In the recent years, there have been increasing public concerns over the harmful effects of petrochemical-derived plastic materials in the environment. Petroleum-based plastics have been used for more than seventy years in a variety of industrial and day-to-day applications due to their versatility and durability (Ojumu and Solomon, 2004; Zinn et al., 2001). They are used in every manufacturing industry ranging from automobiles to medicine. The synthetic polyethylene, polyvinyl chloride and polystyrene having molecular weights from 50,000 to 1,000,000 Da are largely used in the manufacture of plastics because they can be easily molded into almost any desired shape including fibers and thin films (Madison and Huisman, 1999). They have high chemical resistance and are more or less elastic, hence popular in many durable, disposable goods and as packaging materials. Forty percent of the 75 billion pounds of plastics produced every year is discarded into landfills. Several hundred thousand tones of plastics are discarded into marine environments every year and accumulate in oceanic regions (Fig. 1.1) (Swift, 1993). Nature’s built-in mechanisms and self-regulation ability cannot tackle this novel pollutant since these are unfamiliar to it. Excessive molecular size seems to be mainly responsible for the resistance of these materials to biodegradation and their persistence in soil for a long time (Atlas, 1993). Community faces the difficulty in disposal of synthetic plastics as they are being xenobiotic. Incineration of plastics has been one option in dealing with non-degradable plastics, but other than being expensive it is also dangerous. Harmful chemicals like hydrogen chloride and hydrogen cyanide are released during incineration (Johnstone, 1990; Atlas, 1993). Synthetic plastic bear negative attributes including recalcitrance to biodegradation (Reddy et al., 2003), toxicity after incineration and massive waste accumulation into the landfills and the marine environment. Another option is recycling however this also presents some major
disadvantages, as it is difficult sorting the wide variety of plastics and there are also changes in the plastic material such that its further application becomes limited (Johnstone, 1990; Flechter, 1993).

Figure 1.1: Plastic waste accumulation in oceanic region

In response to the problems associated with the synthetic polymers, the public tendency, the scientific interest and the governments’ determination has worked in unison over the past two decades to support the development of a new class of plastics (Veeramanikandan, 2013). The challenge for the new polymers is that it has to retain the physico-chemical characteristics of the traditional plastics with additional benefit of biodegradibility. Making eco-friendly product such as bioplastic is one such reality that can help us to overcome the problem of pollution caused by non-degradable plastics. This has prompted many countries to start developing biodegradable plastics.

The polymer industry, however, is well aware of these problems, and significant research has been done and is continuing to develop biodegradable polymers (Steinbuchel, 2001). Many polymers were proposed and tested for their possible industrial applications and their biodegradability, e.g. cellulose, starch, blends of those with synthetic polymers, polylactate, polyester-amide, and polyhydroxyalkanoates (PHAs). PHAs gained particular interest since they were shown to be biodegradable and biocompatible (Brandl et al., 1990, 1995). Both
properties can best be achieved by production in bacteria, thus, guaranteeing complete stereospecificity, which is essential for their biodegradability and biocompatibility. It has also immense potential for medical applications and therefore, attracts increasing attention. The type of bacterium and growth conditions determine the chemical composition of PHAs and the molecular weight, which typically ranges from $2 \times 10^5$ to $3 \times 10^6$ Da (Byrom, 1987; Lee, 1996).

Unfortunately, the current production costs are much higher as compared to petroleum-based plastics (e.g., 1 kg of polyhydroxybutyrate was about US$ 15-10) (Witholt and Kessler, 1999). Thus, a widespread usage of this high-quality product as a bulk-packaging material cannot be expected in the near future. Researchers are working on cost effective production of PHA using renewable sources.
1.1 Historical outline

Poly(3-hydroxybutyrate) [P(3HB)] is the most common PHA and was first described by Lemoigne, a French scientist in year 1926 (Doi, 1990) (Fig. 1.2). This natural polyester remained unknown to a wider scientific community and it was revealed only when their discoverer, Maurice Lemoigne published his results in little-read French journal. Lemoigne and co-workers reported their PHB studies in 27 publications from 1923 until 1951, and in their later work they found that the cells of *Bacillus megaterium* could contain as much as 44% of their dry weight of PHB depending on growth conditions (Marchessault and Yu, 2002).

