Research was carried out to inform the scientific society with new knowledge or discovery. However, it is not to be expected that everybody would willingly believe what we tackle in our whole research work. Thus, to make our research more convincing we support it with other works which have spoken about the same topic that we have for our research. This is where literature review comes in. Literature review establishes a clear tie between the works that we have cited and the topic that we have written about. Literature review justifies the reason for research and allows establishing our theoretical framework and methodological focus. It acts as a springboard for the whole thesis and shows the originality and relevance of our research problem. It is a critical discussion and summary of literature that is of ‘general’ and ‘specialized’ relevance to our particular area and topic of the research problem.

This chapter will present an overview of the previous work carried out by different researchers and knowledge about the PHA producing bacteria, their diversity, inexpensive carbon sources for the culture of biopolymer producing bacteria, PHAs biodegradability and their application in different areas. The chapter is divided in following sections to cover all the aspects.

PHAs are polyesters of various hydroxyalkanoates accumulated in the form of intracellular granules by a large variety of bacteria when, bacterial growth is limited by depletion of nitrogen, phosphorus\textsuperscript{136} or oxygen and an excess amount of carbon source. If the general physiological fitness of bacteria is not affected they can store excess nutrients inside their cells and by polymerizing soluble intermediates into insoluble molecules and the cells do not undergo alterations in its osmotic state. Peters and Rehm\textsuperscript{43} have reported the prevention of leakage of these PHA compounds from bacterial cells thereby allowing the availability of the nutrient stores at a low maintenance.

2.1 Screening of PHA production in bacteria

According to Byrom\textsuperscript{51} about 8-13 granules per cell having diameter of 0.2-0.5 \(\mu m\) were observed in \textit{A. eutrophus}. These granules appear as highly refractive inclusion bodies under electron microscope. Simbert\textsuperscript{137} has reported the presence of PHB granules in bacterial cells which can be detected by staining with Sudan Black B. However, Ostle and Holt\textsuperscript{109} advocated the use of Nile Blue A, a water soluble
basic oxazine dye that has a greater affinity and higher specificity than Sudan Black B for PHB, and that gives a bright orange fluorescence at a wavelength of 460 nm, as other inclusion bodies do not stain with Nile Blue A, thereby emphasizing its usefulness. The oxazine dye Nile blue A and its fluorescent oxazone form, Nile red, were used to develop a simple and highly sensitive staining method to detect PHB and other PHAs directly in growing bacterial colonies. PHB granules of Nile red-stained living cells of Caryophanon latum at the early stage of PHB accumulation were frequently found at or close to the cytoplasmic membrane which were determined by confocal laser scanning fluorescence microscopy. The identity of polyhydroxyalkanoates (PHA) storing bacteria selected under aerobic dynamic feeding conditions, using propionate as carbon source, was determined by applying reverse transcriptase–polymerase chain reaction (RT-PCR) on micromanipulated cells and confirmed by fluorescence in situ hybridization (FISH).

2.2 PHA production in specific bacteria

Production of PHA has been reported by numerous bacteria. R. eutropha is by far the most extensively studied bacteria used for PHB and PHBV production. Vaneechoutte reported that R. eutropha has the ability to produce PHB with the use of simple carbon source. R. eutropha NCIMB 11599 can produce 121 gL\(^{-1}\) PHB under controlled glucose and nitrogen limitation conditions in fed-batch culture. Mixtures of glucose, propionic acid and either 4-hydroxybutyric acid or β-butyrolactone as carbon sources in fed-batch cultures of R. eutropha led to production of a P (3HB-4HB-3HV) terpolymer. Volova reported R. eutropha B8562 produced PHAs up to 90% of the cell dry weight by using different carbon sources (CO\(_2\), fructose and glucose).

By the virtue of their ability to use methanol as a carbon substrate, Methyllobacterium species are of interest for the production of PHB. M. rhodesianum, M. extorquens, M. organophilum, M. rhodenium, M. zatmanii, M. radiotolerans, Mycoplana rubra, Paracoccus denitrificans and Proteomonas extorquens are some of the bacterial species which uses methanol as a carbon source for PHB accumulation. Bourque reported M. extorquens accumulated 30% (CDW) of P (3HB) with a molecular mass of 250 kDa with methanol concentration of 1.7 gL\(^{-1}\), and addition of complex nitrogen source.
P. putida can efficiently incorporate monomers in the range of C₈–C₁₀ during PHA synthesis. De Smet⁵⁴ observed the presence of intracellular granules consisting of poly (3-hydroxyoctanoate) in P. oleovorans strain ATCC29347 grown in two-phase medium containing 50% v/v octane. Haywood¹⁵⁰ examined various Pseudomonas spp. for growth and polyester accumulation with C₆–C₁₀ straight-chain alkanes, alcohols and alkanoic acid as the sole carbon source. The accumulation of PHAs containing mcl-3HB (C₆–C₁₂), but not 3-HB, appears to be a characteristic of the fluorescent Pseudomonas spp. to accumulate these PHAs¹⁵¹. Several studies have shown that P. putida and P. aeruginosa strains are able to convert acetyl-CoA to medium-chain-length monomers for PHA synthesis¹⁵². Ayub¹⁵³ reported that Pseudomonas spp. 14-3 strain accumulated large quantities of PHB when grown on octanoate isolated from Antarctic environment. This isolate was characterized on the basis of phenotypic features and partial sequencing of its 16s ribosomal RNA gene. Pseudomonas spp. 14-3 showed increased tolerance to both thermal and oxidative stress.

Other important strains recently studied include- Bacillus spp., Alcaligenes spp., Rhodopseudomonas palustris, E. coli, Burkholderia spp. and Halomonas boliviensis.

