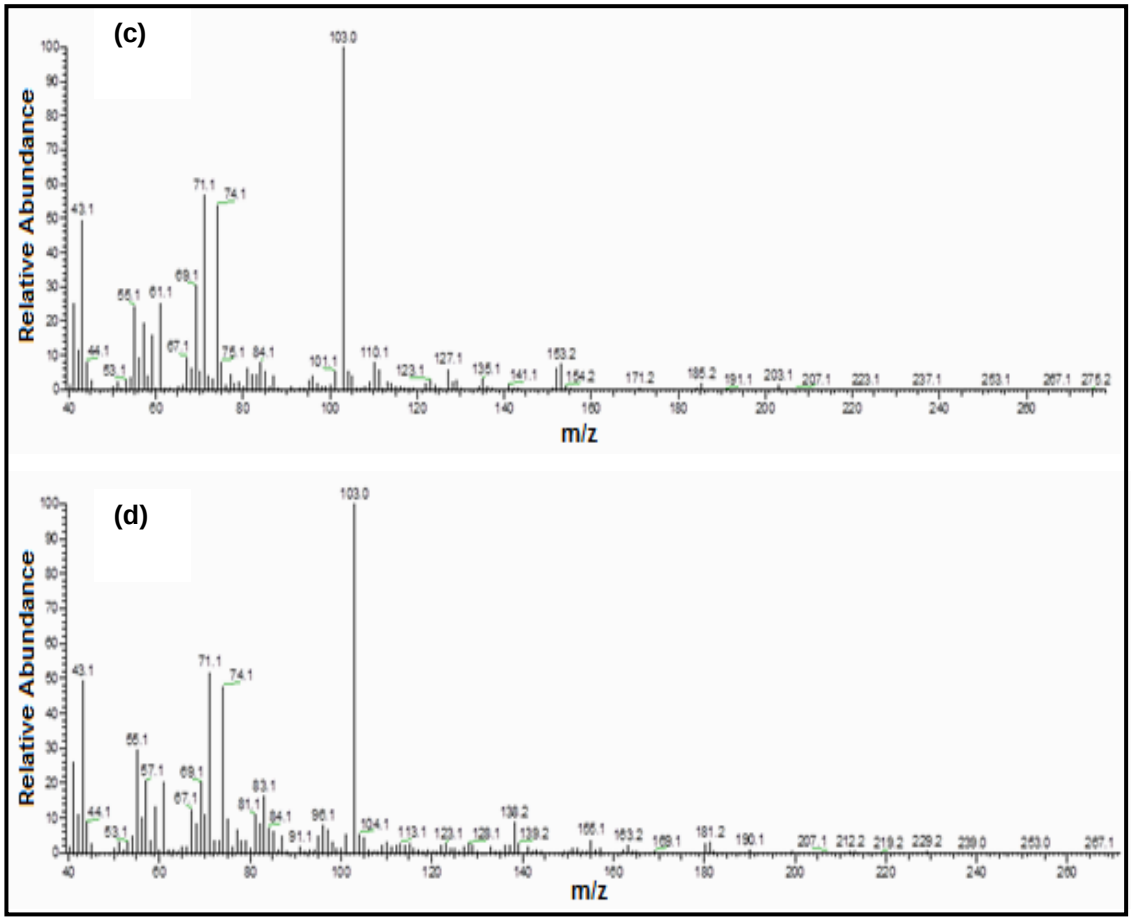
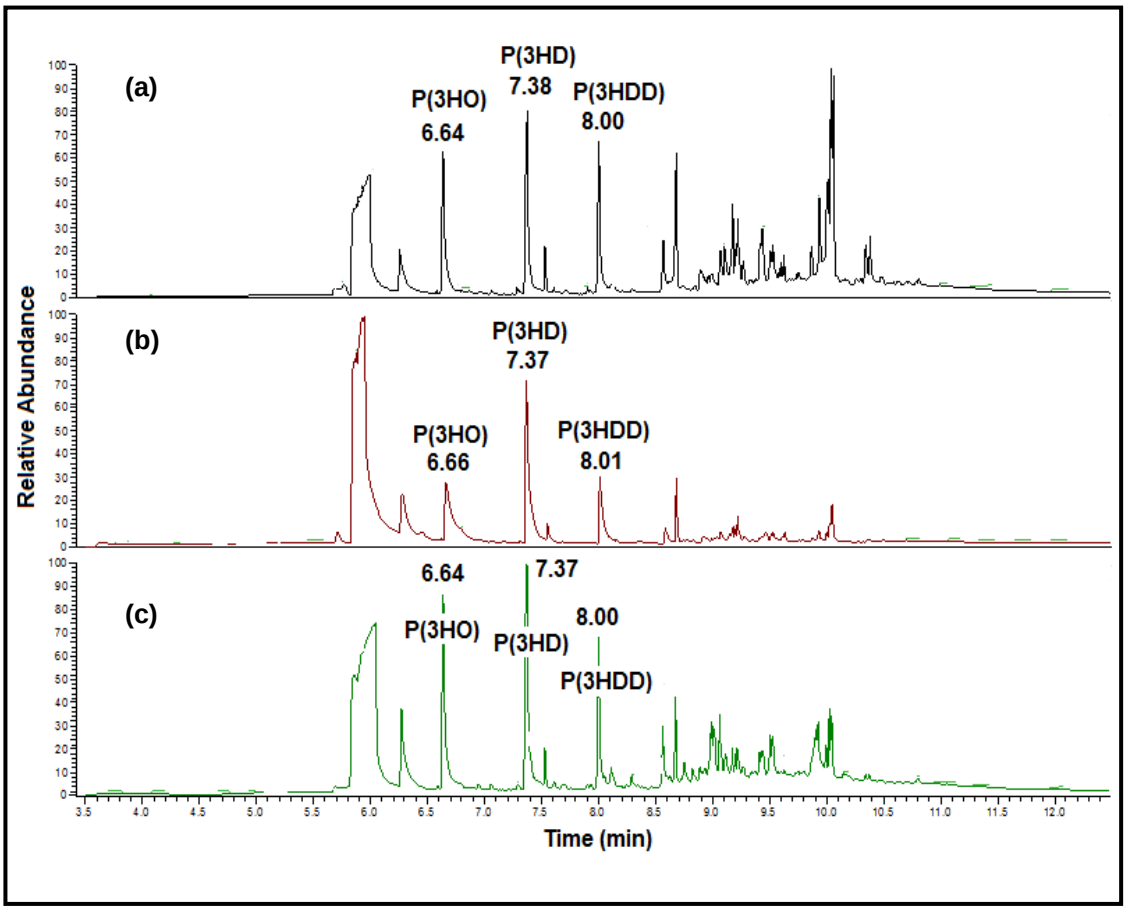


Figure 4.4: GC-MS analysis of the polymers produced when *P. mendocina* was grown on coconut oil, (a) Total ion chromatogram of the methanolysis product of PHA (b) Mass spectrum of the methylester of 3-hydroxyoctanoic acid with a R_t of 10.73 minutes (c) Mass spectrum of methylester of 3-hydroxydecanoic acid with a R_t of 12.14 and (d) Mass spectrum of methylester of 3-hydroxydodecanoic acid with a R_t of 13.39 minutes.

The peaks at R_t of 7.38 and 8.00 minutes in groundnut, olive and corn oil polymers, showed excellent similarity to the mass spectra of the methyl esters of 3-hydroxydecanoate and 3-hydroxydodecanoate respectively in the MS (NIST) library. In case of polymer produced from coconut oil, these peaks were observed at R_t of 12.14 and 13.39. The fragmentation pattern of 3HD showed (**Figure 4.4 (c)**) a peak at m/z 74.1, which originates from the carbonyl end of the molecule due to the cleavage between C_3 and C_4 carbon atoms following McLafferty rearrangement (Rai 2010, Basnett 2014, McLafferty 1956). The peak at m/z 103.0 occurred due to the fragmentation ion of the hydroxyl end of the molecule following the cleavage between C_3 and C_4 carbon atoms; similarly the alkyl end of this cleavage resulted in the peak at m/z 71.1. The peak at m/z 43.1

occurred due to the alkyl end of the molecule following the cleavage between C_7 and C_8 carbon atoms. 3HD fragmentation patterns for polymers produced from groundnut, olive and corn oils showed similarity with the methyl ester of 3HD produced from *P. mendocina* using coconut oil. Finally, the fragmentation pattern for 3HDD produced from groundnut, olive and corn oils, **Figure 4.5 (f)**, showed the m/z peak at 103.0 which originated from the carbonyl end of the molecule. This was due to the cleavage between C_3 and C_4 carbon atoms following McLafferty rearrangement. The peaks at m/z 43.2 occurred due to the alkyl end of the molecule following cleavage between C_9 and C_{10} carbon atoms. Polymer produced from coconut oil also showed similar fragmentation pattern of the methyl ester of 3HDD. Hence, these results confirmed the production of the terpolymer of poly(3HO-co-3HD-co-3HDD) when coconut, groundnut, olive, and corn oil were used as the sole carbon sources.