**Figure 1.2: Stages of PHA discovery (Sudesh et al., 2000)**
In 1974, Wallen and Rohwedder reported the discovery of other monomers such as 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HHx) and 3-hydroxyheptanoate (3HHp) as the major and minor constituents, beside 3HB from activated sewage sludge. In 1982, Imperial Chemical Industries Ltd. (ICI) in England announced a product with a trade named “Biopol”, a copolyester containing randomly arranged units of [R]-3-hydroxybutyrate, HB, and [R]-3-hydroxyvalerate, HV produced by *Alcaligenes eutrophus*, since renamed *Ralstonia eutropha* (more recently changed again to *Cupriavidus necator*). Successively in 1983, De Smet et al. identified new monomer 3-hydroxyoctanoate (3HO) with trace amount of 3HHx in *Pseudomonas oleovorans* when fed with *n*-octane and Findlay and White (1983) identified 3HHp in *B. megaterium*. *Cupriavidus necator* (formerly known as *Alcaligenes eutrophus* or *Wautersia eutropha*) is a well studied bacterium capable of producing PHA SCL and it has been identified to produce PHA polymers consisting of 3HB, 3HV and 4HB monomers (Doi, 1990; Kunioka et al., 1989; Saito et al., 1996). The studies revealed that the production of various PHA monomers was dependent on the substrate fed. To date, about 150 different monomer constituents of PHA have been found (Steinbuchel, 2001; Steinbuchel and Valentin, 1995). Witholt and Kessler (1999) have contributed in PHA discovery by compiling the knowledge about large variety of PHA monomers with straight, branched, saturated, unsaturated and also aromatic structures. PHA can be classified according to the monomer size into two major class of PHA; short-chain-length (SCL) PHA_{SCL} with 5 or <5 carbon atoms in a monomer, and medium-chain-length (MCL) PHA_{MCL} with 6 to 14 carbon atoms in a monomer. The synthesis of PHA_{MCL} consisting of 3HO and 3-hydroxydecanoate (3HD) monomers as major components was reported in *P. oleovorans* and *Pseudomonas putida*. 
Introduction to polyhydroxyalkanoates

1.2 Occurrence of PHAs in bacteria

As mentioned earlier the occurrence of PHB as intracellular inclusions was first reported for *Bacillus megaterium* by Maurice Lemoigne in 1925, One year later, he discovered a polyester from *B. megaterium* with the empirical formula of \((C_4H_6O_2)_n\), which was referred as PHB (Lemoigne, 1926). By the end of the 1950s, sufficient evidence had accumulated from physiological studies to suggest that PHB functions as an intracellular reserve for carbon and energy. In 1961, Schelgel et al. suggested that microbial accumulation of PHA is implemented in the presence of an excess of carbon source with concomitant restriction of another essential nutrient, such as nitrogen. Macrae and Wilkinson (1958) noticed that *Bacillus megaterium* initiated the accumulation of P(3HB) homopolymer when the ratio of glucose to nitrogen in the culture medium was high and its subsequent intracellular degradation (also referred to as mobilization) of P(3HB) occurred in the absence of carbon and energy sources. The large variety of microorganisms capable of synthesizing PHAs comprises diverse heterotrophic, chemolithotrophic and phototrophic, aerobic and anaerobic Eubacteria, as well as Archeaebacteria (Steinbuchel, 1991). Since the discovery of PHB more than 90 genera have been detected in aerobic and anaerobic habitats which are able to produce PHA (Steinbuchel and Valentin, 1995; Findlay and White, 1983).

Merrick and Doudoroff, (1961) have seen that PHB granules are surrounded by a membrane containing both the PHA synthase and the PHA depolymerase. The physical properties of PHB granules are very different, and two physical states can be distinguished represented by intracellular native PHB granules and partially crystalline PHB granules (Jendrossek and Handrick, 2002). Intracellular native PHB granules are in the amorphous rubbery state, and the surface is surrounded with a
layer consisting of phospholipids and granule-associated proteins (Lundgren et al., 1965; Mayer et al., 1996). In the cytoplasm of \textit{W. eutropha}, PHA granules occur as water – insoluble inclusions with a size of 0.2-0.3 µm, and the PHA content can contribute up to more than 90 % of the cellular dry weight (Pedros-Alio et al., 1985). Here Fig. 1.3 shows an electron microscopic photograph of a thin section of a \textit{W. eutropha} cell packed with poly (3HB).

\begin{figure}[h]  
\centering
\includegraphics[width=0.5\textwidth]{image1.png}
\caption{Electron micrograph of \textit{W. eutropha} strain H16 accumulating poly (3HB) (Potter et al., 2002)}
\end{figure}