A number of Bacillus spp. has been reported to accumulate 9-44% dry cell weight of PHB. By comparison, B. mycoides RLJ B-017 contained 69.4±0.4% dry cell weight PHB. Therefore, this strain has been considered as a potent organism for industrial interest¹⁵⁴. The tropical marine and mangrove microflora from the mid-west coast of India possessed a high potential to accumulate important polymers such as PHA. Among these isolates, seven cultures accumulated more than 1 gm of PHA per litre of culture broth¹⁵⁵. Tajima¹⁵⁶ reported the isolation of a PHA accumulating gram-positive bacterium Bacillus sp. INT005 from gas field soil. The species was identified by comparing its morphological and physiological properties and partial nucleotide sequence (500 bp) of its 16s rRNA. Construction of recombinant B. subtilis was also done for the production of PHA copolymer¹⁵⁷.

Lakhman¹⁵⁸ reported that strains of Rhizobium spp. isolated from leguminous plants and standard strains accumulated 27% to 57% PHA of their cell biomass while using sucrose as a sole carbon source. Van-Thuoc¹⁵⁹ produced PHB by
utilizing *H. boliviensis* culturing in cheap and readily available agro-residue as cheap carbon source in order to reduce the cost of production.

The use of recombinant *E. coli* as PHA producer has drawn tremendous scientific attention as it is genetically well characterized. *E. coli* is not being a natural PHA accumulator; its production has to be metabolically engineered. Moreover it does not have any depolymerase activity to degrade the accumulated PHA. Although *R. eutropha* produces high levels of P (3HB), but they have certain limitations which impedes genetic manipulations. Therefore, the expression of PHA biosynthetic genes of *R. eutropha* in *E. coli* for P (3HB) synthesis opened up the possibility for PHA production by recombinant organisms\(^\text{160,161,162}\). Genetically and metabolically engineered *E. coli* can synthesize a variety of polymers, such as P (3HB-3HV), P (3HB-4HB), P (4HB) and P (3HO-3HH). Expression of *P. aeruginosa* PHA synthases, phaC1 and phaC2 in *E. coli* fadB mutant resulted in msc-PHA accumulation when grown in presence of C\(_8\)-C\(_{14}\) fatty acids\(^\text{163, 164}\). Recombinant *E. coli* DH5\(\alpha\) (pQKZ103) has been shown to accumulate PHB up to 85% of the cell dry weight when cultured in minimal glucose medium\(^\text{165}\). PHA synthase gene (pha C1) from indigenous *Pseudomonas* spp. LDC-5 was amplified by PCR and cloned in *E. coli* which is a potential candidate for the large-scale production of polymer\(^\text{166}\).

### 2.3 Production of PHA in higher organisms

#### 2.3.1 Yeasts

PHA production from yeast is still in its infantile stage. Only a few reports of PHA production by different metabolically engineered yeasts are available\(^\text{167, 168}\). Poirier\(^\text{169}\) reported transformation of PHA synthase from *P. aeruginosa* modified at the carboxy-end for peroxisomal targeting in methylotrophic yeast *Pichia pastoris*. The PHA synthase was expressed under the control of the promoter of *P. pastoris* acyl-CoA oxidase gene and accumulated up to 1% medium-chain-length PHA per g dry weight as inclusions within the peroxisomes. Buelhmann\(^\text{170}\) reported production of PHAs in a transgenic yeast *Saccharomyces pombe* harboring the PHB synthesis genes encoding β-ketothiolase (phbARe), acetoacetyl-CoA reductase (phbBRe) and PHB synthase (phbCRe) of *R. eutropha*. The transgenic yeast accumulated about 9%
(w/w) of PHB under optimized conditions. Abu-Elreesh\textsuperscript{167} reported PHA accumulation of about 7% (w/w) by metabolically engineered *Kloeckera* yeast cells.

### 2.3.2 Insects

Williams *et al.*\textsuperscript{171} reported a novel pathway for the synthesis of P-3HB which was engineered by simultaneous delivery of two genes into insect cells *Spodoptera frugiperda* by use of individual baculovirus vectors. This system includes expression of a dehydrase-domain mutant rat fatty acid synthase cDNA and the *phbC* gene encoding polyhydroxyalkanoate synthase from *A. eutrophus*. Approximately 1 mg of PHB was isolated from a one-litre culture of these cells corresponding to 0.16% of cell dry weight.

### 2.3.3 Plants

Plants are capable of producing significant amounts of numerous useful chemicals at a low cost, when compared to bacteria or yeast\textsuperscript{172}. Commercialization of plant derived PHA will require the creation of transgenic crop plants with the addition of high product yields\textsuperscript{173}. Transgenic plants can produce PHAs from photosynthetically fixed CO\textsubscript{2} and water which after disposal degrade back to CO\textsubscript{2} and water. Therefore, it might be possible to produce PHA at a similar low costs which are comparable to those of other biopolymers already obtained from plants. *Arabidopsis thaliana* was the first targeted model for transgenic studies in plants\textsuperscript{37}. Poirier\textsuperscript{174} reported synthesis of PHA in plants which was initially explored by the expression of PHA biosynthetic genes of the bacterium *R. eutropha* in the well-studied plant *A. thaliana*. But scanty (0.1%) accumulation of PHB was achieved from the plant. Recently all three genes necessary for PHB biosynthesis were transformed to *A. thaliana* in a single transformation event\textsuperscript{175}. These plants accumulated more than 4% of their fresh weight (approximately 40% of their dry weight) of PHB in leaf chloroplasts. John and Keller\textsuperscript{176} reported the novel perspective on the use of PHA synthesis in cotton fiber cells by the expression of the *R. eutropha* PHB biosynthetic pathway. Analysis of the transgenic fibers showed PHB accumulation up to 0.3% of dry weight exhibiting better insulating properties. Moreover PHA biosynthetic genes also have been expressed in some agricultural crops such as *Brassica napus*, *Gossypium hirsutum*, *Nicotiana tabacum*, *Solanum tuberosum* and *Zea mays*\textsuperscript{176, 177, 178, 179, 29}. By transforming threonine deaminase gene
from *E. coli* and PHB biosynthetic genes from *R. eutropha*, PHBV were produced by engineered Arabidopsis and Brassica plants in their leaves and seeds respectively. An optimized genetic construct for plastid transformation of tobacco plant *Nicotiana tabacum* for the production of polyhydroxybutyrate (PHB) was reported by Bohmert-Tatarev *et al.*. This plant has been designed using an operon extension strategy and bacterial genes encoding the PHB pathway enzymes were selected for use in this construct based on their similarity to the codon usage and GC content of the tobacco plastome.

