4.1 INTRODUCTION

In past few years, several biodegradable plastics have been introduced into market. At present, biodegradable plastic represents just a tiny market as compared with conventional petrochemical derived plastics. PHAs are considered as environmentally-friendly alternatives to petroleum-based polymers since they are synthesized from renewable resources and are biodegradable to water and carbon dioxide or methane by a large variety of ubiquitous microorganisms present in many ecosystems. A few PHAs such as PHB and copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (3HB-co-3HV; BIOPOL®) (Asrar and Gruys, 2002) are already industrially produced at a scale of few hundred tons per year by several plants all over the world. Several other PHAs will enter the commercial market in near future.

Biodegradation is the process by which organic substances are broken down by living organisms. Organic material can be degraded aerobically, with oxygen, or anaerobically, without oxygen. Plastics are biodegraded aerobically in nature while anaerobically and partly anaerobically in sediments and landfills. Microorganisms such as bacteria and fungi are involved in the degradation of natural plastics through production of enzymes (Fig. 4.1).
4.1 Historical outline

A number of aerobic and anaerobic PHA degrading bacteria and fungi have been isolated from various environments (Anderson and Dawes, 1990; Jendrossek et al. 1996). Bacteria of the genera Bacillus, Pseudomonas and Streptomyces were the organisms isolated more than 40 years ago having the ability to degrade PHA (Chowdhary, 1963). Two years later Delafield et al. (1965) isolated 52 different organisms on the basis of their polymer decomposing capability and differentiated them into five principal groups. Out of the five groups they chose Group III for further studies because these bacteria were among the most active polymer decomposers and at the same time were remarkably restricted in their ability to use different organic nutrients. They identified one of the bacterium as new species and it was named Pseudomonas lemoignei (in honor of Maurice Lemoigne, who discovered PHB in Bacillus megaterium). After 1965, no additional PHA degrading strains
except *Alcaligenes faecalis* (Tanio et al., 1982) were isolated for nearly three decades. However, because of the commercialization of PHA and the discovery of monomers other than 3-HB as constituents of PHA, the investigation of extracellular PHA degradation was resumed and numerous PHA degrading bacteria, in particular PHB degrading bacteria have been isolated and characterized since 1990. Table 4.1 describes the classification of PHA degrading bacteria according to their polymer degrading ability.

**Table 4.1: Classification of PHA-degrading bacteria according to their polymer-degrading ability (Schirmer et al., 1995)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Degraded Polymer</th>
<th>No. of isolates</th>
<th>Representative strains (source)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>P(3HB)</td>
<td>&gt;20</td>
<td><em>Alcaligenes faecalis</em> T1, <em>Comamonas</em> sp.</td>
<td>Tanio et al., 1982, Jendrossek et al., 1993</td>
</tr>
<tr>
<td>II</td>
<td>P(6HX)</td>
<td>1</td>
<td>Isolate SK850</td>
<td>Schirmer et al., 1995</td>
</tr>
<tr>
<td>III</td>
<td>P(3HO)</td>
<td>4</td>
<td>Isolate SK814</td>
<td>Schirmer et al., 1995</td>
</tr>
<tr>
<td>IV</td>
<td>P(3HB), P(3HV)</td>
<td>10</td>
<td><em>P. lemoignei</em></td>
<td>Muller and Jendrossek 1993</td>
</tr>
<tr>
<td>V</td>
<td>P(3HB), P(6HX)</td>
<td>3</td>
<td>Isolae SK860</td>
<td>Schirmer et al., 1995</td>
</tr>
<tr>
<td>VI</td>
<td>P(3HO), P(3HD-co-3HO)</td>
<td>7</td>
<td><em>Pseudomonas fluorescens</em> GK13</td>
<td>Schirmer et al., 1993</td>
</tr>
<tr>
<td>VII</td>
<td>P(6HX), P(3HO), P(3HD-co-)</td>
<td>2</td>
<td>Isolate SK853</td>
<td>Schirmer et al., 1995</td>
</tr>
<tr>
<td></td>
<td>3HO)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------------------------------------------</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
</tbody>
</table>
| VIII | P(3HB), P(3HO), P(3HD-co-3HO)                                         | 1 | Isolate SK827 
Schirmer et al., 1995 |
| IX  | P(3HB), P(3HB-co-3HX-co-4HX), P(3HO), P(3HD-co-3HO)                   | 3 | Streptomyces exfoliatus K10 
Klingbeil et al., 1996 |
| X   | P(3HB-co-3HX-co-4HX), P96HX, P(3HO), P(3HD-co-3HO)                    | 2 | Isolate SK801 
Schirmer et al., 1995 |
| XI  | P(3HB), P(3HB-co-3HX-co-4HX), P(6HX), P(3HO), P(3HD-co-3HO)           | 5 | Isolate SK844 
Schirmer et al., 1995 |