1.3 Structure and synthesis of PHA granules

First investigations on purified PHA granules were done by Williamson and Wilkinson (1958) and also by Griebel et al., (1968) who demonstrated that the granules contained proteins and lipids besides the PHB. Recent studies on the structure of PHA granules and granule-associated proteins have been especially carried out in \textit{P. oleovorans} by Witholt and co-workers (De Smet et al., 1983) and in \textit{R. eutropha} by Steinbuchel (Weiczorek et al., 1995; Potter et al., 2002). Bacterial PHA granules are mostly between 200 and 500 nm in diameter (Anderson and Dawes, 1990) with thickness of membrane 15-20 nm (Lundgren et al., 1964). Chemical analyses have shown that inclusion bodies contain approximately 97.5% PHA, 2% protein, and 0.5% lipid (Griebel et al., 1968). PHA synthesis occurs intracellularly in multiple inclusions which are surrounded by a membrane to which proteins are
bound. Four types of granule-associated proteins are found within bacterial genera producing PHAs (i) PHA synthase, (ii) PHA depolymerases and 3HB-oligomer hydroxylase, (iii) phasins (PhaPs), which are thought to be the major structural proteins of the membrane surrounding the inclusion, and (iv) the regulator of phasin expression PhaR (Fig. 1.4).

![Granular structure of PHA (Rehm, 2003)](image)

Figure 1.4: Granular structure of PHA (Rehm, 2003)

1.3.1 PHA synthase or PHA polymerase (phaC)

Since the cloning of the PHA biosynthesis operon from *W. eutropha* H16 about 27 years ago (Schubert et al., 1988; Slater et al., 1988; Peoples and Sinskey, 1989), more than 60 different PHA synthase genes have been sequenced from different bacteria (Rehm and Steinbuchel, 2002). According to the substrate specificities and sequence homologies, four different classes of PHA synthases were distinguished. Class I PHA synthases synthesize PHAs of hydroxyalkanoates of short-chain-length (PHASCL). Class II PHA synthases prefer coenzyme A thioesters of hydroxyalkanoates of medium-chain-length (HAMCL) comprising 6-14 carbon atoms as substrates. The third class of PHA synthases also exhibit substrate specificities with hydroxyalkanoates of
short-chain-length (HASCL). Class IV PHA synthases are composed of two different types of subunits and occur in species belonging to the genus *Bacillus* (McCool and Canon, 2001). The class I and III PHA synthases of β-proteobacterium *W. eutropha* and of the γ-proteobacterium *Allochromatium vinosum*, respectively, are regarded as model enzymes for studying PHA<sub>SCL</sub> biosynthesis in bacteria, whereas the PHA<sub>MCL</sub> synthases of *P. oleovorans* and *P. putida* represent the most detailed studied class II PHA synthases.

### 1.3.2 PHA depolymerase (*phaZ<i>i</i>*)

PHA depolymerases are the enzymes responsible for the degradation of PHAs. There are two types of PHA depolymerases; intracellular and extracellular PHA depolymerases. In contrast to the well-studied extracellular PHA depolymerases, intracellular PHA depolymerases (*phaZ<i>i</i]*) have been far less investigated although they play an important role for the overall PHA metabolism. Western blot experiments employing polyclonal antibody raised against *phaZ* demonstrated that this PHB depolymerase is expressed in *W. eutropha* in a nitrogen-starved and carbon-rich medium. It is worth mentioning that an intracellular D-(-)-3-hydroxybutyrate-oligomer hydrolase of *W. eutropha* is also granule-associated (Saegusa et al., 2002).

### 1.3.3 Phasins (*phaP*)

Phasins represent a class of most probably non catalytic proteins consisting of a hydrophobic domain, which associates with surface of the PHB granules, and of a predominantly hydrophilic/amphiphilic domain exposed to the cytoplasm of the cell. This layer of phasins stabilizes PHA granules and prevents coalescence of separated granules (Steinbuchel and Valentin, 1995). *PhaP* from *W. eutropha* is the best studied phasin.
1.3.4 Transcriptional repressor (phaR)

Transcriptional repressor phaR in *W. eutropha* regulates the expression of *phaP* (York et al., 2001). PhaR has the capability to bind to at least three different targets in cells of *W. eutropha*: (i) the promoter region of *phaP*, (ii) the promoter region of phaR, and (iii) the surface of PHB granules. All these data support the following simple but elegant and efficient model explained in five situations for the regulation of *phaP* expression in *W. eutropha* with phaR functioning as a transcriptional repressor protein as shown in Fig. 1.5 (Potter and Steinbuchel, 2005).
Figure 1.5: Sequence of events occurring during PHA granule formation that ensure appropriate expression of phasin PhaP1 in *R. eutropha* through regulation by the transcriptional regulator protein PhaR (Potter and Steinbuchel, 2005)