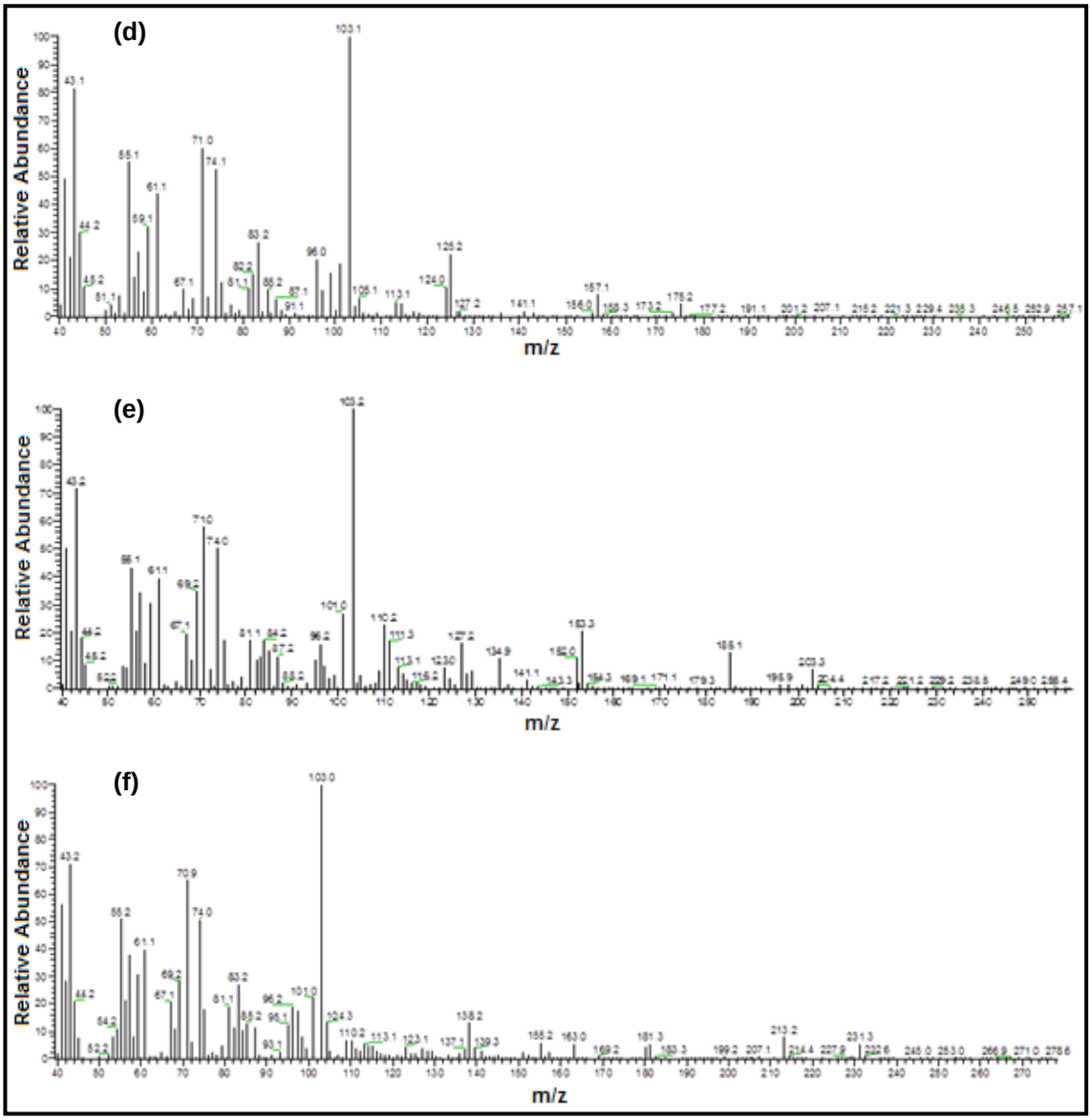


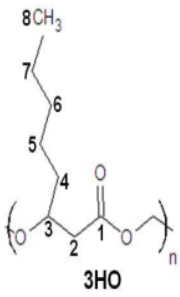
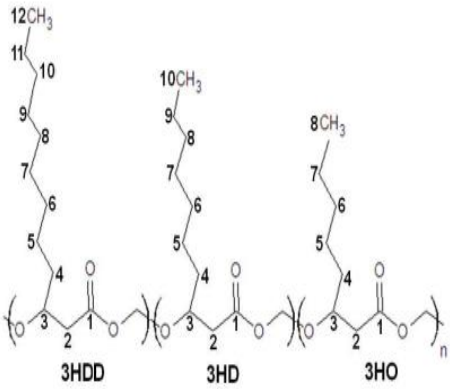
Figure 4.5: GC-MS analysis of the polymers produced when *P. mendocina* was grown on groundnut oil, olive oil, and corn oil, (a)-(c) Total ion chromatogram of the methanolysis product of PHA produced using groundnut oil, olive oil, and corn oil, (d) Mass spectrum of the methylester of 3-hydroxyoctanoic acid with a R_t of 6.64 minutes (e) Mass spectrum of methylester of 3-hydroxydecanoic acid with a R_t of 7.38 and (f) Mass spectrum of methylester of 3-hydroxydodecanoic acid with a R_t of 8.00 minutes.

All the other peaks obtained were due to impurities and some of the fatty acids used as the carbon source. In case of P(3HO) the fragment peaks at a retention time (R_t) of 7.57 and 8.73 minutes were identical to the mass spectrum of the methyl ester of undecanoic acid and hexadecanoic acid in the MS (NIST) library. Moreover, some additional peaks of impurities were also obtained in the P(3HO) sample. In case of polymers produced using olive, ground and corn oil, additional peaks of hexadecanoic acid and octadecanoic acid were observed.

Hence, further purification of the polymers was carried out before carrying out further characterisation. The compositions of the PHAs produced are calculated as follow and summarised in **Table 4.1**.

$$\% \text{ Monomer composition} = \frac{\text{Height of the monomer peak X 100}}{\text{Total height of the monomers' peaks}}$$

Table 4.1: The monomer composition of polyhydroxyalkanoates produced from *P. mendocina* using sodium octanoate, coconut oil, groundnut oil, olive oil, and corn oil as the sole carbon sources. Abbreviations used: 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), and 3-hydroxydodecanoate (3HDD).

Type of the carbon source	PHA composition (mol%)			Molecular structure	
	3HO	3HD	3HDD	3HO	3HO-3HD-3HDD
Sodium Octanoate	100	-	-		
Coconut oil	30.43	60.87	8.70		
Groundnut oil	21.15	55.77	23.08		
Olive oil	29.76	38.10	32.14		
Corn oil	33.65	39.42	26.92		

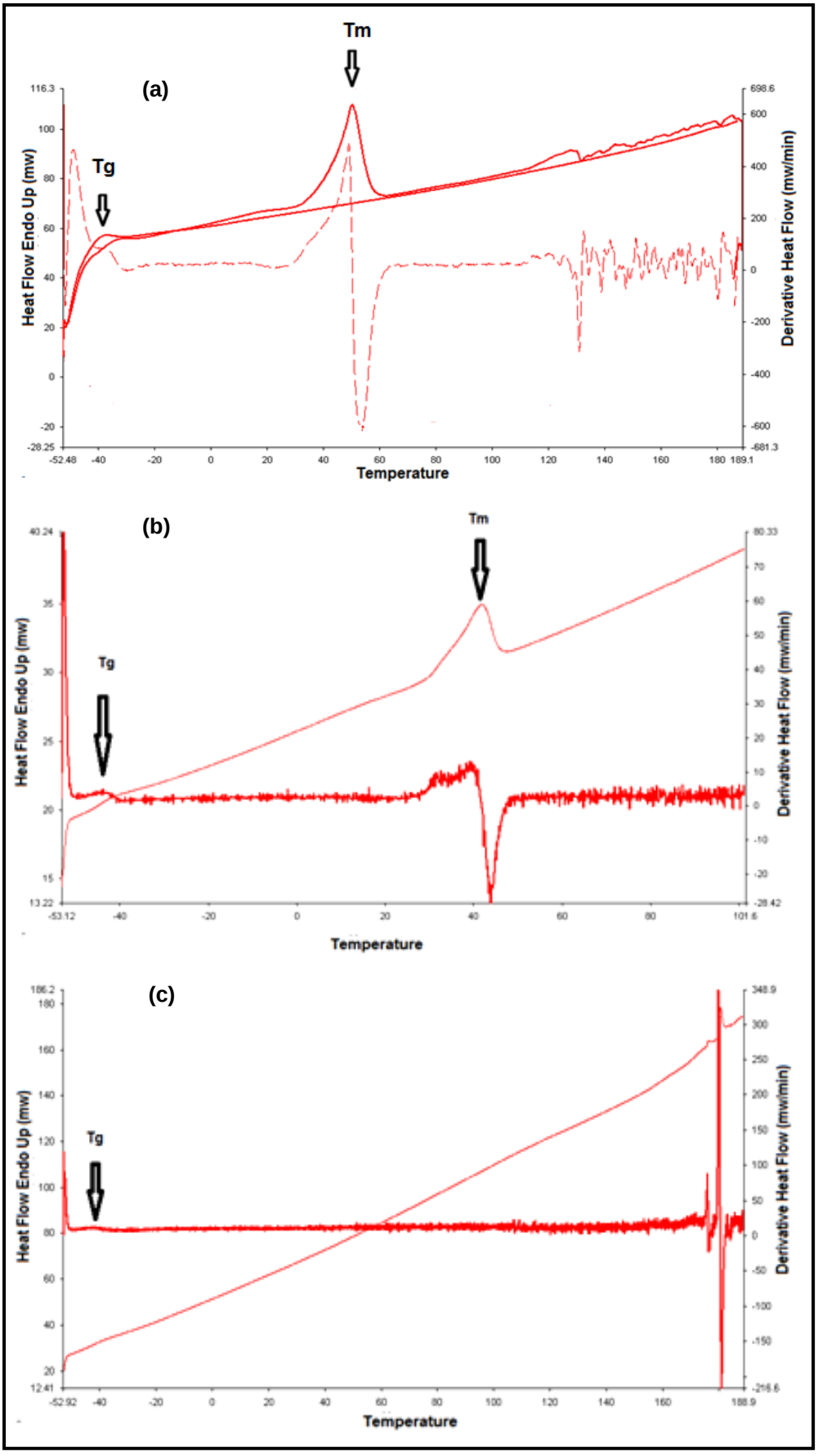
4.2.3 DSC

A representative DSC thermograms of MCL-PHAs are shown in **Figure 4.6**. Corresponding values of glass transition (T_g) and melting temperature (T_m) have been compiled in **Table 4.2**. All terpolymers except that produced using corn oil as carbon source were observed to have lower T_g values compared to the homopolymer P(3HO). The presence of large pendant alkyl chain in terpolymers increases the polymer free volume allowing polymer segment movements at lower temperatures than in P(3HO). Higher glass transition of terpolymer produced with corn oil might be due to alternating sequence of monomer units since this copolymer has similar content of various monomers compared to the other terpolymers.

During the first heat scan, the polymer chains in the crystalline phase of the polymer melted and became disordered. P(3HO) melted around 50^{o}C. Terpolymers can melt at higher temperature compared to P(3HO), for example, the terpolymer produced with olive oil or lower temperature than P(3HO) as in case of polymer produced with coconut oil. Two of the terpolymers did not show a melting event. As can be seen, from the **Figure 4.6**, there is no crystallisation during the cooling cycle for semi-crystalline P(3HO). This indicates that MCL-PHA crystallise very slowly. Thus the absence of melting peaks in case of two terpolymers cannot be attributed to their amorphous nature. Crystalline phases might develop in these copolymers with the increase of storage time. An additional longer term study will need to be conducted to clarify the feasibility of crystallisation of these copolymers. The molar fraction of each monomer unit plays a very important role in controlling the polymer's thermal properties. Therefore, although all the polymers produced using vegetable oils are terpolymers containing 3HO, 3HD and 3HDD monomer units, they all have different T_g and T_m values probably depending on the molar fraction of these monomer units.