3HB 3-hydroxybutyrate, 6HX 6-hydroxyhexanoate, 3HO 3-hydroxyoctanoate, 3HV 3-hydroxyvalerate, 3HD 3-hydroxydecanoate, 3HX 3-hydroxyhexanoate, 4HX 4-hydroxyhexanoate, SK refers to a culture collection of the institute of author

4.1.2 *Pseudomonas lemoignei*: A model PHB depolymerase producing strain

The most intensively studied PHA-degrading organism is *Paucimonas* (*Pseudomonas*) lemoignei (Jendrossek, 2001). It belongs to the β-subclass of the Proteobacteria. *P. lemoignei* is unique among PHA-degrading bacteria because it
synthesizes at least seven extracellular PHB depolymerases \([\text{phaZ1 to phaZ7}]\). A cooperative effect on PHB hydrolysis in the presence of two or more PHB depolymerases with different substrate specificities and \(K_m\) values for different chain-length molecules might exist. The presence of several isoenzymes of a respective polymer hydrolase within a single degrading strain seems to be a common theme for degradation of natural polymers such as cellulose or chitin. Glycosylation in \(P.\ lemoignei\) denatured PHB depolymerase is not well understood. Reports suggested that the carbohydrate moiety becomes noncovalently attached to depolymerase during secretion of the enzyme across the cell wall (Briese et al., 1994). The presence of carbohydrate is not essential for activity of \(P.\ lemoignei\) depolymerases because recombinant \(E.\ coli\) or \(B.\ subtilis\) strains harboring the corresponding depolymerase gene synthesized the nonglycosylated form of the enzyme but had almost the same specific activity. It is possible that glycosylation enhances the resistance of the exoenzyme to elevated temperature and/or to hydrolytic cleavage by proteases of competing microorganisms. PHB depolymerase of \(P.\ lemoignei\) apparently catalyze the reverse reaction, i.e. the synthesis of esters for transesterification if the reaction is performed in the absence of water (e.g. in solvents) (Kumar et al. 2000; Suzuki et al., 2001).

### 4.1.3 Microorganisms degrading plastic

A number of aerobic and anaerobic microorganisms that degrade PHA, particularly bacteria and fungi, have been isolated from various environments (Lee, 1996). \(\text{Acidovorax faecilis, Aspergillus fumigatus, Comamonas sp., Pseudomonas lemoignei}\) and \(\text{Variovorax paradoxus}\) are among those found in soil, while from activated sludge \(\text{Alcaligenes faecalis}\) and \(\text{Pseudomonas}\) have been isolated. \(\text{Comamonas testosteroni}\) has been found in seawater, \(\text{Ilyobacter delafieldii}\) is present in the anaerobic sludge.
Different microorganisms reported to degrade different types of plastics are presented in Table 4.2.

**Table 4.2: List of different microorganisms reported to degrade different types of plastics (Shah et al., 2008)**

<table>
<thead>
<tr>
<th>Plastic</th>
<th>Microorganism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyethylene</td>
<td><em>Brevibacillus borstelensis</em></td>
<td>Hadad et al., (2005)</td>
</tr>
<tr>
<td>Polyurethane</td>
<td><em>Curvularia senegalensis</em></td>
<td>Howard (2002)</td>
</tr>
<tr>
<td>Poly(3-hydroxybutyrate-co-3-hydroxypropionate)</td>
<td><em>Acidovorax sp. TP4</em></td>
<td>Wang et al., (2002)</td>
</tr>
</tbody>
</table>