**Situation A:** If the cells are cultivated under conditions not permissive for PHB biosynthesis, phaR cannot bind to PHB granules because they do not exist in the cells. The cytoplasmic concentration of phaR is sufficiently high to repress transcription of phaP. No phaP protein is formed and detectable in the cytoplasm.
Situation **B**: If conditions are permissive for PHB biosynthesis, the constitutively expressed PHA synthase \((\text{phaC})\) starts to synthesize PHB molecules that remain covalently linked to the enzyme. At the beginning small micelles are formed which become larger and constitute the nascent PHB granules. \(\text{PhaC}\) no longer covers the PHB granule surface entirely, and proteins with a binding capacity to the hydrophobic surface like \(\text{phaR}\) bind to the granules. This lowers the cytoplasmic concentration of \(\text{phaR}\).

**Situation C**: From a certain point the cytoplasmic concentration of \(\text{phaR}\) becomes so low that it can no longer repress transcription of \(\text{phaP}\). \(\text{PhaP}\) is then synthesized and binds subsequently to the PHB granules. The concentration of soluble \(\text{phaP}\) in the cytoplasm remains beyond a detectable level.

**Situation D**: The PHB granules grow and reach their maximum size; \(\text{PhaP}\) protein is being continuously synthesized in sufficient amounts.

**Situation E**: When the PHB granules have reached the maximum possible size according to the physiological conditions, almost the entire surface will be covered by \(\text{phaP}\) protein, and the latter is displacing \(\text{phaR}\) protein from the PHB granules. Consequently, the cytoplasmic concentration of \(\text{phaR}\) increases and it will exceed the threshold concentration required to repress again transcription of \(\text{phaP}\).

### 1.4 Physiology and biosynthesis of PHA_{scL} (PHB)

The biochemistry of PHB biosynthesis is very well studied. The PHB biosynthesis process involves the three enzymes and their encoding genes. Study of Witholt and Kessler, (1999) revealed that PHB synthesis in \(W.\ eutropha\) occur in three steps, reaction starting with acetyl CoA when cultivated on carbohydrates, pyruvate or
acetate (Fig.1.6). 3-ketothiolase, first enzyme of the reaction encoded by *phbA* gene, catalyzes the condensation reaction. In condensation reaction two acetyl-CoA molecules are coupled to form acetoacetyl-CoA, the product is subsequently stereoselectively reduced to (R)-3-hydroxybutyryl-CoA in a reaction catalyzed by NADPH-dependent acetoacetyl-CoA reductase which is encoded by *phbB* gene. Finally, PHB is synthesized by polymerization of (R)-3-hydroxybutyryl-CoA molecules by the PHB synthase encoded by *phbC* gene (Haywood et al., 1989).

**Figure 1.6: Polyhydroxybutyrate (PHB) synthesis genes and pathway in *W. eutropha* (Witholt and Kessler, 1999)**

PHB synthesis is regulated at the enzymatic level. The availability of suitable substrates for PHB synthesis routes results from an imbalanced supply of nutrients to
the cell via physiological regulation pathways. These pathways have been studied in *Azotobacter beijerinckii* (Senior and Dawes, 1971) and *W. eutropha* (Oeding and Schelgel, 1973). It was found that the intracellular concentration of acetyl-CoA and free coenzyme A play a central role in the regulation of polymer synthesis (Haywood et al., 1988). Under balanced growth conditions acetyl-CoA is oxidized via the tricarboxylic acid (TCA) cycle and is used for biosynthetic purposes. When growth ceases the NADH concentration increases, which reduces the activity of TCA cycle enzymes citrate synthase and isocitrate dehydrogenase. As a result acetyl-CoA cannot be oxidized via the TCA cycle and enters the PHB synthetic pathway. The 3-ketothiolase enzyme of this pathway is inhibited by free CoA, which is generated by oxidation of acetyl-CoA via the TCA cycle during normal growth.