### 2.4 Fermentation process

Fermentation strategies for the production of high concentration of P (3-HB-co-3-HV) with different 3-HV fractions by recombinant *E. coli* harboring *A. latus* PHA biosynthesis genes were developed. *A. latus* when transformed with its own cloned *phaC* gene, exhibited increase in PHB synthesis as well as increase in PHB content. The recombinant *A. latus* synthesized maximum concentration from 3.1-3.7 gL\(^{-1}\) and content of PHB from 50.2-65% of cell dry weight, respectively, as compared to the untransformed *A. latus*.

PHA-producing bacteria are classified into two groups based on culture conditions required for efficient PHA synthesis. One group requires the limitation of an essential element such as N, P, Mg, O, or S for the efficient synthesis of PHA and the other group requires no nutrient limitation. Both the groups can accumulate polymer accumulation during growth. Therefore, these characteristics should be considered in developing culture methods for the efficient production of PHAs. Either fed-batch or continuous culture techniques can be used for the production of PHA with high productivity. An initial growth phase in nutrient enriched medium yields sufficient biomass, followed by a product formation phase in nitrogen-depleted medium. Single fed-batch fermentation that is nitrogen limited leads to low amounts of polymer, because there is not enough accumulation of biomass.

Patnaik reported the use of open mixed cultures, such as activated sludge which can contribute to decrease the cost of PHAs and therefore increase their commercial potential. A stable methane-utilizing mixed bacterial culture was used for the development of viable large-scale production of PHB using cheap substrates like methane from natural or renewable sources in an open system. PHB content
could be increased to 87% by applying nitrogen limitation in fed-batch culture, which was considerably higher than that of typically obtainable 50% under nitrogen-sufficient conditions\textsuperscript{185}. Greater research capabilities are needed to investigate whether continuous culture can truly give higher productivity than fed-batch cultures, without any process problems, such as, culture instability and contamination. Kang\textsuperscript{165} developed a stress-induced system by which the PHB biosynthesis pathways can be induced under stress conditions in which fermentation results showed that recombinant \textit{E. coli} DH5\(\alpha\) (pQKZ103) harboring this system was able to accumulate polyhydroxybutyrate up to 85.8\% of cell dry weight in minimal glucose medium without adding any inducer.

Currently PHA production processes based on mixed microbial cultures (MMC) are being investigated as a possible technology to decrease production costs, as no sterilization is required and bacteria can adapt quite well to the complex substrate present in low-cost substrates. In spite of this ‘feast and famine’ or aerobic dynamic feeding (ADF) is developed which is based on the supply of a short period of excess carbon (feast) followed by a long period of starvation\textsuperscript{186}. Serafim\textsuperscript{187} showed that the intracellular PHA content reached 65.4\% cell dry weight under ADF conditions, with carbon addition (acetate) by sequential pulses.

In the bacterial cell, carbon substrates are metabolized by 3 different pathways. Pathway I, which generates PHB homopolymer from acetyl-CoA processed from sugars and has been found extensively in a wide range of bacteria like \textit{R. eutropha}. Pathways II and III, which generates mainly mcl-(\textit{R})-3HA monomers from fatty acid β-oxidation intermediates and fatty acid biosynthesis intermediates,\textsuperscript{188,189} respectively, have been found in various fluorescent \textit{P. sp. Xi} \textit{et al.}\textsuperscript{190} reported the \textit{P. stutzeri} strain 1317 was found to produce PHA up to 77\% of cell dry weight when grow on various fatty acids, alcohols, diols as well as glucose and gluconate. Impallomeni \textit{et al.}\textsuperscript{191} reported that \textit{P. aeruginosa} ATCC 27853 synthesize random co-polyhydroxyalkanoates (co-PHAs) using Tween 20 as the sole carbon source although it is a mixed carbon source. Tween 20 and its three major fatty acids support both cellular growth and PHA production and its emulsifying and solubilizing properties seems to facilitate its use as carbon source by cells, leading to production of the highest polymer yield. Moreover it could provide fatty acids substrates at lower cost than that of purified fatty acids. Halami\textsuperscript{192} reported a native
Chapter 2: Review of Literature

strain *B. cereus* CFR06 which produces amylase and polyhydroxyalkanoate (PHA) (48% CDW) from the starch containing medium.

Plant oils or their derived fatty acids are good carbon sources for the production of PHA as they are inexpensive renewable carbon sources. A large-scale production of P (3-HB-co-3-Hx) from lauric acid was carried out using *Aeromonas hydrophila* with a final PHA content of 50%.