### 4.1.4 Types of Enzyme

Enzymes which are actively involved in biological degradation of polymers are extracellular or intracellular depolymerases. Extracellular degradation is the utilization of exogenous PHA by a not necessarily PHA accumulating microorganism. The ability to degrade extracellular PHA is widely distributed among microorganisms and depends on the secretion of extracellular PHA depolymerases (e-PHA depolymerases) (*phaZ*<sub>e</sub>) that hydrolyze the water-insoluble polymer to water-soluble monomers and/or oligomers (Jendrossek and Handrick, 2002). On the other hand, intracellular degradation of PHA means active mobilization (hydrolysis) of the polymer by the accumulating bacterium itself and is catalyzed by specific intracellular PHA depolymerases (i-PHA depolymerase) (*phaZ*<sub>i</sub>). An exception is the periplasm-
located PHB depolymerase of *Rhodospirillum rubrum* (p-PHA depolymerases) (Handrick et al., 2004).

PHA depolymerases are specific for either short-chain-length PHA (PHA$_{SCL}$, three to five carbon atoms per monomer, EC 3.1.1.75) or for medium-chain-length PHA (PHA$_{MCL}$, six or more carbon atoms per monomer, EC 3.1.1.76).

Most i-PHA depolymerase known so far are unrelated in primary amino acid sequence to e-PHA depolymerases with recently described *phaZ* from *Wautersia eutropha* as the first exception (Abe et al., 2005). Explanations for the existence of distinct properties of e-PHA depolymerase and i-PHA depolymerase lies in the finding that PHAs *in vivo* and outside the bacteria are present in different conformations; *in vivo*, the polymer molecules are in the native, amorphous “rubbery” state (nPHA granules). nPHA granules consist of highly mobile chains of the carbon back bone in disordered conformation and are covered by a surface layer of mainly proteins and phospholipids (Fig. 4.2) (Potter and Steinbuchel, 2005). Upon extraction from the cell, or after cell lysis, the surface layer of nPHA granules is rapidly damaged or lost and the polyester chains tend to adopt an ordered helical conformation and develop a crystalline phase. This polymer is referred as denatured (crystalline) PHA (dPHA).
Extracellular dPHB, for example, is a partially crystalline polymer (typical degree of crystallinity 50 – 60 %) with an amorphous fraction characterized by the glass transition temperature ($T_g\sim 0 \, ^\circ C$) and a crystalline fraction that melts in the range 170-180 $^\circ C$ (Scandola et al., 1988).

Enzymes that hydrolyze PHB are often specific for one of the two forms (nPHB or dPHB). For example, extracellular e-PHB depolymerases hydrolyze dPHB, whereas intracellular i-PHB depolymerases are specific for nPHB and possibly for aPHB (artificial PHB); i-PHB depolymerases do not hydrolyze dPHB. More than 80 e-
dPHA depolymerases (phaZs), most from prokaryotic and eukaryotic microorganisms (Jendrosseck, 2001) have been isolated.

4.1.5 Molecular biology of dPHASCL depolymerases

Twenty genes for extracellular bacterial dPHA depolymerases (phaZ) have been cloned and analyzed since 1989; *Acidovorax* sp. (Kobayashi et al., 1999), *A. faecalis* (strains AE122 and T1) (Kita et al., 1997; Nojiri and Saito, 1997; Saito et al., 1989), *Delftia (Comamonas) acidovorans* strain YM1069 (Kasuya et al., 1997), *Comamonas testosteroni* (Shinomiya et al., 1997), *Comamonas* sp. (Jendrossek et al., 1995), *Leptothrix* sp. strain HS (Takeda et al., 2000), *Pseudomonas* sp. strain GM101 (accession no. AF293347), *P. fluorescens* strain GK13 (Schirmer et al., 1995), *P. lemoignei* (six genes) (Briese et al., 1994; Jendrossek et al., 1995; Schober et al., 2000), *P. stutzeri* (Ohura et al., 1999), *R. pickettii* (strains A1 and K1, accession no. JO4223, D25315), *S. exfoliatus* K10 (Klingbeil et al., 1996). Nineteen genes coded for depolymerase with specificity for only PHASCL, especially dPHB.