**1.5 Molecular biology and enzymology of PHA<sub>SCL</sub> synthesis**

In *W. eutropha*, the structural genes for PHA synthesis are organized in the phb CAB operon, coding for PHB synthase, β-ketothiolase, and NADPH-dependent acetoacetyl-CoA reductase, respectively. A putative model for the polymerization process based on *de novo* fatty acid synthesis is presented in Fig. 1.7. This model illustrates one possible reaction mechanism, and other models are also possible. In an initiation or priming process (Fig. 1.7a), the 3-hydroxybutyric acid moiety from 3-hydroxybutyryl-CoA is transferred to the thiol group of the phosphopantothein-modified serine of PHA synthase coupled with the release of coenzyme A. This 3-hydroxybutyryl moiety is then translocated to the thiol of the cysteine. It has been shown that acylation of cysteine<sub>319</sub> causes a shift of the monomeric form of the synthase to this dimeric form, and this shift is accompanied by an increase in its specific activity and a decrease in the lag phase of polymer formation (Wodzinska et al., 1996). A second monomer is then again added to the thiol group of the
phosphopantotheine-modified serine, and its 3′ hydroxy group attacks the thiol ester of the first monomer to form a covalently bound dimer (Fig. 1.7b). After translocation of the formed dimer to the thiol group of the cysteine (either of the same subunit as shown in Fig. 7b or of the other enzyme subunit), the thiol group of the postranslationally modified serine can accept a new 3-hydroxybutyric acid moiety, and by repeated propagation steps the covalently bound oligomer or polymer grows by one unit during each cycle. It is assumed that the termination of polyester biosynthesis occurs if a chain transfer with water occurs (Fig. 1.7c). Doi et al., (1990) have postulated that a chain transfer agent, which might be an enzyme with a water molecule in its active site, could be involved in the termination process (Hori et al., 1994; Kusaka et al., 1997).
A combined chemical and enzymatic procedure has been developed to synthesize PHB granules in vitro (Gerngross and Martin, 1995). Purified PHB synthase from A. eutrophus was exposed to synthetically prepared (R)-3-hydroxybutyryl-CoA, and PHB was formed, thereby establishing the minimal requirements for PHB formation. The in vitro polymerization system yielded PHB with a molecular mass higher than $10 \times 10^6$ Da, exceeding by an order of magnitude the mass of PHB typically extracted from microorganisms. Furthermore, the molecular mass of the polymer could be controlled by the initial PHB synthase concentration.
1.6 Physiology and biosynthesis of PHA_{MCL}

*Pseudomonads* not only synthesize PHA_{MCL} from aliphatic alkanes or fatty acids, but also from sugars or other unrelated carbon sources (Fig.1.8) (Haywood et al., 1990; Timm and Steinbuchel, 1990).

**Figure 1.8: PHA biosynthesis from unrelated substrates (Steinbuchel and Fuchtenbusch, 1998)**

Alkanes such as octane, are oxidized to the corresponding fatty acids, the latter are activated by thiokinases and degraded by the β-oxidation pathway. Under cultivation conditions promoting PHA accumulation, intermediates of the β-oxidation cycle can be converted to R-(−)-3-hydroxyacyl-CoA by enoyl-CoA hydratases, epimerases or ketoacyl-CoA reductases, and subsequently polymerized by the PHA_{MCL} synthase (Huisman et al., 1991). Several Pseudomonads such as *P. putida* are also able to synthesize PHA_{MCL} from unrelated carbon sources, e.g., glucose by the involvement of the fatty acid *de novo* synthesis pathway (Huijberts et al., 1992, 1994). The linking enzyme (PhaG), which transfers the R-(−)-3-hydroxyacyl residue from the acyl carrier protein to coenzyme A has been identified in *P. putida* and other Pseudomonads.
Introduction to polyhydroxyalkanoates

(Rehm et al., 1998). A cryptic phaG homologous gene has been detected in *P. oleovorans*, explaining the inability of this strain to synthesize PHA$_{MCL}$ from sugars (Hoffmann et al., 2000).

*In vitro* PHA synthesis has also been demonstrated with purified type-II and type-III *phaC* genes, which were obtained from recombinant strains of *E. coli* (Jossek et al., 1998; Qi et al., 2000). Referring to these findings, efforts were made on the establishment of more efficient *in vitro* systems employing CoA recycling systems (Jossek et al., 1998; Liu and Steinbuchel, 2000; Steinbuchel, 2001).