Table 4.2: Compilation of the thermal properties of the polymer produced from different sole carbon sources: Abbreviations used: T_g = glass transition temperature, T_m = melting temperature, and (-) = not observed.

Type of the carbon source	Polymer produced	T_g	T_m	ΔH J/g
Sodium Octanoate	P(3HO)	- 40.9^{o}C	49.8^{o}C	11.9
Coconut oil	P(3HO)-co-P(3HD)-co-P(3HDD)	- 45.1^{o}C	41.7^{o}C	15.4
Groundnut oil	P(3HO)-co-P(3HD)-co-P(3HDD)	- 42.9^{o}C	-	-
Olive oil	P(3HO)-co-P(3HD)-co-P(3HDD)	- 45.7^{o}C	65.8^{o}C	1.0
Corn oil	P(3HO)-co-P(3HD)-co-P(3HDD)	- 35.5^{o}C	-	-


(a)
Tm
Tg
Heat Flow Endo Up (mw)
Derivative Heat Flow (mw/min)
Temperature
(b)
Tm
Tg
Heat Flow Endo Up (mw)
Derivative Heat Flow (mw/min)
Temperature
(c)
Tg
Heat Flow Endo Up (mw)
Derivative Heat Flow (mw/min)
Temperature

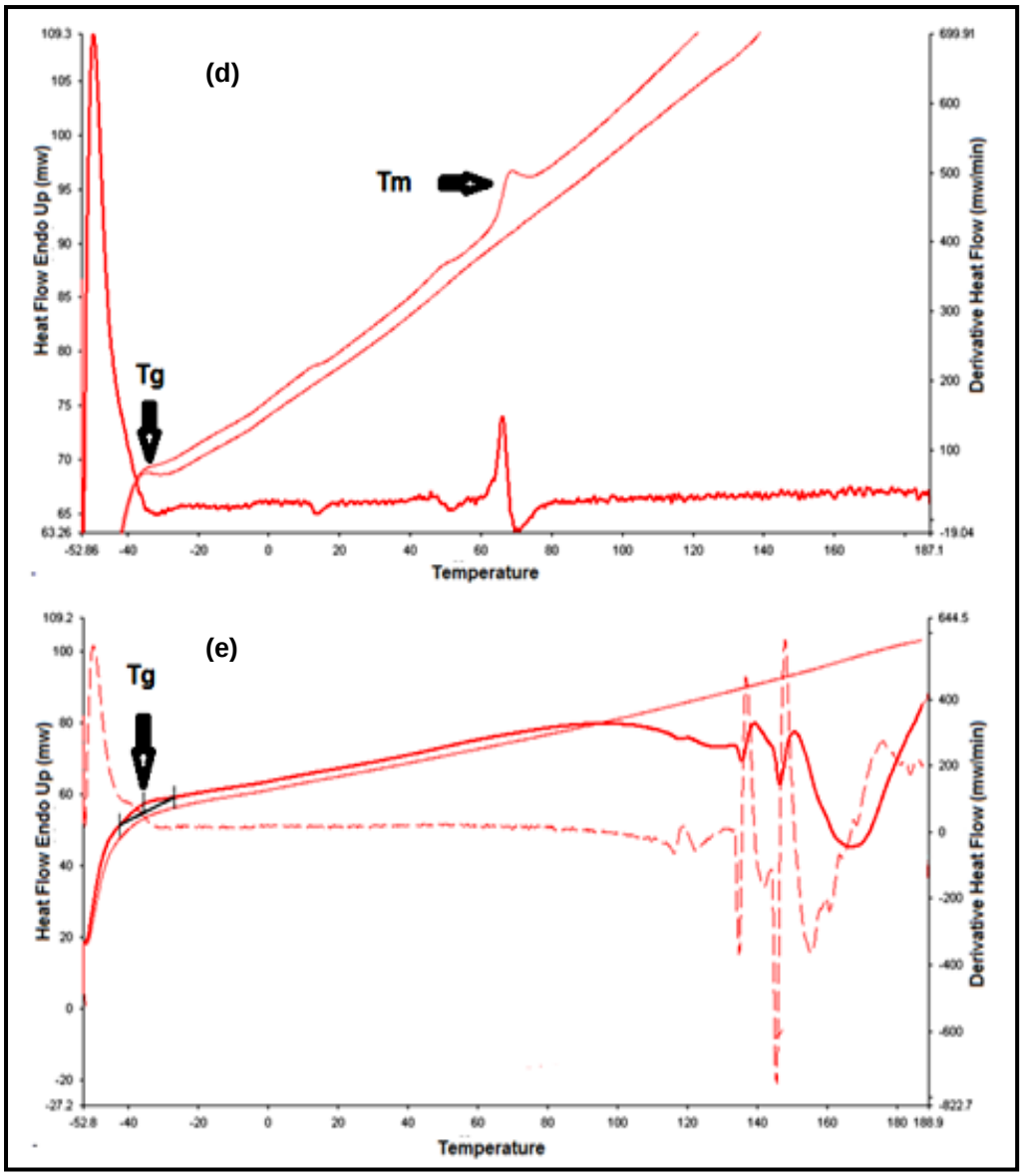


Figure 4.6: Thermal profiles of the polymers extracted from lyophilised *P. mendocina* cells grown on sodium octanoate (a), coconut oil (b), groundnut oil (c), olive oil (d), and corn oil (e). The figures show the normalised DSC heating curve with mathematical derivatives to detect the smallest changes in the curves.

4.2.4 Tensile testing

Tensile testing on the solvent cast thin films (**Figure 4.7**) of the polymers produced using sodium octanoate and coconut oil as the sole carbon source was performed and stress v/s strain curve was plotted (**Figure 4.8**) in order to determine their strength and stiffness (Young's modulus) (**Table 4.3**). The polymer produced using coconut oil exhibited a slightly higher elastic modulus of 4.4 MPa compared to 3.6 MPa Young's modulus of the polymer obtained

using sodium octanoate. Moreover, the tensile strength and the percentage elongation values of the polymer produced using coconut oil were also higher than the P(3HO) produced using sodium octanoate. These results indicated that the stiffness of the polymer from coconut oil was higher compared to that of the polymer from sodium octanoate. This lower stiffness of P(3HO) homopolymer obtained using sodium octanoate could be due to the long uniform carbon backbone containing the C_8 side chains as compared to the random terpolymer with the carbon backbone containing the random C_8,C_{10} and C_{12} side chains (Rai 2010).

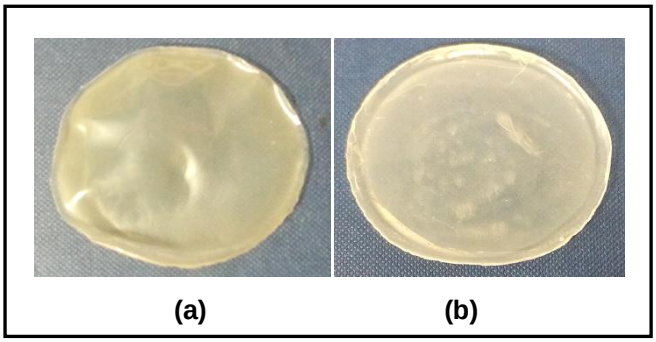


Figure 4.7: Solvent cast films prepared using PHAs produced from (a) sodium octanoate and (b) coconut oil.

Table 4.3: Compilation of Young's modulus (E), tensile strength and elongation at break values for the polymers obtained using sodium octanoate and coconut oil (n=2, ± =SD).

Type of the carbon source	Polymer produced	Young's modulus	Tensile strength	Elongation at break
Sodium Octanoate	P(3HO)	3.62 MPa ±2.26	4.27 MPa ±0.13	341.64 % ±30.56
Coconut oil	P(3HO)-co-P(3HD)-co-P(3HDD)	4.42 MPa ±0.28	4.89 MPa ±0.01	393.70 % ±3.37

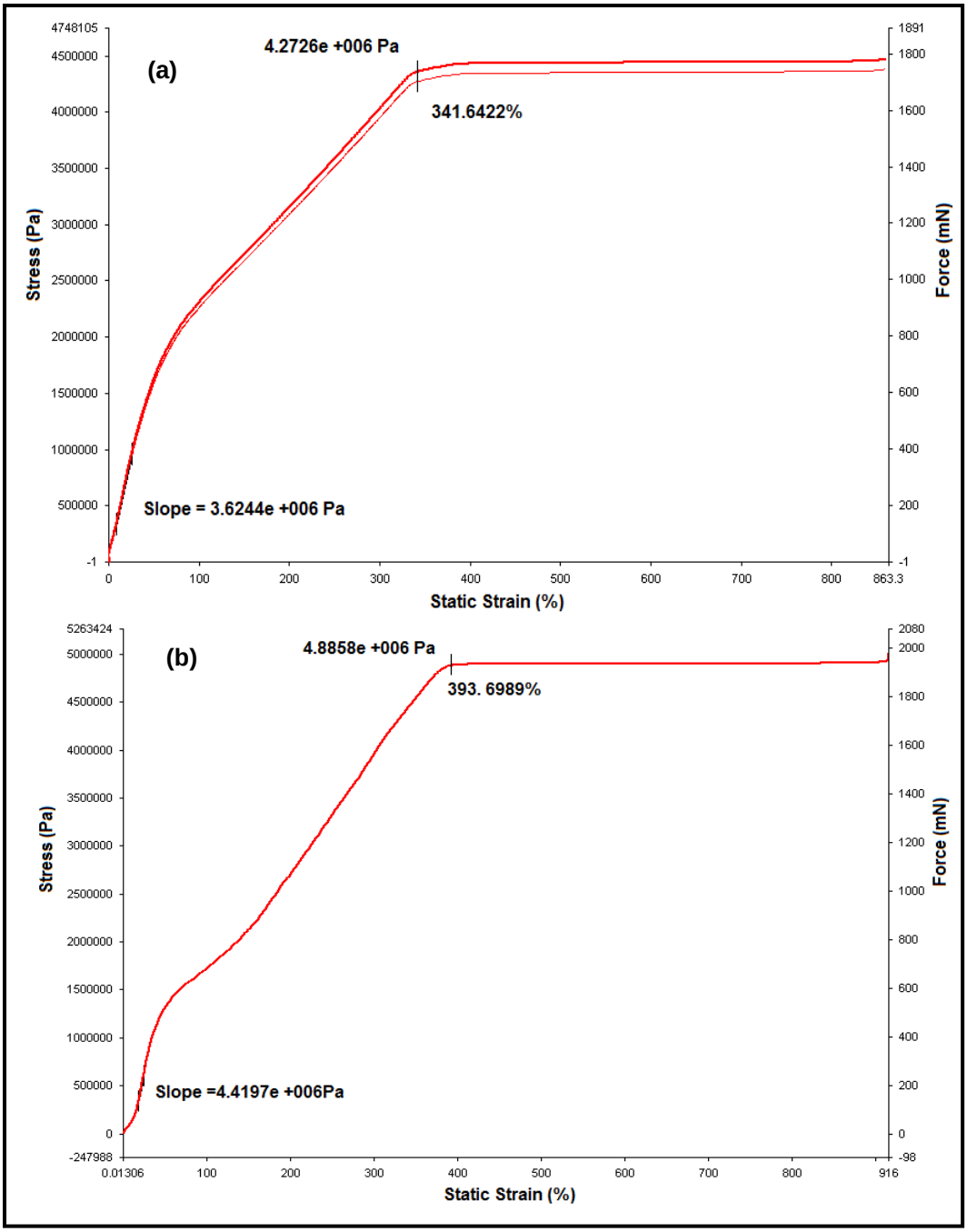


Figure 4.8: Stress–strain profile of the fabricated 5 wt% PHA films produced using (a) sodium octanoate, and (b) coconut oil, as the sole carbon source.