4.1.6 Structure of dPHASCL depolymerases

dPHA depolymerases are highly specific with respect to the length of the monomer carbon side chain of the PHA substrate. All extracellular dPHASCL depolymerase proteins have a composite domain structure typically comprised of three functional domains: a catalytic (320-400 aa), a linker (50-100 aa), and a substrate binding (40-60 aa) domain along with a signal peptide (Fig. 4.3a,b) (Jendrossek and Handrick, 2002; Hisano et al., 2006). Domain model of dPHASCL depolymerases also reported by Bhatt et al., (2011).
Depending on the microbial origin, depolymerase consists of; a signal peptide (SP), one of two possible types (type 1 or 2) of catalytic domains having lipase box (LB), one of three possible types of linking domains (Fn3, Thr, or Cad), and one of the two types of substrate-binding domains (SBD 1 or 2).
a) A 22 to 58 amino acid – long signal peptide is necessary for secretion of the polypeptide across the cytoplasmic membrane and is cleaved off during the secretion process by signal peptidases.

b) A large catalytic domain (320 - 400 aa) is situated at the N terminus of the mature protein. Three strictly conserved amino acids, serine, aspartate, and histidine, constitute the active center of the catalytic domain. The serine is part of a lipase box pentapeptide Gly-Xaa1-Ser-Xaa2-Gly, which is present in almost all known serine hydrolases such as lipases, esterases, and serine proteases. Site-directed mutagenesis of the lipase-box amino acids from two PHA<sub>SCL</sub> and one PHA<sub>MCL</sub> depolymerases resulted in inactive proteins and confirmed the requirement of the catalytic triad amino acids for activity (Nojiri and Saito, 1997; Schirmer et al., 1995; Shinoher et al., 1996; Suzuki et al., 2001). A histidine residue is also conserved in dPHB depolymerases. The region around this histidine resembles the oxyanion hole known from lipases (Jaeger et al., 1994). Two types of catalytic domains can be differentiated by the arrangement of the catalytic active amino acids within the primary amino acid sequence: In many PHA depolymerases and lipases the sequential order is histidine (oxyanion)-serine-aspartate-histidine (type 1, Fig.4.3). In <i>S. exfoliatus</i> and in several Comamonas and Leptothrix species, the depolymerases have the putative histidine (oxyanion) at C-terminal of the catalytic triad (type 2, Fig.3), and the sequential order is serine-aspartate-histidine-histidine (oxyanion). This indicates a different primary structure of the protein (Shinomiya et al., 1997; Klingbeil et al., 1996).

c) A linking domain (40-100 amino acids) links the catalytic domain to the C-terminal domain. The function of the linking region between the catalytic and
the C-terminal domain in PHA\textsubscript{SCL} depolymerases is unknown. In five dPHB depolymerases, namely phaZ1, phaZ2, phaZ3, phaZ5 and phaZ6 of \textit{P. lemoignei}, this region consists of about 40 amino acids with repeating clusters of two to six threonine residues. This threonine (Thr)-rich region is replaced by a fibronectin type III (Fn3) fingerprint in phaZ4 of \textit{P. lemoignei} and in most other sequenced dPHA\textsubscript{SCL} depolymerases. Fn3-sequences have been described for many eukaryotic extracellular matrix proteins and in several prokaryotic polymer-hydrolyzing proteins such as chitinases, cellulases, and several glucoamylases (Little et al., 1994). The Fn3-sequence of the \textit{Bacillus circulans} chitinase is not essential for activity (Watanabe et al., 1994). However, the Fn3-domain of the \textit{A. faecalis} dPHB depolymerase was essential because its deletion resulted in a protein that had lost depolymerase activity but not the 3HB dimer hydrolase activity (Nojiri and Saito, 1997). Interestingly, the Fn3-domain of the \textit{A. faecalis} dPHB depolymerase could be functionally replaced by a Thr-rich region of the dPHB depolymerase A (phaZ5) of \textit{P. lemoignei}. These findings support the hypothesis that Fn3-sequences and/or the Thr-rich regions are necessary to provide a proper distance (linker) between the substrate-binding domain (SBD) and the catalytic center of the protein, but the nature of this linker appears to be less important (Nojiri and Saito, 1997). Third type of linker consisting of ~ 100 amino acid-long cadherin-like sequence (Cad) has been described for the dPHB depolymerase of \textit{P. stutzeri} (Ohura et al., 1999). Cad sequences have also been found in other polymer hydrolases, e.g., in chitinases. Thus linker domain may function as a spacer that introduces a flexible region between the
catalytic and substrate-binding domains to increase the hydrolytic efficiency of the catalytic domain.