Different kinds of agricultural products like starch and wastes like beet and cane molasses, wheat bran, malt waste, and dairy wastes like cheese whey with or without nitrogen supplement have been used as raw materials for PHA production. Liu et al. reported that recombinant *E. coli* strain was capable of producing 39.5 gL⁻¹h⁻¹ PHB using molasses as the carbon source. *B. megaterium* was grown on various carbon sources such as date syrup and beet molasses. Currently efforts are being made to grow bacteria on different renewable vegetable oils and various waste products. The use of these inexpensive carbon sources to produce PHAs could lead to significant economical advantages. Huang et al. reported *Haloferax mediterranei*, an archaean which could produce a PHA content of 55.6 wt.% and 38.7 wt.% by a repeated fed-batch fermentation process using low-cost raw materials like extruded rice bran (ERB) and extruded cornstarch (ECS) respectively. *P. putida* can produce medium chain length PHA (28 gL⁻¹) using corn oil hydrolysate (an inexpensive and renewable carbon source) in fed-batch culture.

Moreover novel processes have been investigated to produce PHAs from organic wastes in wastewater, industrial wastes and municipal wastes. Different wastes containing volatile fatty acids like palm oil mill effluent, banana pseudostem, damaged food grains, pea shells, apple pomace are used as carbon sources. A large number of bacteria like *A. eutrophus, B. megaterium, P. oleovorans, Azotobacter* sp, *Beijerinckia* sp, *Rhizobium* sp, *Nocardia* sp utilize this waste as substrates for PHA production. Production of PHAs from organic wastes can provide multiple benefits to the environment and promote sustainable development. But Lee and Yu reported that PHA-producing microbes like *R. eutropha* cannot directly utilize organic wastes as they are usually in the complex form. So, the first step to overcome this problem is hydrolysis and acidogenesis of
the wastes producing short-chain volatile fatty acids such as acetic, propionic and butyric acids that can be used by *R. eutropha* for synthesis of P (HB-co-HV)\(^{52}\).

CO\(_2\) in the atmosphere is the ultimate feedstock for PHA production. Some wild-type cyanobacteria are capable of accumulating small amounts of P (3-HB) (approx 6%) in the cells from CO\(_2\). Ishizaki *et al*\(^{212}\) reported that *R. eutropha* can assimilate CO\(_2\) and produce P (3-HB) in the absence of light energy, but with oxidization of hydrogen. It has been also reported by Volova *et al*\(^{213}\) that the carbon monoxide (CO)-resistant strain *R. eutropha* B5786 is able to synthesize PHAs upto 70-75% in the presence of CO under autotrophic conditions. *Cupriavidus necator* H16 can metabolize a mixture of H and CO\(_2\) to form PHAs\(^{214}\).

The use of methanol as a carbon substrate is significant because it is a cheap carbon source and can significantly reduce the production costs of PHB. It can also be considered as a renewable substrate since it could be derived from woody materials or from natural gas obtained after anaerobic digestion of organic substances. A new bacterial strain *Methylobacterium* sp. strain GW2 isolated from groundwater was found to be capable of producing the homopolymer P-3HB from various carbon sources such as methanol, ethanol, and succinate. Yezza *et al*\(^{215}\) reported that the bacterium *Methylobacterium* GW2 showed best PHB production (40% w/w dry biomass) in methanol containing medium.

### 2.5 Recovery of PHA

In addition to the cost of maintaining pure cultures and the high cost of organic substrates, the recovery of PHA contributes significantly to the production cost of the polymer. In the past two decades, a number of economic methods have been suggested for recovery of purified PHA. After fermentation, bacterial cells containing PHAs are separated from the medium by centrifugation, washed, dried and finally disrupted to recover the polymer. Most of the methods to recover intracellular PHA involve chloroform, methylene chloride, propylene carbonate and dichloroethane which can result in purified PHA\(^{216}\). Chen and Wu\(^{12}\) reported that solvent extraction is a good method for medical application as it gives higher percentage of purified PHAs.

Jiang *et al.*\(^{217}\) reported the use of cheaper and less toxic solvents such as hexane, acetone and dimethylcarbonate for the PHA recovery process. The extracted
polymer solution containing more than 5% (w/v) P (3HB) is very viscous and the removal of cell debris is difficult. Additionally, the process needs large quantities of toxic and volatile solvents, which increases the total production cost despite being hazardous to the environment\textsuperscript{218}.

Digestion using sodium hypochlorite has been proposed as an alternative to the unfavorable extraction with organic solvents\textsuperscript{116}. Even though this method is effective in digestion, it causes severe degradation of PHA. Surfactant pretreatment and hypochlorite digestion under optimized conditions results in pure isolation of P (3HB) with less degradation and improved molecular weight\textsuperscript{219, 220}. An enzymatic digestion method developed by Zeneca has been used for the production of Biopol, but the use of expensive chemicals and complex processes does not seem to be economically feasible.

Fidler and Dennis\textsuperscript{221} reported a system for PHB recovered from \textit{E. coli} cells by expressing T7 bacteriophage lysozyme gene. In this system, the lysozyme penetrated and disrupted the bacterial cells resulting PHB granules to be released. Choi and Lee\textsuperscript{222} reported a simple alkaline digestion method for the recovery of P (3HB) from the recombinant \textit{E. coli} cells. Recombinant \textit{E. coli} cells having a P (3HB) content of 77% when treated with 0.2 M NaOH for 1 h, recovers P (3HB) of 98.5% purity\textsuperscript{223}. Hampson and Ashby\textsuperscript{224} developed a simple two-step process to extract and purify mcl-PHAs from \textit{P. resinovorans} cells. In this method supercritical fluid extraction (SFE) of the lyophilized cells was done using CO\textsubscript{2} to remove lipid impurities, followed by chloroform extraction of the cells. By this method a maximum of 42.4% mcl-PHA was obtained which designate that this process saves time, uses much less organic solvent, and produces a pure mcl-PHA biopolymer than previous extraction and purification methods.