4.2.5 GPC

Samples of the produced polymers were sent to Nottingham University for GPC analysis. The molecular weight and the polydispersity index (PDI) values obtained are compiled in **Table 4.4**. PDI is a measure of the distribution of the molecular masses in a given polymer sample. The PDI values of the polymers

produced were found to be between 1.10 - 1.84, indicating a reasonably mono-disperse polymer preparation. Interestingly, the homopolymer of P(3HO) produced from *P. mendocina* using sodium octanoate as the sole carbon source showed the highest M_w and M_n values of 2.25 x 10^5 and 1.22 x 10^5 respectively, compared to the rest of the polymers produced using vegetable oils as the sole carbon sources. Terpolymers produced using the vegetable oils as sole carbon sources exhibited M_w and M_n values in the range of 0.1-0.6 x 10^5 and 0.1-0.5 x 10^5 respectively as shown in **Table 4.4**. These polymers are hence much lower in molecular weight compared to the P(3HO) homopolymer obtained using sodium octanoate.

Table 4.4: Molecular weight analysis of the PHAs produced using sodium octanoate, coconut oil, groundnut oil, olive oil, and corn oil.

Type of the carbon source	Polymer produced	M_n (X10^5)	M_w (X10^5)	PDI
Sodium Octanoate	P(3HO)	1.22	2.25	1.84
Coconut Oil	P(3HO)-co-P(3HD)-co-P(3HDD)	0.11	0.14	1.29
Groundnut Oil	P(3HO)-co-P(3HD)-co-P(3HDD)	0.31	0.37	1.17
Corn Oil	P(3HO)-co-P(3HD)-co-P(3HDD)	0.16	0.22	1.49
Olive Oil	P(3HO)-co-P(3HD)-co-P(3HDD)	0.48	0.53	1.10

4.2.6 XRD

To investigate the effect of the different carbon sources on the crystallinity of the PHAs produced, XRD studies were carried out. The XRD pattern (**Figure 4.9**) showed wide peaks at around 2θ=20° for each polymer which is indicative of the amorphous nature of the polymers produced. Hence, it was confirmed that the polymers produced in this study were predominantly amorphous in nature (Rai 2010). An increase in the variety of side chains within one polymer chain of MCL-PHA can decrease its ability to crystallise and therefore there are some distinct differences in the crystallinity of MCL-PHAs as compared to SCL-PHAs. Sanchez *et al.* (2003) observed that for MCL-PHAs and its copolymers low crystallinity is possibly 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 to form a crystalline array. The terpolymer produced using

groundnut oil showed the highest intensity of the peak followed by polymers produced using olive oil, corn oil, sodium octanoate and coconut oil respectively. This result indicated highly amorphous nature of the polymer produced using groundnut oil as compared to all the other polymers produced in this study.

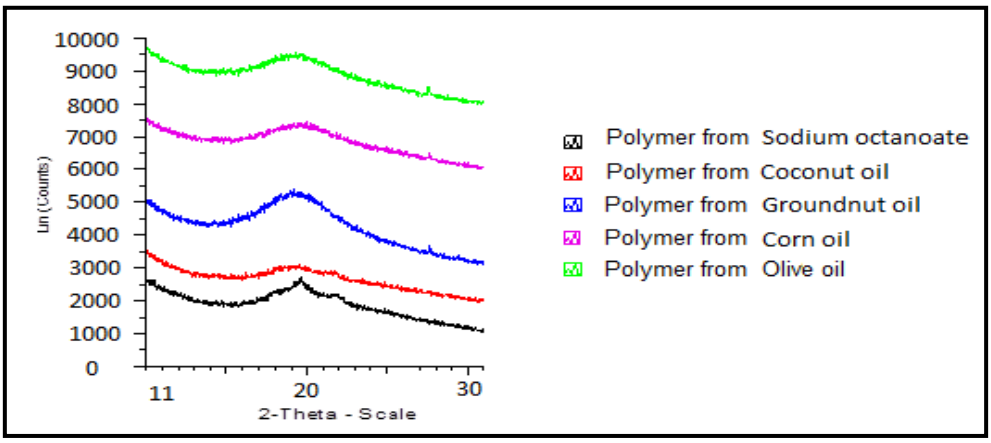
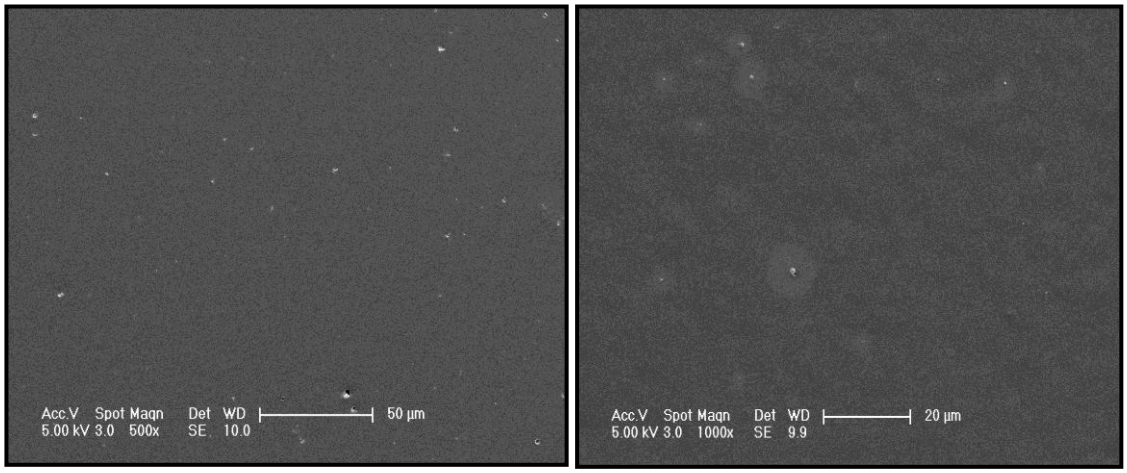


Figure 4.9: X-ray diffraction patterns of PHAs produced using sodium octanoate, coconut oil, groundnut oil, olive oil and corn oil.

4.2.7 SEM

Surface and microstructural features of a biomaterial can have important implications on its biocompatibility and hence its applications. Therefore, to evaluate these microstructural properties and understand its impact on the biocompatibility, the surface of the fabricated films were observed using scanning electron microscopy (SEM). The SEM micrographs (**Figure 4.10**) showed that the polymer produced from corn oil had highly rough surface when compared to the polymers produced from sodium octanoate, coconut oil, groundnut oil and olive oil.



(a) **(b)**

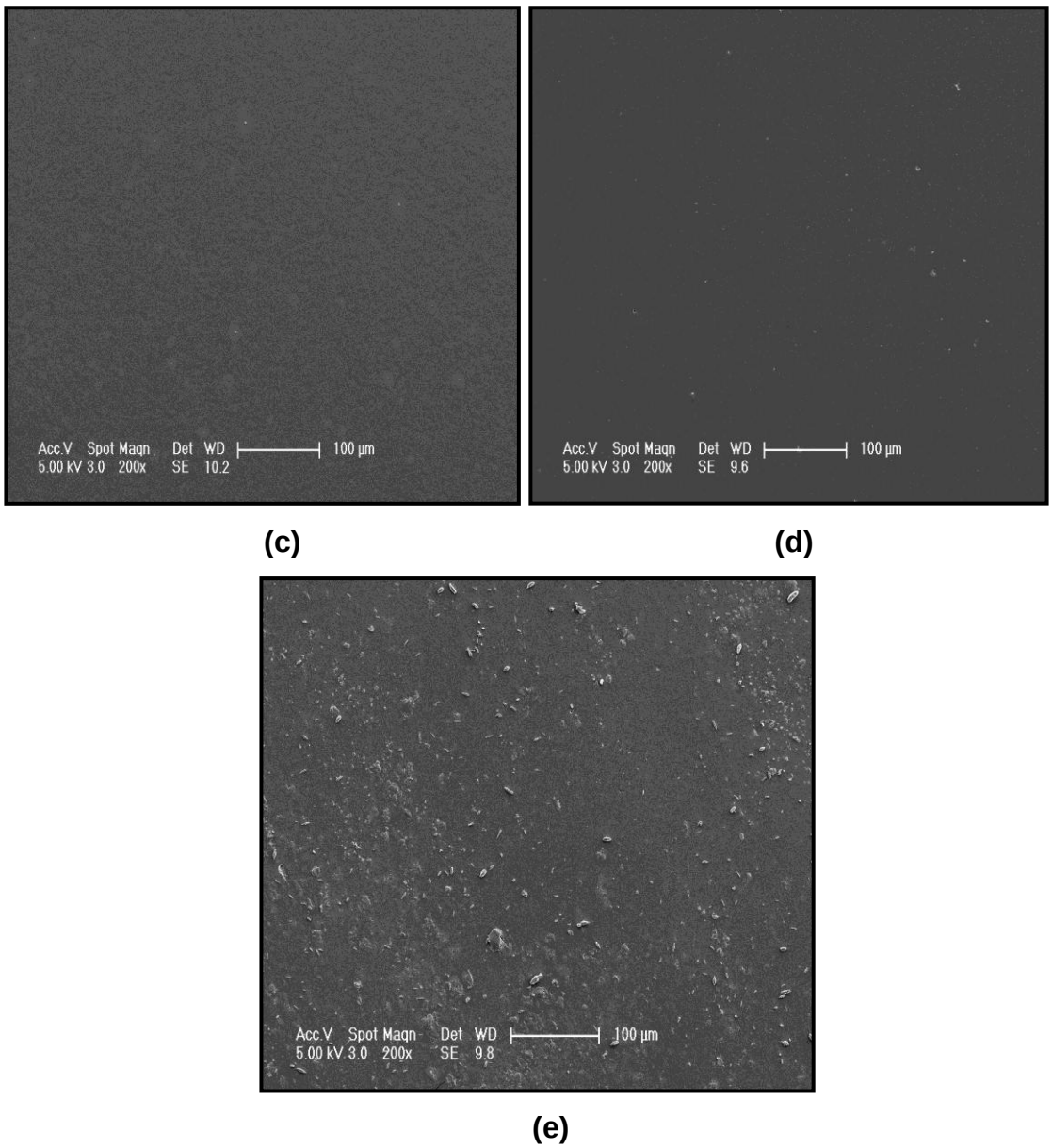


Figure 4.10: SEM micrographs of polymer films produced from (a) sodium octanoate, (b) coconut oil, (c) groundnut oil, (d) olive oil, (e) corn oil taken at different magnifications (3 samples were scanned for each polymer).