d) C-terminal substrate binding domain (SBD) of dPHAS\textsubscript{SCL} depolymerases bind to dPHA-granules and consists of 40 to 60 amino acids (Fig. 4.3). The SBD apparently has some specificity for PHA because dPHB depolymerases do not bind to chitin or to crystalline cellulose (Behrends et al., 1996; Kasuya et al., 1999; Ohura et al., 1999). The dPHB-binding ability can be used for affinity purification of dPHB depolymerases. The dPHB binding ability is lost in proteins that lack the C-terminal domain (60 amino acids), and these truncated enzymes do not hydrolyze dPHB any more. However, the ability to hydrolyze soluble esters (e.g., p-nitrophenylesters) is unaffected (Behrends et al., 1996; Briese and Jendrossek, 1998; Fukui et al., 1988; Hiraishi et al., 2000; Nojiri and Saito, 1997). Obviously SBD is responsible and sufficient for dPHB binding. The function of SBD for binding the water-insoluble substrate was confirmed by analysis of fusion proteins consisting of the maltose-binding protein Mal E or the glutathione-S-transferase (GST) with SBD (Kasuya et al., 1997; Ohura et al., 1999; Shinomiya et al., 1997). The fusion proteins were able to bind specifically to dPHA in all cases. The dPHB depolymerase sequences of \textit{A. faecalis} AE122, \textit{P. stutzeri} and \textit{Pseudomonas} sp. GM 101 which contains two instead of only one dPHB-binding domain. (Kita et al., 1997; Ohura et al., 1999).

Two types of SBD can be differentiated (types 1 and 2, Fig. 4.3). However several amino acids including positively charged amino acids such as histidine (His\textsubscript{49}), arginine (Arg\textsubscript{44}) and a cysteine residue (Cys\textsubscript{2}) are conserved in both types of binding domains. In type 1, a second cysteine at position -57 or -59 is present. It is not known...
whether the conserved amino acids are necessary to constitute a particular three-dimensional structure or whether these amino acids are directly involved in binding to the polymer chain, e.g., by hydrophobic, hydrophilic, and/or electrostatic interaction (Yamashita et al., 2001). Computer analysis of the putative secondary structures of SBD predicted the absence of any α-helices and the presence of mainly coiled structures with one or two β-sheets. The sensitivity of dPHB depolymerases to DTT suggests that at least some of the cysteine residues participate in a disulfide bridge(s) (Brucato and Wong, 1991).

Since these three functional domains namely; catalytic domain, linker domain and substrate binding domain are essential for the enzymatic degradation of water-insoluble polymers, it has been proposed that the degradation of the crystalline region of the polymer should proceed in three steps: adsorption of the enzyme to the polymer, non-hydrolytic disruption of the structure of the polymer, and hydrolysis (Hisano et al., 2006).