1.7 Production of PHA by genetically engineered plants

PHA production in bacteria and yeast requires growth under sterile condition in a costly fermentation process with external energy sources. In contrast, PHA production in plant systems is considerably less expensive because the system only relies on carbon dioxide and light, represents a more cost-effective approach to produce this biopolymer in large quantities. In addition, a plant production system is much more environmentally friendly. Production of PHA in agricultural crops is likely to be economically viable if it can be produced as a byproduct with some other plant constituents such as oil or starch. PHA produced in an oilseed crop can potentially be recovered along with the oil fraction leaving the remaining meal for use as animal feed. Oil seed crops are considered as good targets for seed specific polyhydroxyalkanoate production also. As PHB and oil are derived from acetyl-CoA, metabolic engineering of plants for the diversion of acetyl-CoA towards PHB accumulation can be more directly achieved in the seeds of crops having a naturally high flux of carbon through acetyl-CoA. Thus, many oil crops such as rapeseed, sunflower and soyabean could be potentially engineered for the production of PHA.
Synthesis of PHA in plants was first demonstrated in 1992 by the accumulation of PHB in the cytoplasm of cells of *Arabidopsis thaliana* (Poirier et al., 1992a). Since then, a range of different PHAs had been synthesised in various species through the creation of novel metabolic pathways either in the cytoplasm, plastid or peroxisome. PHA synthesis in plants has more recently emerged as a useful and novel tool to study fundamental aspects of plant metabolism. It was known that PHB was synthesised in bacteria from acetyl-CoA. Since acetyl-CoA is present in plant cells in the cytosol, plastid, mitochondrion and peroxisome, the synthesis of PHB in cytoplasm was targeted as the first site for PHB synthesis because it had the advantage that the bacterial enzymes could be directly expressed in this compartment without any modification of the proteins. Moreover, the enzyme 3-ketothiolase was present in the cytoplasm of higher plants, where it is involved in the synthesis of mevalonate, the precursor to isoprenoids. The same enzyme is involved in the synthesis of PHB in *R. eutropha*. Thus, the production of PHB in the cytoplasm of plants required the expression of two additional enzymes, reductase and synthase. So the *R. eutropha* phbB and phbC genes, encoding acetoacetyl-CoA reductase and PHB synthase, respectively, were expressed into plants to complete the PHB biosynthetic pathway under the control of the cauliflower mosaic virus (CaMV) 35S promoter, allowing a relatively high expression of the enzymes in a broad range of tissues. PHA could potentially be produced at a cost of US $ 0.20-0.50/kg if they could be synthesized in plants to a level of 20-40% dry weight and thus be competitive with the petroleum based plastics. Poirier et al. (1992a) reported that the reductase and synthase genes of *W. eutropha* can be inserted into a plant, *Arabidopsis thaliana*, which can also produce acetoacetyl-CoA, and the transgenic plant can then accumulate PHB granules (Fig. 9), to approximately 14% of its dry weight (Poirier et al., 1992b).
The other plants used for PHA production are *Gossypium hirsutum* and *Zea mays*. The advantage looks more with the starch producing crops than oil crops in terms of yield (kg/hectare) but the diversion of acetyl-CoA towards PHB synthesis is likely to be more complex in starch crops since the flux of carbon is primarily directed towards sucrose instead of acetyl-CoA. In UK, ZENECA Seeds, is focusing its efforts on rapeseed while in USA, Monsanto is working on both rapeseed and soyabean. Many other companies are also intensely involved in the manufacture and marketing of biodegradable plastics. The global biodegradable plastics market in terms of volume is expected to grow from 664,000 metric tons in 2010 to 2330,000 metric tons by 2016, at an estimated CAGR of 20.24% from 2011 to 2016. Various PHA producing companies are mentioned in table 1.1.
Table 1.1: Companies carrying out research and producing PHA in different countries. (Guo-Qiang, 2009)

<table>
<thead>
<tr>
<th>Company</th>
<th>Types of PHA</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICI, UK</td>
<td>PHBV</td>
<td>1980 to 1990</td>
</tr>
<tr>
<td>BASF, Germany</td>
<td>PHB, PHBV</td>
<td>1980 to 2005</td>
</tr>
<tr>
<td>Metabolix, USA</td>
<td>Several PHA</td>
<td>1980 to present</td>
</tr>
<tr>
<td>Mredian, USA</td>
<td>Several PHA</td>
<td>2007 to present</td>
</tr>
<tr>
<td>Mitsubishi, Japan</td>
<td>PHB</td>
<td>1990</td>
</tr>
<tr>
<td>Bio-on, Italy</td>
<td>PHA</td>
<td>2008 to present</td>
</tr>
<tr>
<td>Tianjin Northen food, China</td>
<td>PHB</td>
<td>1990</td>
</tr>
<tr>
<td>Shenzhen O’Bioer, China</td>
<td>Several PHA</td>
<td>2004 to present</td>
</tr>
</tbody>
</table>

1.8 Properties and applications of PHA

Marchessault and Yu (2002) showed that PHB is a compact right-handed helix with a two fold screw axis and a fiber repeat of 0.596 nm. It is optically active, with the chiral center of the monomer unit always in the R absolute configuration [D-(-) in the traditional nomenclature]. The similarity of the PHB structure to that of polypropylene, which also has a compact helical configuration and a melting point near 180°C, attracted the attention of ICI to the potential of PHB for fiber and plastics applications (Holmes et al., European patent, 1985), particularly in the biomedical field, where biocompatibility and biodegradability are important features.