4.2.8 Static contact angle

The surface of a material is crucial in determining its compatibility with other materials as well as its ability to interact with the surrounding environment; especially cell adherence and viability is determined to a large extent by the surface properties of the material. Hence, investigating the material's surface properties is very important to assess its suitability in a particular application, particularly in biomedical applications. The water contact angle (θ_{H2O}) is a measure of the hydrophilicity/hydrophobicity of a material surface. Surfaces with

θ_{H2O} less than 90^o are considered to be hydrophilic in nature whereas, surfaces with θ_{H2O} greater than 90^o are considered to be hydrophobic in nature (Peschel *et al.,* 2007). Water contact angle measurements of the fabricated polymer films are shown in **Figure 4.11**. The θ_{H2O} value obtained for most of the produced polymer was greater than 90^o, hence confirming their hydrophobic nature. Polymer produced using groundnut oil showed the highest water contact angle indicating its highest hydrophobicity followed by polymers produced using sodium octanoate, corn oil, olive oil, and coconut oil. These measurements showed that the presence of 3HO, 3HD and 3HDD resulted in the presence of long aliphatic side chains on the surface of the polymer, leading to the hydrophobic nature of the surface of the polymers.

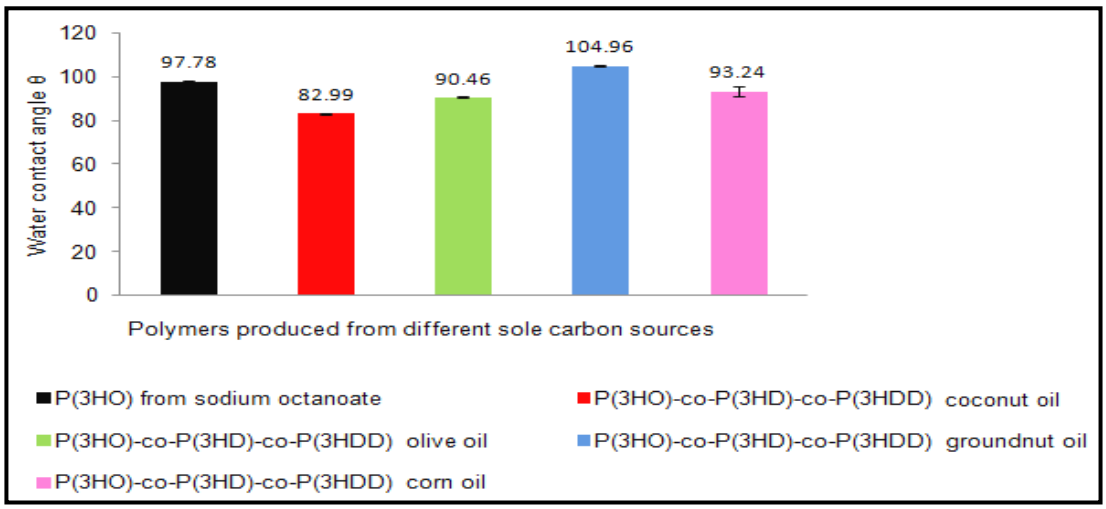


Figure 4.11: Comparison of the water contact angle values of the fabricated PHA films. (n=3; error bars=±SD, error bars are based on the measurements of different samples from same batch)

4.2.9 Cell culture study

Human Mesenchymal Stem Cells (hMSCs) were used in this study since they are multipotent stromal cells that can differentiate into a variety of cell types including: osteoblasts, odontoblasts, ameloblasts, cementoblasts, fibroblasts and vascular endothelial cells. These cell types can ultimately lead to the formation of different tooth parts such as bone, dentin, enamel, cementum and pulp ligaments respectively. To investigate cell attachment, migration and proliferation, hMSCs were cultured (2x10^4 cells/sample) for 1, 4, 7, and 14 days on the surface of the polymer films and MTT assays were performed to evaluate cell attachment and proliferation. The results obtained are shown in **Figure**

4.12. Although cell growth was higher on tissue culture plastic compared to that on the polymer films, a gradual increase in cell growth was observed during the 14 days of measurements. These results indicated that these polymers are not cytotoxic towards the human mesenchymal stem cells and could support cell attachment and proliferation. Highest cell growth was observed on the polymer samples produced from corn oil, followed by the polymer produced using coconut oil. Cell growth observed on the polymer films produced using groundnut oil, sodium octanoate and olive oil showed comparatively lower values than the cell growth on polymer films produced using corn and coconut oils. **Figure 4.12(a)** shows the cell proliferation results highlighting a significant increase in the growth of hMSCs during the 14 days of incubation, on all the polymer samples. **Figure 4.12(b)** shows the cell proliferation results of all the samples compared to the control (control was normalised to 100%). Thus the polymer produced using corn oil as the sole carbon source was found to be the most cytocompatible. SEM analysis of the test samples also confirmed that these polymer materials were able to support hMSC attachment and proliferation (**Figure 4.13**).

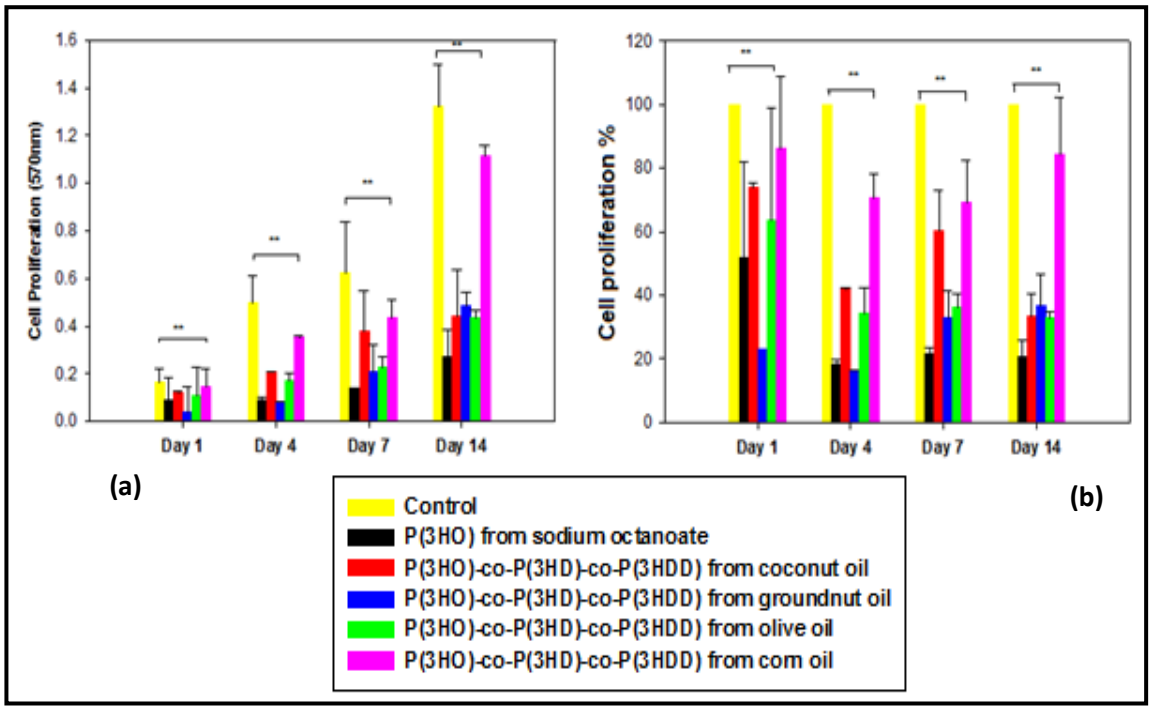
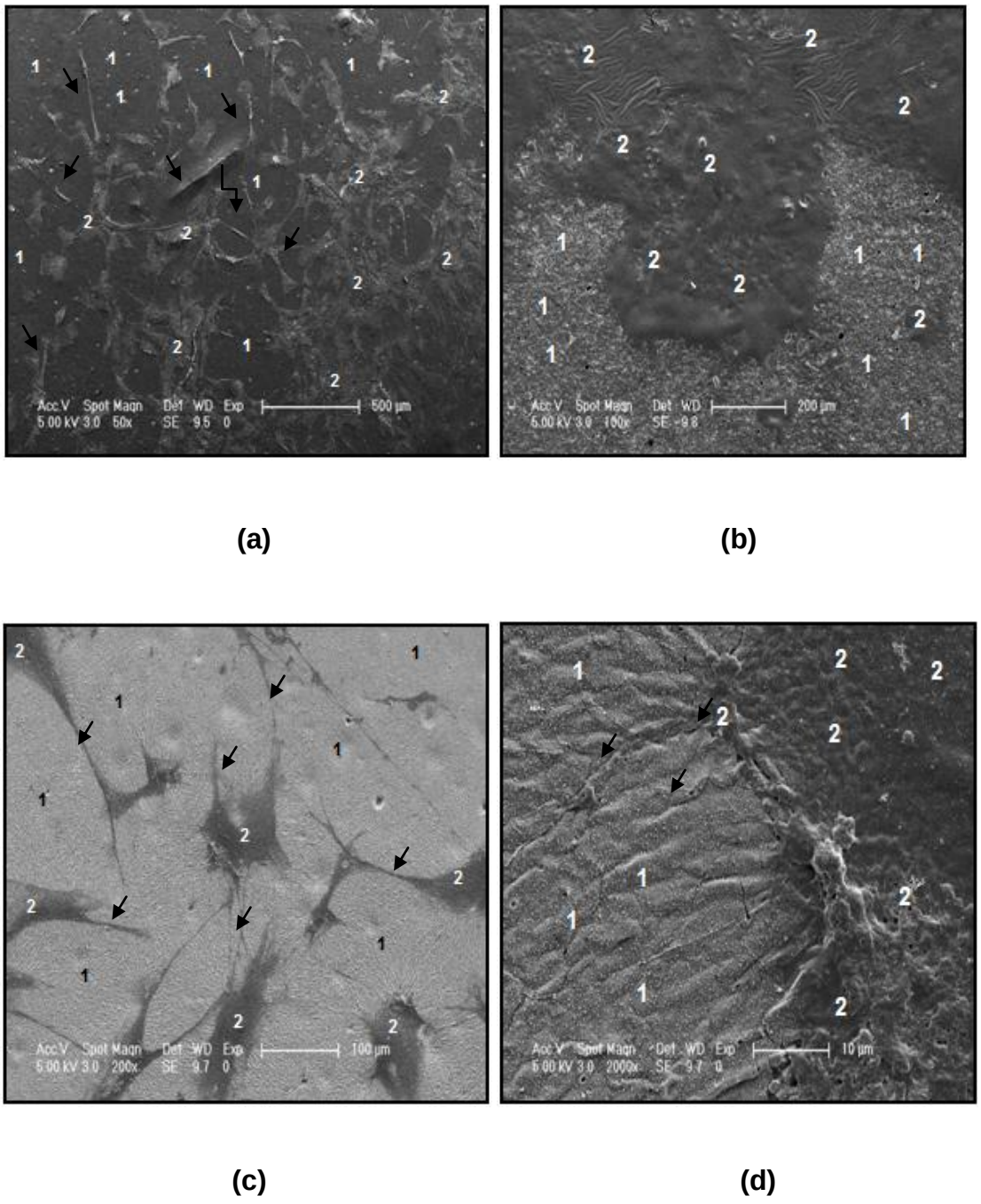