4.1.7 Molecular biology and functional analysis of dPHA_{MCL} depolymerases

In contrast to a large variety of isolated and more or less well-characterized dPHA_{SCL} depolymerases, only few dPHA_{MCL} depolymerases have been described so far. The dPHO depolymerase of *P. fluorescens* GK13 is the only one that has been purified and studied at the molecular level (Jendrossek et al., 1997; Schirmer et al., 1993). The dPHO depolymerase of *P. fluorescens* significantly differs from dPHA_{SCL} depolymerases. It is specific for PHA_{MCL} and for soluble esters such as p-nitrophenylacyl esters with six or more carbon atoms in the fatty acid moiety. dPHB and other dPHA_{SCL} are not hydrolyzed. The enzyme is not inhibited by DTT or EDTA and is not dependent on Ca^{2+} or other divalent cations. A PHA_{MCL} depolymerase was
isolated from a *Xanthomonas*-like strain that hydrolyzed a copolyester of 3-hydroxy-5-phenoxyvalerate and 28% 3-hydroxy-7-phenoxyheptanoate (Kim et al., 2000). The purified enzyme could not hydrolyze PHASCL; the ability to hydrolyze PHO was not tested. Another bacterium with extracellular PHAMCL activity is *Comamonas* sp. P37C (Quinteros et al., 1999). Cell-free culture fluid of PHO-grown *Comamonas* sp. P37C cells showed high PHO depolymerase and weak PHB depolymerase activity. It is not known whether the bacterium synthesizes two enzymes with different substrate specificities for PHASCL and PHAMCL or only one enzyme with broad specificity for both types of polyesters. The deduced amino acid sequence of the cloned dPHO depolymerase gene of *P. fluorescens* GK13 has no significant similarity to PHASCL depolymerases except for small regions in the neighbourhood of a lipase-box (Gly-Xaa1-Ser172-Xaa2-Gly), an aspartate (Asp228), and a histidine (His260) (Jendrossek and Handrick, 2002).

### 4.1.8 Regulation of PHA depolymerase synthesis

The synthesis of PHA depolymerases in bacteria is generally repressed if suitable soluble carbon sources such as glucose or organic acids are present. However, after exhaustion of the soluble nutrients synthesis of PHA depolymerases is derepressed in many strains (Jendrossek et al., 1993). At least in some bacteria dPHB depolymerase is expressed even in the absence of the polymer after cessation of growth. Therefore, an induction mechanism by the polymer itself is not necessary.

In most known dPHA-degrading bacteria high levels of dPHA depolymerase are produced only during growth on dPHA but are repressed on succinate. In contrast, production of PHASCL depolymerases by *P. lemoignei* is maximal during growth in batch culture on succinate. Therefore, isolation of PHASCL depolymerase from *P.*
**PHB depolymerase and biodegradation**

*P. lemoignei* is usually performed from succinate-grown cells (Delafield et al., 1965; Muller and Jendrossek, 1993; Nakayama et al., 1985). Synthesis of dPHB depolymerase on succinate is pH-dependent and occurs only above pH 7 (Stinson and Merrick, 1974). Recently, the relationship between depolymerase synthesis and growth as well as uptake of succinate has been elucidated: it was shown that transport of succinate into the bacteria is pH-dependent and does not work well above pH 7 in *P. lemoignei*. As a consequence, the bacteria starve even in the presence of residual succinate at pH above 7, and depolymerase synthesis is derepressed. Analysis of the succinate transport system of *P. lemoignei* revealed that it utilizes only the monocarboxylate form of succinate (H-succinate\(^1\)) but is not able to take up the dicarboxylate (succinate\(^2\)). The pH of the culture fluid increases during growth of *P. lemoignei* in batch culture on succinate owing to the uptake of succinic acid. As a consequence, the concentration of the succinate \(^1\)-ion (pK\(_A2\) = 5.6) decreases. The ignition of dPHB depolymerase synthesis above pH 7 can be considered as induced by carbon starvation because of insufficient uptake of H-succinate\(^1\) at high pH (Terpe et al., 1999).

Analysis of the DNA region adjacent to depolymerase genes revealed a putative transcriptional regulator gene, *phaR*, in front of *phaZ2* in *P. lemoignei*. Based on analysis of *phaZ*, *lacZ* transcriptional fusions in *P. lemoignei* wild-type and in *phaR* null mutants, it was demonstrated that *phaR* is a negative regulator of *phaZ2* (dPHB depolymerase B) but does not affect the expression of other depolymerases (Jendrossek and Handrick, 2002).
4.1.9 Influence of physicochemical properties of the polymer on its biodegradability

PHA depolymerases differ highly in their specificities towards various PHAs. The polymer itself also influences the biodegradability. The most important factors are:

1) **The stereoregularity of the polymer**: only ester linkages of monomers in the (R) configuration are hydrolyzed by the depolymerases (Hocking et al., 1995).