In the present scenario, the states of art technology for PHA recovery are supercritical fluid disruption\textsuperscript{225} dissolved air floatation\textsuperscript{226} and selective dissolution of cell mass\textsuperscript{227} has been established. These results highlighted the importance of developing an economical and efficient recovery method.

In most of the organisms so far investigated, the PHB is synthesized from acetyl-coenzyme A (acetyl-CoA) by a sequence of three reactions catalyzed by three biosynthetic enzymes. In the first step, 3-ketothiolase (Pha A) combines two molecules of acetyl-CoA to form acetoacetyl-CoA. Acetoacetyl-CoA reductase (Pha
B) allows the reduction of acetoacetyl-CoA by NADH to 3-hydroxybutyryl-CoA. Finally, PHB synthase (Pha C) polymerizes 3-hydroxybutyryl-CoA to PHB and coenzymeA being liberated (Fig.1.5). During the normal bacterial growth, the 3-kitothiolase will be inhibited by free coenzyme-A coming out of the Krebs cycle. But, when entry of actyl-CoA into Krebs cycle is restricted, the surplus acetyl-Co A is channeled in to PHB biosynthese. In *Rhodospirillum rubrum*, two stereospecific enoyl-Co A hydratases are also involved; these enzymes catalyze the conversion of L-(+)-3-hydroxybutyryl-Co A via crotonyl-Co A to D-(-)-3-hydroxybutyryl-Co A, which is polymerized to yield PHB.

### 2.6 Chemical structure of PHAs

Although the microbial origin, structure and physicochemical properties of PHAs is quite variable (Fig. 1.1), most PHAs could be grouped in to three. The repeat units of scl-PHAs are composed of hydroxyfatty acids. The pendant group (R) varies from methyl (C\(_1\)) to tridecyl (C\(_{13}\)). Fatty acids with the hydroxyl group at position 4, 5 or 6 and pendant groups containing substituents or unsaturations are also known. Within bacterial metabolism, carbon substrates are converted to hydroxyacyl-CoA thioesters. In all PHAs that have been characterized so far, the hydroxyl-substituted carbon atom is of the stereochemical (R) configuration. Haywood *et al.* and Williams *et al.* reported that a few bacteria are known to synthesize scl-PHAs containing monomers other than 3HB when grown on simple sugar. It is suggested by Sato *et al.* that the most common PHB homopolymers, synthesized by bacteria, always contain less than 1 mol of 3-HV monomers. Steinbuchel reported that some *Pseudomonas* spp have been reported to accumulate PHA copolymers containing mcl monomer. The composition of PHA produced is usually related to the substrate used for growth mostly 2\(_n\) carbon shorter than the substrate used. Copolymers of PHB are formed when mixed substrates are used, such as, a mixture of glucose and valerate and convert the carbon substrates to PHBV or PHB4B. Pederson *et al.* reported that when substrates were alternated overtime, it was possible to obtain PHA block copolymers synthesized by bacteria. Labuzek and Radecka reported that gram–positive *B. cereus* UW85 strain could produce tercopolymers by using 3-HB, 3-HV and 6-hydroxyhexanoate units’ ε-caprolactone, or ε-caprolactone and glucose as carbon
source. *R. eutropha* synthesized a copolymer of 3-hydroxybutyrate and 3-mercaptpropionate, poly (3-HB-co-3MP) containing sulfur in the backbone, when 3-mercaptpropionic acid or 3, 3'-thiodipropionic acid was provided as carbon source in addition to fructose or gluconic acid under nitrogen-limited growth conditions\(^{237}\).

But in contrast to the normal production of copolymer when using mixed substrates by the bacteria, the strain *P. nitroreducens* isolated from oil-contaminated soil demonstrated some unusual ability to synthesize PHB homopolymer from medium-chain-length (mcl) fatty acids including hexanoate and octanoate\(^{238}\).

The surface of a PHA granule is coated with a layer of phospholipids and proteins. Phasins, a class of predominant protein of the PHA granule influence the number and size of it\(^ {239}, 240\). Karen *et al.*\(^ {241}\) has reported that *P. putida* strain CA-3 accumulates polyphosphate (poly P) and it appears that poly P is not the rate-limiting step for mcl-PHA accumulation in *Pseudomonas* strains.

### 2.7 Physical properties of PHAs

Among all the reported biopolymers, PHB are the most extensively studied polymer and as such, the properties of PHAs have been elucidated by taking it into consideration\(^ {242}\). The weight average molecular weight (Mw) of scl-PHAs, like PHB produced from wild-type bacteria is usually in the range of 1x10\(^4\) to 4x10\(^6\) Da with a polydispersity of around 2\(^ {243}, 244\). But according to Valappil *et al.*\(^ {245}\), the Mw in mcl-PHAs, lies between the range of 60,000 and 412,000. The polydispersity index value of scl-PHAs to be around 1.75, while in mcl-PHA copolymers the polydispersities are in the range of 1.6 to 4.4\(^ {245}\).