Figure 4.12: Cell proliferation study for 1, 4, 7, and 14 days, using MTT assay on PHAs produced using sodium octanoate, coconut oil, groundnut oil, olive oil, and corn oil, (a) cell proliferation measured using the MTT assay, (b) % cell viability for all tested samples relative to the control (control set at 100%). The data (n=3; error bars=±SD, based on experimental replication) were compared using ANOVA and differences were considered significant when *p < 0.05, **p < 0.01 and ***p < 0.001.



(a) **(b)**

(c) **(d)**

Figure 4.13: SEM images of the seeded hMSCs on to the polymer films prepared from; (a) sodium octanoate polymer: showed hMSCs attachment, proliferation and spreading (b) coconut oil polymer: showed hMSCs spreading on the film forming a cell layer, (c) olive oil polymer: showed hMSCs attachment and proliferation, (d) corn oil polymer: showed hMSCs spreading on the film forming a cell layer. 1) Polymer surface, 2) hMSCs, arrows indicate filopodia.

4.3. Discussion:

Using all five sole carbon sources investigated in this study, a range of PHAs were produced. Preliminary characterisation using FTIR suggested that the polymers produced in this study are medium chain length PHAs. There are several factors which define the monomer composition of MCL-PHAs produced using long-chain fatty acids. The major factor is the fatty acid used as carbon feedstock, the specificity of the PHA-synthesising system, the degradation systems for fatty acids and finally the PHA-synthesising pathways (Eggink *et al.,* 1993). Triglycerides contain different proportions of saturated and unsaturated fatty acids, hence MCL-PHA produced using these triglycerides should vary in their monomer composition. This reflects the substrates and the enzymatic make-up of the organism (particularly those involved in β-oxidation of unsaturated fatty acids) (Ashby and Foglia 1998). GC and GC–MS analysis of the purified PHAs obtained from *P. mendocina* during cultivation on different vegetable oils showed the presence of 3-hydroxyalkanoic acids with chain length ranging from C_8 to C_{12}. The composition and structure of each constituent of the polymers were confirmed by GC–MS analysis (Thakor *et al.,* 2005). Vegetable oils used in this study always contained long chain fatty acids (LCFAs) like, myristic acid (C_{14}), palmitic acid (C_{16}), stearic acid (C_{18}), arachidic acid (C_{20}), palmitoleic acid (C_{16}), oleic acid (C_{18}) and linoleic acid (C_{18}) as constituents, and coconut oil also contained caproic acid (C_6), caprylic acid (C_8), capric acid (C_{10}) and also lauric acid (C_{12}) along with LCFAs (Thakor *et al.,* 2005). PHAs obtained from *P. mendocina* during growth on coconut oil, groundnut oil, olive oil and corn oil showed almost similar patterns of 3HAs incorporation into a heteropolymer. 3-hydroxyoctanoaic acid, 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid were found common in PHAs obtained from *P. mendocina* using different vegetable oils in this study. Coconut oil used in this study contained a high concentration of saturated fatty acids (86%) and low concentration of unsaturated fatty acids (6%) (Ashby and Foglia 1998). Whereas groundnut oil, olive oil and corn oil are mainly composed of unsaturated fatty acids ranging from 78-85%. When coconut oil was used as a feedstock, 3-hydroxyoctanoic acid, 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid were incorporated at concentrations of 30.43, 60.87,

and 8.70 mol% respectively. From this result, it can be seen that the least incorporated 3HA moiety was 3-hydroxydodecanoic acid when coconut oil was used as the sole carbon source. Thus, it demonstrated the high concentration of C_8 and C_{10} moieties in the MCL-PHA produced from coconut oil sole carbon source, suggesting the enhanced PHA synthase specificity for 3-hydroxyoctanoyl-CoA and 3-hydroxydecanoyl-CoA. Moreover, this could be the result of the low concentration of unsaturated fatty acids in coconut oil (Ashby and Foglia 1998). In a previous study when *Ps. saccharophila* was grown using coconut oil, it showed that the organism grew well and gave maximum MCL-PHA production at 48 hours. This study also observed the incorporation of the 3-hydroxyoctanoic acid and 3-hydroxydecanoic as the major monomer repeat units with higher concentrations (Daniel *et al.,* 1999, Ashby and Foglia 1998). Thus, it was confirmed that when coconut oil was used as the sole carbon source, higher concentrations of the saturated hydroxy fatty acids (HFA) monomers were incorporated in the PHA produced (Daniel *et al.,* 2006, Chenyu *et al.,* 2012). Moreover, *P. resinovorans* also showed the synthesis of MCL-PHA with intact oils and fats, reflecting the monomer repeat unit composition with the fatty acids of the oil or fat substrate used for their synthesis (Daniel *et al.,* 2006, Chenyu *et al.,* 2012). When groundnut oil, olive oil and corn oil were used as the carbon source, 3-hydroxydodecanoic acid was incorporated at concentrations of 23.08, 32.14 and 26.92 mol% respectively. The high degree of polyunsaturation resulted in the additional incorporation of 3-hydroxydodecanoic acid repeat units into the PHAs produced when groundnut oil, olive oil and corn oil were used as the feedstock. It was probably due to the higher specificity of the β-oxidation enzymatic system for unsaturated fatty acids of the groundnut, olive, and corn oils.

The material properties of PHAs are very important criteria to determine the applications of the particular type of polymer. For example, the MCL-PHAs have elastomeric behaviour with an elongation at break of about 300%. This, together with other properties such as high cytocompatibility, means MCL-PHAs could be used as a scaffold material in tissue engineering (Chenyu *et al.,* 2012, Hazer *et al.,* 2012).

PHAs are normally crystalline polymers. Their thermal properties are presented in terms of the glass transition temperature (T_g) of the amorphous phase and the melting temperature (T_m) of the crystalline phase. The thermal properties of each MCL-PHA are given in **Table 4.2**. During the first heat scan, the polymer chains in the crystalline phase of the polymer melted and became disordered. This absorption of energy for the melting of the polymer chains in the crystalline phase was reflected as the T_m peak. MCL-PHAs produced in this study using sodium octanoate, coconut oil, and olive oil exhibited some degree of melting transition, indicating that each of these PHAs had some degree of crystallinity. In contrast, the thermal properties of the PHAs produced from groundnut and corn oil showed no melting transition, indicating that they could be completely amorphous or semi-crystalline which needs further confirmation. In previous studies, the T_g value of MCL-PHA is found to decrease with the increase in average length of the pendant group, caused by the increased mobility of the polymer chains. When *P. oleovorans* was grown on a range of n-alkanoates from C_6 to C_{10}, a decrease in T_g of the PHA produced using hexanoate (T_g = -25°C) to that produced from decanoate (T_g = -40°C), was observed, corresponding to an increase in the average length of the predominant side-chain. A similar observation was made with a decrease of T_g value of almost 18°C for MCL-PHAs produced from coconut oil fatty acid (-43.7°C) to that produced from linseed oil fatty acid with a T_g value of -61.7°C (Rai 2010). Moreover, Ouyang *et al.* (2007) found that in the mutant strain of *P. putida* KTOYO6, with the increasing content of 3-hydroxydodecanoate, (3-HDD from 15 to 39 mol%), in the accumulated MCL-PHA, T_m increased from 53 to 65°C and ΔH_f from 18 to 28 J/g. In general, crystallisation occurs slowly in MCL-PHAs and for some copolymers no T_m value is observed because the copolymers do not crystallise at all (Rai 2010). In the present study, relatively low T_g values were obtained for the MCL-PHAs produced, which presented low rigidity and high elasticity. These properties make them potential material of choice for biomedical applications (Hazer *et al.,* 2012).