The Mw of P(3HB) produced from wildtype bacteria is usually in the range of $1 \times 10^4$ to $3 \times 10^6$ g/mol with a polydispersity of around 2 (Doi, 1990). The glass transition temperature of P(3HB) is around 48 °C, measured by calorimetric analysis. The
densities of crystalline and amorphous P(3HB) are 1.26 and 1.18 g/cm³, respectively. Mechanical properties like the Young’s modulus (3.5 GPa) and the tensile strength (43 MPa) of P(3HB) material are close to those of isotactic polypropylene. The extension to break (5%) for P(3HB) is however markedly lower than that of polypropylene (400%). Therefore, P(3HB) appears as stiffer and more brittle plastic material when compared with polypropylene (De Koning et al., 1992). Properties of both polymers are presented in table 1.2.

Table 1.2: Comparison of polymer properties of poly-3-hydroxybutyrate (PHB) with polypropylene (PP) (Witholt and Kessler, 1999)

<table>
<thead>
<tr>
<th>Physical properties</th>
<th>PHB</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting Point (°C)</td>
<td>180</td>
<td>170</td>
</tr>
<tr>
<td>Tensile Strength (MPa)</td>
<td>43</td>
<td>34.5</td>
</tr>
<tr>
<td>Young’s Modulus (GPa)</td>
<td>3.5</td>
<td>1:7</td>
</tr>
<tr>
<td>Elongation to break (%)</td>
<td>5.0</td>
<td>400</td>
</tr>
</tbody>
</table>

The PHAs are non-toxic, biocompatible, biodegradable thermoplastics that can be produced from renewable resources. They have a high degree of polymerization, optically active and isotactic (stereochemical regularity in repeating units), piezoelectric and insoluble in water. PHA\textsubscript{MCL} are semi-crystalline elastomers with low melting point, low tensile strength and high elongation to break (Preusting et al., 1990). These features make them highly competitive with polypropylene, the petrochemical-derived plastic. The family of PHA exhibits a wide variety of mechanical properties from hard crystalline to elastic, depending on composition of monomer units which broadens its application area.
PHAs have a wide range of applications owing to their novel features. Initially, PHAs were used in packaging films mainly in bags, containers and paper coatings. Similar applications as conventional commodity plastics include the disposable items, such as razors, utensils, diapers, feminine hygiene products, cosmetic containers, shampoo bottles and cups. In addition to potential as a plastic material, PHA are also useful for synthesis of optically active compounds (Oeding and Schlegel, 1973; Senior and Dawes, 1971). Such compounds are particularly used as biodegradable carriers for long term dosage of drugs, medicines, hormones, insecticides and herbicides. They are also used as osteosynthetic materials in the stimulation of bone growth owing to
their piezoelectric properties, in bone plates, surgical sutures and blood vessel replacements. However, the medical and pharmaceutical applications are limited due to the slow biodegradation and high hydraulic stability in sterile tissues (Wang and Bakken, 1998). The PHAs are raw materials for the production of paints.

PHA were reported to have 150 (R)-hydroxyalkanoic acid (R-HA or HA) monomers. Owing to the monomer chiral centers and at least two functional groups including a hydroxyl group and a carboxyl group in each monomer, HA can be used as precursors or intermediates for the synthesis of many fine compounds such as antibiotics, vitamins, aromatics, and pheromones (Ren et al., 2010). Among HA, (R)-3-hydroxyalkanoates (R-3HA) are the most common monomers. 3HB was the most widely available monomer which has shown some potential medical applications related to Ca-channel activation and memory enhancement (Cheng et al., 2006; Zou et al., 2009), as well as biofuel (Zhang et al., 2009). Other HA, if conveniently available, will open a wide area for application development. Basically, the production of HA can be accomplished by chemical synthesis, chemical and enzymatic hydrolysis, or through metabolic engineering of microorganisms (Ren et al., 2010). Chemical synthesis is usually very expensive to make chiral HA, while enzymatic hydrolysis of PHA can conveniently lead to chiral HA formation. Metabolic engineering of PHA producing bacteria allows the production of chiral HA directly without the PHA in vitro hydrolysis, which is more energy efficient. The application of PHA as a source of biofuel looks very promising since it does not require highly purified PHA, and thus, the PHA can possibly be obtained from activated sludge or nutrient-rich wastewater, which does not compete with human or animals for food, resulting in cost reduction. Recent development on PHA production from open and continuous mixed cultures will allow the PHA to be produced with very low cost for the biofuel
applications (Johnson et al., 2009; 2010). Market of biodegradable plastic in 2016 and various applications of PHA are mentioned in Fig. 1.10 and table 1.4, respectively.