The family of PHAs exhibits a wide variety of mechanical properties from hard crystalline to elastic, depending on composition of monomer units which extends its application. Scl-PHAs like P (3HB) homopolymer are highly crystalline, stiff, and brittle material\(^ {127}, 246\). When spun into fibres it behaves as a hard-elastic material\(^ {247}, 248\). The glass transition temperature (Tg) and melting temperature (Tm) is 180 °C and 4 °C, respectively. These polymers become fluid and can be moulded above their melting temperature. Mechanical properties like Young’s modulus (3.5 GPa) and tensile strength (40 MPa) are close to that of polypropylene, although the elongation to break is about 5%, which is significantly lower than that of
polypropylene, 400%\(^{36}\). Ashby et al.\(^{249}\) also reported that the film of mcl PHAs synthesized by \(P.\) \textit{resinovorans} from coconut oil, tallow and soybean oil can be improved when subjected to 50 kGy of \(\gamma\)-irradiation. This resulted in the formation of crosslink based on the number of olefinic groups present in the polymer side-chains and improved the tensile strength (104% and 63%), percent elongation (49% and 13%), and Young’s modulus (30% and 76%) of the polymer film.

The mcl-PHAs have their mechanical properties similar to that of PHB, but they are less stiff and brittle. Khanna and Srivastava\(^{49}\) reported that the physical and thermal properties can be regulated by varying the copolymer compositions. Mcl-PHAs are semi-crystalline elastomers due to their low melting point (40-60 °C), low glass transition temperatures (-50 to-25 °C). Sanchez \textit{et al.}\(^{250}\) reported mcl-PHAs also have low crystallinity due to the presence of large and irregular pendant side groups. In case of 3HV copolymer, the copolymer becomes tougher and more flexible with the increase of 3HV unit. However, the melting temperature becomes decreasing and elongation to break becomes increasing with increasing 3HV fraction\(^{49}\). Gunaratne and Shanks\(^{251}\) reported the melting behavior and crystallization of PHAs which showed multiple melting peak behavior and melting-recrystallization-remelting. Carrasco \textit{et al.}\(^{252}\) reported that the thermal biodegradation of PHB (Biopol) starts at 246.3 °C, while the value for PHBV (Biopol) is 260.4 °C as the side chain increases in the later which in turn increases the thermal stability. This is due to the presence of valerate in the polymer chain which increases the thermal stability of the polymer. Introduction of co-monomers other than 3HV into PHB chain also give copolymers of improved mechanical properties. The physical properties of some of polymers are compared with polypropylene (PP) and polystyrene in Table 2.1
Table 2.1 Physical properties of some polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Melting point (°C)</th>
<th>Glass-transition temp (°C)</th>
<th>Crystalline (%)</th>
<th>Young’s modulus (GPa)</th>
<th>Tensile strength (MPa)</th>
<th>Elongation to break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P (3HB)</td>
<td>179</td>
<td>4</td>
<td>80</td>
<td>3.5</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td>P (4HB)</td>
<td>53</td>
<td>-</td>
<td>-</td>
<td>149</td>
<td>104</td>
<td>1000</td>
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<tr>
<td>P (3HB-co-3HV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mol% 3HV</td>
<td>170</td>
<td>-</td>
<td>2.9</td>
<td>38</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>9 mol% 3HV</td>
<td>162</td>
<td>-</td>
<td>1.9</td>
<td>37</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>14 mol% 3HV</td>
<td>150</td>
<td>-</td>
<td>1.5</td>
<td>35</td>
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<td>-</td>
<td>1.2</td>
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<tr>
<td>25 mol% 3HV</td>
<td>137</td>
<td>-1</td>
<td>40</td>
<td>0.7</td>
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<th>Polymer</th>
<th>Melting point (°C)</th>
<th>Glass-transition temp (°C)</th>
<th>Crystalline (%)</th>
<th>Young’s modulus (GPa)</th>
<th>Tensile strength (MPa)</th>
<th>Elongation to break (%)</th>
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<tr>
<td>P (3HB-co-4HB)</td>
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<tr>
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<td>-</td>
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<td>159</td>
<td>-</td>
<td>-</td>
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<td>30</td>
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<td>100</td>
<td>65</td>
<td>1080</td>
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<tr>
<td>P(3HB-10%3HHx)</td>
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<td>-1</td>
<td>34</td>
<td>-</td>
<td>21</td>
<td>400</td>
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<tr>
<td>Polypropylene (PP)</td>
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<td>Polystyrene</td>
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<td>21</td>
<td>3.1</td>
<td>50</td>
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Data adapted from Poirier et al.\textsuperscript{24} , Lee\textsuperscript{47} , Tsuge\textsuperscript{57} , Khanna and Srivastava\textsuperscript{49} and Verlinden et al.\textsuperscript{248} .
2.8 Biodegradation of PHA

The stereochemistry of PHA fits well with its biodegradability. They are fully biodegradable and innocuous to the environment. Jendrossek et al.\textsuperscript{253} reported that these degradations enable \( \text{CO}_2 \) and organic compound recycling in the ecosystem which provides a buffer to climate change. The polymers can degrade both under aerobic and anaerobic conditions. They can also be subjected to thermal degradation and enzymatic hydrolysis.

Different factors like stereoregularity, molecular mass, monomeric composition and crystallinity are accountable for the biodegradability of PHAs. Mochizuki \textit{et al.}\textsuperscript{254} and Tokiwa \textit{et al.}\textsuperscript{255} reported that biodegradation of PHAs is influenced by the chemical structure i.e. presence of functional groups in the polymer chain. Nishida and Tokiwa\textsuperscript{256} described that the development of crystallinity evidently depresses the microbial degradability of PHB. Moreover, Boopathy\textsuperscript{257} has reported that the rate of polymer also depends on a variety of factors including surface area, microbial activity of the disposal environment, \( \text{pH} \), temperature, moisture and presence of other nutrient materials. Wang \textit{et al.}\textsuperscript{258} reported that the rate of degradation of PHAs depend upon the crystallinity and surface area of the polymer. Surface morphology affects degradation by facilitating contact between water, enzyme molecules or bacteria and polymer chains. When exposed to hydrolysis, the degradation starts on the surface and at physical lesions on the polymer and proceeds to the inner part of the material.