2) **The crystallinity of the polymer**: the degradability of a polyester decreases as the overall crystallinity or its crystalline phase perfection increases (Kumagai et al., 1992).

3) **The molecular mass of the polymer**: low-molecular-mass polymers are generally degraded more rapidly than high-molecular-mass polyesters

4) **The monomeric composition of PHA**: e.g. the rate of degradation by purified *A. faecalis* PHB depolymerase was slower for PHB than for copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (Doi et al, 1990). However, similar experiments with other PHB copolymers having 41% 3-hydroxyvalerate content as well as *in situ* studies with compost soils, showed the reverse order (Kanewasa et al., 1994; Mergaert et al., 1994). Therefore, degradation of PHA in complex ecosystems cannot be predicted from laboratory experiments using pure culture and/or purified enzymes alone. In addition, the position of the hydroxyl group, and thus the length of the side-chain, highly influences the rate of degradation; polymers of ω-hydroxy fatty acids, which do not have side-chains, are good substrates for many lipases and thus are likely to be more susceptible to biodegradation in complex ecosystems (Mukai et al., 1993).
Aim and scope of the chapter

The environmental pollution caused by discarded petrochemical plastics has inspired us for the synthesis, modification and biodegradation of PHAs. As biodegradability is one of the most prominent characteristics of PHA, their biotechnological development should always be accompanied by an investigation on their biodegradation. Hence detailed studies on bacterial PHB depolymerases have been carried out.

Poly-3-hydroxybutyrate (PHB) is the most widespread and best characterized member of PHAs. PHB is an excellent biocompatible material suitable for several biomedical applications. In this chapter, production, purification and characterization of PHB depolymerase from *Brevibacterium mcbrellneri* DP1, a natural soil isolate, was carried out. This research work has led to the purification of a PHB depolymerase which is alkalophilic as well as thermophilic enzyme. Hence it can also be considered as a good candidate for application in the composting processes for bioplastic degradation where temperature may be higher.
4.2 MATERIALS AND METHODS

Materials

PHB was obtained from Sigma (St. Louis, USA). Bushnell Haas Minerals (BHM) medium, Luria Agar, electrophoresis grade acrylamide and bis-acrylamide were obtained from Hi-media Laboratories Pvt. Ltd. (India). Sephadex G-100 powdered matrix was obtained from Sigma (St. Louis, USA). Protein molecular weight marker PMWH was obtained from Bangalore Genei (India).

4.2.1 Isolation of PHB degrading organisms

PHB degrading cultures were isolated from various soil samples collected from garbage areas. The standard liquid enrichment technique using BHM medium in 250 ml Erlenmeyer flasks containing 100 ml medium with PHB (0.1% w/v) as sole carbon source was used for isolation. The BHM medium contains (g/l): MgSO₄.7H₂O 0.20; CaCl₂ 0.02; KH₂PO₄ 1.00; K₂HPO₄.3H₂O 1.00; NH₄NO₃ 1.00; FeCl₃ 0.005. These flasks were incubated on a rotary shaker at 37 °C till the growth was observed. Isolation of PHB degrading organisms was carried out by streaking the loop full of broth onto Luria agar plate. Morphologically different colonies were selected and screened for PHB depolymerase production.

4.2.2 Screening for PHB depolymerase producers:

Media used for the screening and cultivation of PHB depolymerase producer composed of (g/L): Bushnell Hass minerals medium 3.27; PHB 1; agar powder, 30. The ability of isolates to produce PHB depolymerase was determined by inoculating them on PHB agar plates. The isolates showing transparent halos on PHB agar plates were transferred onto Luria agar slants and preserved. The isolates were then tested
for their ability to hydrolyze PHB in liquid culture at 37 °C and 150 rpm on environmental shaker in BHM medium having PHB as a sole carbon source. Four isolates DP1, DP2, DP3 and DP4 showing zone of clearance on PHB agar plate were transferred on Luria agar slants and preserved at 4 °C. Cultures were preserved by periodic transfer on Luria agar slants.