An increase in the variety of side chains within one polymer chain of MCL-PHA can hamper its ability to crystallise and therefore there are some distinct differences in crystallinity of MCL-PHAs. Previously Barbuzzi *et al.* (2004) confirmed that crystallisation of the MCL-PHAs is possible due to the formation

of layerlike order of both the backbone and the side chains. Moreover, the structural regularity of the repeating units will affect the crystalllinity of the polymers (Ashby and Foglia 1998). Sanchez *et al.* (2003) reported that for MCL-PHAs and its copolymers low crystallinity is possibly 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 to form a crystalline array. In fact, saturated MCL-PHAs, which are able to crystallise due to their isotactic configuration, are also seen to crystallise with alkyl side chains in an extended conformation to form ordered sheets, but they still show a reduced degree of crystallinity. Copolymers of P(3HO) are amongst the common MCL-PHAs studied and in the study carried out by Gagnon *et al.* (1992) using wide angle X-ray diffraction (WAXD) and ^{13}C nuclear magnetic resonance (^{13}C NMR), it was found that the copolymer of MCL-PHA containing 86% of HO and minor quantities of 3-hydroxydecanoate and 3-hydroxyhexanoate was approximately 30% crystalline. A two phase morphology, was therefore proposed to be present in P(3HO), the amorphous phase and the crystalline phase (Rai 2010).

MCL-PHAs are elastomers with the crystalline parts acting as physical crosslinks and therefore have mechanical properties very different from those of SCL-PHAs and its copolymers. The introduction of a comonomer into the polymer backbone is seen to significantly increase the flexibility (elongation to break) and toughness of the polymer (Rai 2010). Results obtained in this study indicated that the flexibility of polymer from coconut oil was higher compared to the polymer from sodium octanoate. This higher rigidity of the polymer from sodium octanoate could have been due to the homopolymeric nature of the P(3HO) films, which have long carbon backbones of the same type (all C_8 monomers) making layerlike order arrangements of the polymer. Hence, this study showed that the fabricated P(3HO) films were less stiff than the copolymer produced from coconut oil (Basnett 2014).

Molecular weights of the produced polymers are likely to be affected by several criteria such as the producing organism, the state of inoculums, production media composition, fermentation conditions and downstream processes. Molecular weights can vary depending on the stage of the cultivation.

Downstream processing such as biomass pre-treatment, polymer extraction and purification methods also affected the molecular weights of the produced polymers (Ranjana *et al.,* 2011). In this study, high cell and polymer yields using vegetable oils as the sole carbon source indicated that the triglycerides are used by the bacterium as carbon sources. This was further suggested by the similar molecular masses of each PHA produced from vegetable oils. It has been shown that the molecular masses of microbially produced polymers vary as a function of the stage of growth when the cells are harvested (Birrer *et al.*, 1994). Because the polymerisation efficiency of a bacterial system is based on enzyme activity or number of enzymes present, it is likely that relatively equal molecular masses are the result of a similar polymerisation process (Daniel *et al.,* 1999, Ashby and Foglia 1998). The P(3HO) produced using sodium octanoate as the sole carbon source showed the highest molecular weight compared to the polymers produced using vegetable oil sole carbon sources. Hydrolysis of oils by lipases produces free fatty acids and glycerol. It has been observed that the presence of glycerol in the culture medium decreases the molecular weight of the PHA (Madden *et al.,* 1999). Thus, in this study lower molecular weight polymers were obtained using vegetable oil as the sole carbon sources compared to the polymer synthesised using sodium octanoate as the sole carbon source.

Water contact angle measurements (also an indication of the wettability) showed that addition of the comonomers in the polymeric backbone and increase in the length of the side chains decreased the wettability of the polymers. Hence confirmed the hydrophobic nature of the polymers produced from vegetable oils (Superb *et al.,* 2008).

Many studies have been carried out to understand cell and material interfacial relationships, particularly related to biocompatibility of the material used. This is because most mammalian cells are anchorage dependent and need a biocompatible surface for attachment, differentiation, and migration to form new tissue. For tissue regeneration applications, the scaffold should be biocompatible, biodegradable and the degradation products should be non-toxic and resorbable. Moreover, the scaffold should facilitate the attachment, migration, proliferation, and three-dimensional spatial organisation of the cell

population required for structural and functional replacement of the target tissue. Biocompatibility is of utmost importance to prevent adverse tissue reactions (Galler *et al.,* 2011). The biocompatibility of PHAs, like for any other biomaterial, is dependent on factors such as shape, surface porosity, surface hydrophilicity, surface energy, chemistry of the material, the environment where it is incorporated and its degradation products. Interestingly, the cytotoxicity of oligo-hydroxyalkanoates (OHA) decreased with increasing OHA side chain length thus indicating that medium chain length OHAs containing PHA, such as poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), P(3HB-co-3HHx) and MCL-PHAs are more biocompatible then short chain length hydroxyalkanoates. These results indicated that these polymers are not cytotoxic towards the human mesenchymal stem cells and could support cell attachment and proliferation. Highest cell growth was observed on the polymer samples produced from corn oil, followed by the polymer produced using coconut oil. Previous studies have shown that the cells prefer to attach to the rough surface of the material compared to the smooth surface. Surface properties of the polymer film samples were studied using SEM which indicated a considerably rough surface of the polymer produced using corn oil compared to all the other produced polymers which explains the higher cell growth on polymer samples produced using corn oil. In case of the polymer produced using groundnut oil the highest water contact angle was observed, indicating its highly hydrophobic nature, followed by polymers produced using sodium octanoate and olive oil. Previous studies have shown that hydrophilic surfaces are favorable for cell attachment and proliferation. Thus, these results could also be correlated to the cell growth observed on the polymer films produced using groundnut oil, sodium octanoate and olive oil (Basnett 2014, Daniel *et al.,* 1999). To increase the biocompatibility of polymer scaffolds, attempts have also been made to increase the hydrophilicity of the polymer which may enable better adherence of the seeded cells on the scaffolds. Another approach to increase the biocompatibility of the polymer is by coating the surface of the polymer with a biocompatible compound (Rai 2010). Moreover, the reaction of cells and tissues to PHAs depends not only on the chemical composition of the material but also on the degree of purity, methods of processing the material into scaffolds, physical mechanical properties of these scaffolds, properties of the surface and methods of its treatment (Shishatskaya and Volova 2004).

CHAPTER 5
Conclusions and Future Works

5.1. Conclusions:

PHAs are a good replacement for the petroleum-based plastics. However, commercialisation of PHAs has been limited due to their overall high production cost. A major factor that adds to their relatively high production cost is the cost of expensive carbon feedstocks for the organism. Hence, to make PHA production more economical, much research is involved in identifying and using renewable and cheap carbon sources, which not only reduces the production costs but also increases polymer yields (Ashby and Foglia 1998). In recent years, the use of organic wastes, agricultural and dairy by-products and vegetable oils have been investigated as alternative substrate, for PHA production. Among these, vegetable oils are considered as the most suitable and desirable feedstocks for PHA production due to their high productivity. Vegetable oils can reduce both production cost and energy consumption when compared with sugar substrates such as glucose or sucrose (Daniel *et al.,* 2006, Chenyu *et al.,* 2012, Chee *et al.,* 2010). However, there are only few reports published using some vegetable oils, including soybean oil, castor oil, sunflower oil, and palm oil, have been used to obtain high yields of PHAs (Daniel *et al.,* 2006, Chenyu *et al.,* 2012, Chee *et al.,* 2010). The bacterial species that have been shown to produce MCL-PHAs from plant oils include *Aeromonas caviae*, *Pseudomonas aeruginosa*, *Pseudomonas resinovorans*, *Pseudomonas saccharophila*, and *Comamonas testosteroni*. Pseudomonads belonging to the rRNA homology group I are particularly known to produce MCL-PHAs from vegetable oils (Ashby and Foglia 1998). In this study we have investigated, for the first time, the PHA production by *Pseudomonas mendocina* using different vegetable oils as the feedstocks for the culture.

The ultimate goal of the first part of this study was to investigate PHA production using vegetable oils as the sole carbon sources. Moreover, there is no report published for PHA production by *Pseudomonas mendocina* using vegetable oils. Hence, *Pseudomonas mendocina* was the chosen organism in this study. Growth and production profiles were investigated at shaken flasks and (2 L) fermenter levels. In order to identify an overall good sole carbon source for PHA accumulation from *P. mendocina,* different parameters such as cell growth, pH, nitrogen concentration, dry cell weight and % PHA yield

measured and compared. The PHA yields obtained using vegetable oils (coconut oil, groundnut oil, olive oil and corn oil) were then compared with that obtained during the production of PHAs using sodium octanoate as the sole carbon source. It was found that, when *P. mendocina* was grown in the MSM media at shaken flask level with different sole carbon sources such as sodium octanoate, coconut oil, groundnut oil, olive oil and corn oil, the organism was able to grow and accumulate a range of different PHAs with varying yields. Moreover, the organism had selective preference for one of the carbon sources used i.e. coconut oil, giving higher values of the cell growth and PHA accumulation. Fermentation profile results for shaken flask level PHA production revealed that coconut oil was by far the best sole carbon source for supporting cell growth as well as PHA accumulation. It resulted in the highest PHA yield of 60.5% dcw at shaken flask level. PHA yield (43.6% dcw) achieved using olive oil as the sole carbon source was significantly higher than the PHA yield (35.1% dcw) using sodium octanoate as the sole carbon source. This was followed by a decreased PHA yield of 31.8% dcw from groundnut oil and the least PHA yield (29.8% dcw) was achieved when corn oil was used as the sole carbon source.

2 L Fermenter level PHA production using all five sole carbon sources also revealed that the highest PHA accumulation was obtained when coconut oil was used as the sole carbon source. Results at fermenter level showed the PHA yield of 34.6% dcw was obtained when coconut oil was used. PHA yield (% dcw) of 27.4, 26.3, 23.6 and 20.5 were obtained when sodium octanoate, olive oil, groundnut oil and corn oil were used respectively at 2 L bioreactor level production.

Overall comparison of the growth and production profiles at shaken flask and fermenter levels showed that when vegetable oils were used as the sole carbon sources, more cell growth was observed in fermenters (2 L) than in shaken flasks. However, higher PHA yields were obtained in shaken flask level fermentations for all five sole carbon sources. When sodium octanoate was used as the main carbon source, more cell growth and PHA yield were observed in shaken flask than in fermenter (2 L) study.

The above results also concluded that the coconut oil as the sole carbon source supported comparatively higher *Pseudomonas mendocina* cell growth and PHA accumulation in both the fermentation conditions. These results are encouraging and show that coconut oil is a promising renewable feedstock for an economical and commercially viable production of PHA by *Pseudomonas mendocina*. This will ultimately decrease the cost of PHA production and could enhance its widespread usage.