Any research on the biodegradation of PHA should clearly distinguish between intracellular and extracellular PHA degradation. Extracellular degradation is the utilization of an exogenous polymer by a not-necessarily accumulating microorganism that secretes extracellular PHA depolymerases. The source of extracellular polymer is PHA released by accumulating cells after their death and cell lysis. A number of microorganisms excrete PHA depolymerases to hydrolyze the ester bonds of a polymer into water-soluble monomers and oligomers small enough to be transported into a microbial cell and metabolized to \( \text{CO}_2 \) and water\textsuperscript{259, 260}.

Degradation of PHA polymer by different gram positive, gram negative bacteria and some methanogenic co cultures were reported by different researchers\textsuperscript{261, 262, 32}. Mabrouk and Sabry\textsuperscript{263} reported that a marine \textit{Streptomyces} sp.
SNG9 utilize PHB and its copolymer P (3HB-co-HV) as the sole carbon source and degraded the polymer particles in 4 days. Many microorganisms like *P. lemoignei*, *P. pseudomallei*, Acidovorax facilis, *A. delafildii*, Comamonas testosteroni, Variovorax paradoxus, Zoogloea ramigera, and Bacillus sp., as well as *Streptomyces* are able to degrade P (3HB) extracellularly\(^{264}\). Colak and Guner\(^{265}\) reported that three *Pseudomonas* sp. namely *P. fluorescens*, *P. aeruginosa* and *P. putida* were isolated from fuel oil contaminated soil to investigate the biodegradation of PHAs where morphological changes in the polymer were observed by the help of scanning electron microscope (SEM). Phithakrotchanakoon *et al.*\(^{266}\) reported that a thermophilic *Streptomyces* sp BCC23167 isolated from a landfill site is capable of degrading various aliphatic polyesters including polyhydroxyalkanoate copolymers, poly(ε-caprolactone) and polybutylene succinate at 50 °C and neutral pH. Another soil bacterium Actinomadura sp. AF-555 has also the potential to degrade P-3HB-co-HV when exposed to soil\(^{267}\).

Conversely, intracellular degradation is the active degradation of an endogenous storage reservoir by the accumulating bacterium itself. Enzymes catalyzing the intracellular degradation of PHA are intracellular PHA depolymerases. In case of intracellular degradation, PHAs are degraded by some PHA producing bacteria intracellularly. In this case, the polymer is broken down to acetyl-CoA which under the aerobic conditions enters the citric acid cycle and is oxidised to CO\(_2\)\(^{36, 268}\). Knoll *et al.*\(^{269}\) reported that the PHA depolymerase enzyme phaZ is involved in the degradation of the PHAs.

Although the anaerobic degradation of PHAs has not been well documented yet, it has been recommended that PHAs can be degraded in an anaerobic environment such as sewage sludge. It was reported that P (3HB) and the copolymer P (3HB-co-3HV) were fermented (upto 83-96%) to methane and CO\(_2\) within 16 days by using anaerobically digested domestic sewage sludge consortium\(^{261}\). Lee\(^{47}\) reported that P (HB-HV) does not degrade under normal conditions of storage as it is insoluble in water and stable in air.

PHAs can be composted over a wide range of temperatures, even at a maximum of around 60 °C. UV radiation also affects the degradation of bacterial biopolyester poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) and significant Mw losses were observed with UV radiation time\(^{131}\).
Since the fungal biomass in soils generally exceeds the bacterial biomass and thus it is demonstrated by Kim and Rhee\textsuperscript{270} that fungi may play a considerable role in degrading bacterial biopolymesters. Lee and Chang\textsuperscript{271} studied the degradation of PHB by fungi from samples collected from various environments and found clear zone below and around the fungal colony in the tested plates. Different fungi such as \textit{Penicillium}, \textit{Cephalosporium}, \textit{Paecilomyces}, and \textit{Trichoderma}\textsuperscript{272}, \textit{Ascomycetes}, \textit{Basidiomycetes}, \textit{Deuteromycetes}, \textit{Mastigiomycetes}, \textit{Myxomycetes}\textsuperscript{2}, \textit{Aspergillus fumigatus}\textsuperscript{273} have also been reported to degrade PHA. Fungal species \textit{Candida albicans} and \textit{Fusarium oxysporum} were reported for the biodegradation of mcl-PHA in our published paper\textsuperscript{274}.  

\textbf{2.9 PHA application}  

PHA has been drawing considerable attention as biocompatible plastics for a wide range of applications\textsuperscript{126}. A number of different companies are developing PHAs for use in the plastic industry. Currently, Metabolix (USA) and Kaeka (Japan) are among the startup companies actively teamed up for the commercialization of PHA biopolymers to a product called Nodax. Nodax has already been made into a variety of different prototype objects such as plastic fiber or twine and molded plastic ware. The extensive range of physical properties of PHA provides them a broad range of potential applications to mankind\textsuperscript{132, 133}.  

Bucci and Tavares\textsuperscript{275} reported that P (HB-HV) could be used for films, blow molded bottles, containers and as a creating agent on paper. Composites of bioplastics have already been used in electronic products, like mobile phones.  