Figure 1.10: Global bio-based biodegradable plastics market, by the year 2016
Table 1.4: Applications of PHA in various fields (Guo-Qiang, 2009)

<table>
<thead>
<tr>
<th>Applications</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packaging Industry</td>
<td>All packaging materials that are used for a short daily consumables, electronic appliances period of time, including food utensils, films</td>
</tr>
<tr>
<td>Printing and photographic industry</td>
<td>PHA are polyesters that can be easily stained</td>
</tr>
<tr>
<td>Block copolymerization</td>
<td>PHA can be changed into PHA diols for block copolymerization with other polymers</td>
</tr>
<tr>
<td>Plastic processing</td>
<td>PHA can be used as processing aids for plastic processing</td>
</tr>
<tr>
<td>Textile industry</td>
<td>Like nylon, PHA can be processed into fibers</td>
</tr>
<tr>
<td>Fine chemical industry</td>
<td>PHA monomers are all chiral r-forms, and can be used as chiral starting materials for the synthesis of antibiotics and other fine chemicals</td>
</tr>
<tr>
<td>Medical implants biomaterials</td>
<td>PHA have biodegradability and biocompatibility, and can be developed into medical implants materials, PHA can also turned into drug controlled release matrices</td>
</tr>
<tr>
<td>Medical</td>
<td>PHA monomers, especially R3HB, have therapeutic effects on Alzheimer’s, Parkinson’s diseases and osteoporosis</td>
</tr>
<tr>
<td>Healthy food additives</td>
<td>PHA oligomers can be used as food supplements for obtaining ketone bodies</td>
</tr>
<tr>
<td>Biofuels or fuel additives</td>
<td>PHA can be hydrolyzed to form hydroxyl-alkanoate methyl esters that are combustible</td>
</tr>
<tr>
<td>Specific drug delivery</td>
<td>Coexpression of PhaP and specific ligands can help achieve specific targeting to diseased tissues</td>
</tr>
</tbody>
</table>

1.9 Economics of PHA production

PHA production from pure carbon substrate has been reported by many scientists but the cost of such carbon sources, fermentation process and the downstream processing...
of the polymer contribute to the high cost of manufacturing process of polymer which is 5-10 times more expensive than petroleum based polymers (Suriyamongkol et al., 2007). PHA price varies from 1.5 to 5 $D per kg, whereas polypropylene price varies from 0.2 to 0.4 $D per kg (Khanna and Srivastava, 2005; Chanprateep, 2010). About 50% of the production costs of the PHA are added by the cost of carbon source (Halami et al., 2008). However, in order to use these polymers as commodity plastics, the cost of production has to be sufficiently lowered without affecting the useful properties of the polymer. A viable strategy for cost minimization can include the utilization of a broad range of waste and surplus materials that can be used as feedstock for the bio-mediated production of desired end product which can help industry to overcome their disposal problems. Starch, cellulose, whey, fat and glycerol are such inexpensive materials which can be used for cost effective production of PHA. Sucrose-rich inexpensive substrate sugarcane liquor was used by Jiang et al. (2008) to produce P(3HB) using Pseudomonas fluorescens.  

1.10 Recent developments

It has become possible to recruit mixed cultures for continuous and unsterile production of PHA (Johnson et al., 2009). However, pure culture is preferred as it usually lead to more reproducible results. Keshavarz and Roy (2010) and Shrivastav et al. (2013) have been reported PHA nanoparticles for drug delivery and biocompatible porous implants made from poly-4-hydroxybutyrate. Various enantiomerically pure R-HAs can be conveniently prepared by depolymerizing the biosynthesized PHA which have applications in the industry including food supplements, pharmaceuticals, cosmetics, fragrances, flavors, and other fine chemicals.
OBJECTIVES OF THE THESIS

As compiled above, studies on PHAs cover various fields of basic and applied research. This thesis deals with the studies on production, chemical modifications, biodegradation and biotechnological conversion of polyhydroxyalkanoates with primary objectives as under.

- Isolation of potential PHA producer and optimization of process parameters for PHA\textsubscript{MCL} production using agro-industrial wastes such as glycerol and tallow.
- Modification of PHA by synthesis of PHA graft with chitosan and acrylic acid. Characterization and biodegradation of this newly synthesized graft.
- Biodegradation of PHB by natural soil isolate and characterization of depolymerase enzyme.
- Synthesis of R-hydroxyalkanoic acids by \textit{in vivo} depolymerization of accumulated PHA inside the cells