The main objective of the **chapter 4** was to characterise PHAs, produced by *Pseudomonas mendocina* using five different sole carbon sources (sodium octanoate, coconut oil, groundnut oil, olive oil and corn oil) for their chemical, mechanical, thermal, physical, surface properties and biocompatibility in order for them to be assessed for biomedical applications, in particular as the scaffold material for dental pulp tissue engineering in future. Using all five sole carbon sources in this study, a range of PHAs was produced. Preliminary characterisation using FTIR suggested that the polymers produced in this study have characteristic marker bonds for medium chain length PHAs. GC and GC-MS analysis of the purified PHAs obtained from *P. mendocina* during cultivation on different vegetable oils showed presence of 3-hydroxyalkanoic acids with chain length ranging from C_8 to C_{12}. Monomer compositions of the polymers produced were confirmed by GC-MS analysis which revealed that 3-hydroxyoctanoaic acid, 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid were found common in PHAs obtained from *P. mendocina* using different vegetable oils in this study. When sodium octanoate was used as the sole carbon source, the organism accumulated a homopolymer of 3-hydroxyoctanoate (P(3HO)). MCL-PHAs produced in this study using sodium octanoate, coconut oil, and olive oil exhibited some degree of melting transition, indicating that each of the PHAs were semi-crystalline. In contrast, the thermal properties of PHAs produced from groundnut, and corn oil showed no melting transition, indicating that they could be completely amorphous or semi-crystalline which needs further confirmation. Copolymers showing low crystallinity could possibly be due to the presence of large and irregular pendant side groups which inhibited close packing of the polymer chains in a regular three dimensional fashion to form a crystalline array. The introduction of a comonomer into the polymer backbone is seen to significantly affect its

mechanical property, hence resulting in an increase in flexibility (elongation to break) and toughness of the polymer (Rai 2010). Therefore, results obtained in this study revealed the higher flexibility of polymers from vegetable oils compared to polymer from sodium octanoate. Because the polymerisation efficiency of a bacterial system is based on enzyme activity, it is likely that relatively equal molecular masses are the result of a similar polymerisation process (Daniel *et al.,* 1999, Basnett 2014, Ashby and Foglia 1998). Hence, GPC study showed the similar molecular masses of each PHA produced from vegetable oils. The P(3HO) produced using sodium octanoate as the sole carbon source showed the highest molecular weight compared to the polymers produced using vegetable oil sole carbon sources. X-Ray Diffraction analysis revealed the amorphous or semi-crystalline nature of the polymers produced using vegetable oils. Moreover, water contact angle measurements (also an indication of the wettability) showed that addition of the comonomers in the polymeric backbone and increase in the length of the side chains decreased the wettability of the polymers. Hence, the hydrophobic nature of the polymers produced from vegetable oils was confirmed (Superb *et al.,* 2008).

Furthermore, biocompatibility studies demonstrated that the polymers were not cytotoxic to the hMSCs and were able to support their attachment and proliferation. The terpolymer produced using corn oil showed the highest hMSCs growth during the 14 days cell culture studies, compared to the polymers produced using other sole carbon sources in this study. The main reason for this could be the comparatively rough surface of the polymer produced using corn oil as observed in SEM analysis. Hence, to further develop these polymers produced using renewable carbon feedstocks, construction of the composite materials using phosphate glass and polymers produced in this study would be carried out in future. This would improve cell growth on all the PHA films. Moreover, phosphate glass is known to have higher biological activity. Therefore, it will be possible to prepare scaffold materials of choice for dental pulp regeneration application in particular.

5.1.1. Concluding remarks

This study has shown for the first time that *Pseudomonas mendocina* is capable of producing copolymers of MCL-PHAs using a range of vegetable oils. The fermentation profiles obtained demonstrated that all the vegetable oils were relatively good feedstock for supporting cell growth and MCL-PHA production. Moreover, their mechanical, physical and surface characterisation revealed that these polymers were elastic, amorphous or semi-crystalline and hydrophobic in nature. Biocompatibility studies demonstrated that the polymers were not cytotoxic to the hMSCs and were able to support their attachment and proliferation. The terpolymer produced using corn oil showed the highest cell growth in this study perhaps due to its rough surface. In future, in order to improve cell growth on all the PHA films, phosphate glass containing composites of the polymers will be produced, leading to higher biological activity and increased surface roughness. The preliminary cell culture data suggests that the polymers produced in this study may encourage the dental tissue regeneration which is one of the targets of this work. If successful, in future, this work will potentially lead to the use of cheap vegetable oils for the production of PHA-based dental polymers.

5.2. Future work:

The application of inexpensive vegetable oils as carbon sources for PHA biosynthesis is predicted to reduce the production cost of bacterial polyhydroxyalkanoates. However, there are many potential areas that could be the focus of the future research to gain more understanding on the different MCL-PHAs produced in this study. Some potential experiments that would need to be performed are:

5.2.1. Optimisation of MCL-PHAs production

In order to identify an overall good sole carbon source for PHA accumulation from *P. mendocina*, initial investigations were carried out. It would be interesting to carry out more research in optimisation of the MCL-PHA production using vegetable oils as the sole carbon sources to enhance PHA yield and further

reduce the cost of production of the polymer. Further optimisation of the physical parameters for MCL-PHA production in the fermenter would need to be performed using pH, C/N and agitation as the varying parameters.

5.2.2. Development of MCL-PHA/SCL-PHA blends

As observed in this study, it was really very difficult to handle the polymers produced from *P. mendocina* using groundnut oil, olive oil and corn oil sole carbon sources due to their very sticky nature. These polymers were sticking to everything they contacted. Therefore, it was impossible to prepare solvent cast films of the PHAs produced using groundnut oil, olive oil and corn oil sole carbon sources, which would limit their possible applications. To overcome this problem, blending of these polymers with brittle nature SCL-PHAs would be advantageous. Using different concentrations of the SCL-PHAs for blending would give appropriate amount of elasticity to these MCL-PHAs and would help in controlling their very sticky nature. This tailor made property could be exploited in their applicability in Dental Tissue Engineering, which is the ultimate goal of this study. Finally, analysis of the prepared blends for their chemical, physical, mechanical, and biocompatibility properties would be another interesting area to look into for their further development.

5.2.3. Composite preparation and characterisation

The polymers produced in this study will be used to prepare composites in combination with phosphate glass for dental pulp regeneration applications. Phosphate glass is known to have, high degree of bioactivity which helps them to bind with living tissues by forming an apatite layer, bioresorption and osteoconduction. Moreover, dissolution products from phosphate glass can enhance gene transduction pathways in cells by increasing cell differentiation and osteogenesis which could be beneficial for dental pulp cell regeneration. The prepared scaffolds will then be analysed and characterised for their chemical, physical, mechanical, and biocompatibility properties.

5.2.4. Development of controlled antimicrobial/bio factor delivery systems

Not only scaffold materials but also the transcription factors, growth factors and a series of ECM molecules are necessary in order to trigger somatic stem cells to differentiate into specific host cell type for pulp tissue regeneration. Therefore, they are very important molecules for controlling cell behaviour and activity in dental tissue repair and regeneration. Injured host tissues or disordered development of the teeth prevents the required growth factor production. Hence, constructing the scaffold materials as the biofactor delivery vehicles would be an interesting and advantageous strategy for appropriate pulp repair. Another interesting area of research would be novel scaffold material preparation with antimicrobial ions released at a controlled level in order to kill residual bacteria to prevent any infection.

5.2.5. Applicability of the composites as the dental pulp tissue engineering material

The multipotency of hMSCs will be investigated for different time periods when grown on the neat polymers and the composite materials. This study will focus on the differentiation of these cells into functional odontoblasts and into vascular endothelial cells. Cellular responses including proliferation, ALP activity (Casagrande *et al.*, 2010, Sakai *et al*., 2010), and expression of indicative markers, such as Dentin sialophosphoprotein (DSPP), Dentin matrix phosphoprotein 1 (DMP-1) or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), will be evaluated for their odontogenic differentiation. Moreover, for the angiogenic potential of hMSC populations can be studied *in vitro* with capillary tube formation assays (Hirschi *et al*., 2008, Sakai *et al*., 2010). Moreover, detection of endothelial-cell-specific markers such as platelet endothelial cell adhesion molecule 1 (PECAM-1), vascular endothelial growth factor receptor-2 (VEGFR2), vascular cell adhesion molecule-1 (VCAM1), intercellular adhesion molecule-1 (ICAM-1), and vascular endothelial cadherin (VE-cadherin) will be investigated for the evidence of the differentiation of stem cell population into endothelial cells (Jazayeri *et al*., 2008, Marchionni *et al*., 2009, Sakai *et al*., 2010).

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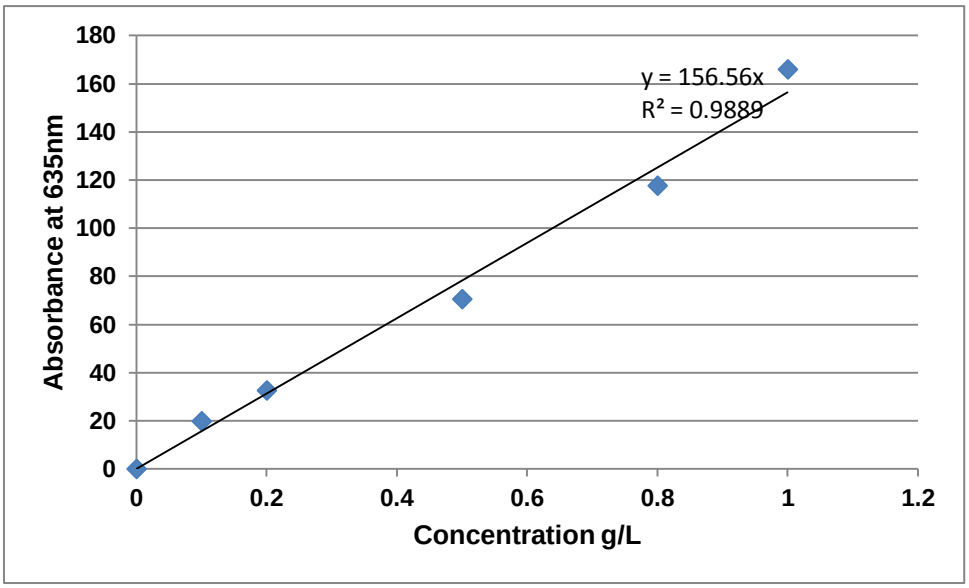
Appendix

1. Standard curve for nitrogen estimation.



Conference attended: 22 European conference at Birmingham for dental material (EDMC) (29th and 30th August 2013) – National conference (Poster Presentation).

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