PHA has the ability to chemically modify their functional groups as well as their biodegradability and biocompatibility properties, making them an attractive material in the biomedical field\textsuperscript{276}. PHAs are mainly composed of different chiral hydroxyacids that have potential as synthons for anticancer drugs, anti-HIV drugs, antibiotics and vitamins. In biomedical and tissue engineering applications, PHAs have mainly been involved for biodegradable implants. PHA together with hydroxyapatite (HA) finds application as bioactive and biodegradable composite in hard tissue replacement\textsuperscript{12}. Kilicay \textit{et al.}\textsuperscript{277} reported that a matrix of PHBHHx nanoparticles has been used to deliver antineoplastic agents to cancer cells. Certain target specific breast cancer cells have also been examined with PHB nanoparticles.
functionalized with tumor-specific ligand. Francis tested the P (3HB) microsphere for releasing the antibiotics gentamycin and tetracycline in vitro. The slow hydrolytic degradation inside the human body makes PHAs more advantageous for use in reconstructive surgery. According to Lee, the degradation product of PHB, D (-)-3-hydroxybutyrate has been detected in relatively large amount in human blood plasma. PHAs can also be used as starting material for other chemicals taking advantage of their uniform chirality. Sudesh et al. reported that the 4HB units of PHB compounds have been used in the treatment of alcohol withdrawal syndrome. Partial digestion of PHA and recombination with other polymers can be used to achieve specific properties. Degra Pol, a block–copolyesters urethane chemically synthesized from PHB-diol showed good biocompatibility. In experimental animals, P (3HBHHx) scaffolds have been assessed for use in eyelid reconstruction. Moreover PHA has been used in other biomedical applications, such as tablet formulations, surgical sutures, wound dressings, controlled release contraceptive devices.

In addition to biomedical applications, PHAs have diverse applications. Recent studies suggest that PHAs can be used as precursors for biofuels production. Hydrolysis of PHAs followed by methyl esterification provides energy containing 3-hydroxyalkanoates methyl esters comparable to that of bioethanol. Foster et al. reported that PHAs has potential role as pollution bioindicator in preliminary assessments of environmental health. Hiraishi and Khan reported that many PHAs used in the solid phase denitrification of water and waste water has several advantages over the conventional system supplemented with liquid organic substrate.

Bourbonnais and Marchessault reported the use of PHA latex in paper industry for surface coating of paper and as a sizing agent. In certain aquaculture applications PHAs have been found useful for controlling bacterial pathogens. PHAs have also been used as controlled-release agents for herbicides in agriculture which can potentially reduce the repeated need of herbicides on non target species.
Chapter 2: Review of Literature

In nano-composites

Silver nanoparticles (SNP) have attracted much attention because of their antibacterial properties. The dominant use of SNP is in cosmetics, antibacterial-water filters and also as drug carriers. But slurries of such particles tend to be unstable and therefore always need some biodegradable but stable polymer support. In this regard, use of a hydrophobic, biodegradable, renewable and non-cytotoxic biopolymer for stabilizing SNP particle would provide advantage for the synthesis, and transportation of the SNP colloids and their use in different biomedical applications. In the light of the above information, an experiment designed for stabilizing the colloidal solution of SNP by using bacterial polyhydroxyalkanoates was reported in our previous studies\(^2^{99}\). The hydrophobic nature of PHA would speed up the process of water filtration as water cannot access the surfaces and pores of these particles where SNP would be in touch with water. In cosmetic technology, the SNP–PHA might act as a consistent stabilizer and the stability may lead to increase in the duration of sun screen protection without adding excess of SNP.

Use in biosensors

Polyhydroxyalkanoates have also been used in the biosensors. Ma et al.\(^3^{00}\) reported that myoglobin immobilized in P (3HB) film provides a model for constructing a third generation \(\text{H}_2\text{O}_2\) biosensor. The side effects from the artemisinin class of medications are similar to the symptoms of malaria. A case of significant liver inflammation has been reported in association with prolonged use of a relatively high-dose of artemisinin for an unclear reason. In the treatment of severe malaria, parenteral artesunate have shown promising results by reducing mortality rate in South East Asian patients by 35% when compared to quinine. Therefore, it is very important to develop a simple, sensitive, fast, portable and reliable method for detection and quantification of artemisinin.

2.10 PCR based identification of the PHA biosynthetic genes

The genetic organization of PHA biosynthesis genes varies among PHA producing organisms. The type II of PHA biosynthetic genetic system consists of two PHA synthase genes (phaC1 and phaC2) separated by depolymerase phaZ gene. The type II system is commonly found in mcl-PHA producing \textit{Pseudomonads}.
Solaiman et al.\textsuperscript{301} reported a rapid and sensitive PCR procedure for the specific detection of phaC type genes using primer pair, I-179L and I-179R, based on the highly conserved sequences found in the coding regions of Pseudomonas phaC1 and phaC2 genes. Solaiman et al.\textsuperscript{302} has reported the presence of PHA biosynthesis gene locus, phaC1 in \textit{P. resinovorans} and this has subsequently been PCR cloned and expressed in \textit{E. coli}. Zhang et al.\textsuperscript{303} designed PCR-based cloning approach by using the highly conserved regions of PHA biosynthesis gene locus for cloning of PHA biosynthesis genes from \textit{Pseudomonads}. A semi-nested PCR method was opted by Solaiman\textsuperscript{304} for the specific and individual amplification of type II subgenomic fragments phaC1 and phaC2. The method was used to show that strains of \textit{Pseudomonas oleovorans} harbor different pha loci. Jamil et al.\textsuperscript{305} reported PCR base amplification of PHA polymerase genes C1 and C2 from chromosomal DNA. A portion of polymerase C1 and C2 genes of the pha operon was cloned and sequenced.