4.2.3 Inoculum preparation

The cultures from slants were transferred to 50 ml sterile Luria broth (Hi-media, India), in 100 ml Erlenmeyer flask and incubated on a rotary shaker at 150 rpm at 37 °C. Overnight grown cultures, were centrifuged and the pellet containing cells were washed twice with sterile distilled water. The cell growth was measured at 660 nm. The cells were then inoculated to 100 ml BHM medium in 250 ml flasks containing PHB (0.1 % w/v) as a sole carbon source.

4.2.4 PHB depolymerase production and extracellular protein content of PHB degrading bacterial isolates

Studies on biodegradation of polymer were carried out using the Bushnell Hass minerals medium containing PHB (0.1% w/w) as a sole source of carbon for the growth of microorganisms. Inoculums were prepared as mentioned above and the flasks were inoculated with DP1, DP2, DP3 and DP4 separately and incubated at 37 °C under shaking as well as static condition. Samples were withdrawn at regular time interval of 24 h over a period of 10 days. The samples were analyzed for PHB depolymerase activity and protein production.
4.2.5 PHB depolymerase assay and protein estimation

PHB depolymerase was assayed from the culture supernatant by measuring the hydrolysis of PHB granules leading to a decrease in turbidity at 660 nm. The assay mixture (3 ml) contained 0.6 ml of PHB granules (300 µg) suspension, 0.5 ml Tris-HCl buffer (100 mM, pH 9.0), and 1.4 ml distilled water. The reaction was initiated by adding 0.5 ml enzyme solution. One unit of PHB depolymerase activity was defined as the degradation of 1 µg of PHB per min (Jendrossek et al., 1993; Kita et al., 1995). Protein estimation was carried out using Folin phenol reagent (Lowry et al., 1951).

4.2.6 Identification of isolate DP1:

The culture was characterized using Biolog Phenotypic Microarray kit on account of its biochemical properties. The culture was grown on Luria agar plates for 24 h and then inoculated in Biolog PM plates. The plates were incubated for 24 h and the results were entered in the software provided to get the probable homology between the test culture known genera of microorganisms, using standard data.

4.2.7 Optimization of cultural conditions for maximum PHB depolymerase production by Brevibactrium mcbrellneri DP1

4.2.7.1 Optimization of pH

The effect of initial pH of the production medium on PHB depolymerase production was tested by growing B. mcbrellneri DP1 with 0.1% (w/v) PHB as sole carbon source in BHM medium having pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0.
4.2.7.2 Optimization of temperature

PHB depolymerase production was carried out at 30 °C, 37 °C and 45 °C with the initial pH of 7.0 in BHM containing 0.1% (w/v) PHB as sole source of carbon.

4.2.7.3 Optimization of substrate concentration

To check the effect of substrate (PHB) concentration on PHB depolymerase production, B. mcbrellneri DP1 was grown in BHM medium containing different PHB concentrations; 0.05%, 0.1%, and 0.15%, 0.2%.

4.2.8 PHB depolymerase purification

4.2.8.1 Enzyme concentration

The entire purification procedure was carried out at 4 °C unless specified. All buffer and solutions were prepared in distilled water. BHM medium (500 ml) containing 0.1% w/v PHB was inoculated. Cells were harvested by centrifugation and supernatant was used for enzyme purification. Ammonium sulphate was added with constant stirring at 4 °C to the supernatant to give a final concentration of 20% saturation. The mixture was then incubated at 4 °C for 1 h. The precipitated proteins were collected by centrifugation at 9000 rpm for 20 min at 4 °C and resuspended in a minimum amount of 100 mM Tris-HCl buffer. The solution was dialyzed (using cellulose dialysis bag) for 24 h against same buffer pH 9. The precipitated proteins were assayed for PHB depolymerase activity and total protein content and used for further purification by gel permeation chromatography.
4.2.8.2 Gel permeation chromatography

Sephadex G-100 powdered matrix (1 g) was regenerated for overnight in Milli Q water and packed in the column. The concentrated protein (2 ml) was loaded on to the column. Same Tris-HCl buffer (100 mM) was used as the mobile phase. The flow rate was maintained at 30 ml h⁻¹. Fractions of 1 ml each were collected and analyzed for its protein content and PHB depolymerase activity. The purified enzyme was used further for characterization studies.

4.2.8.3 Denaturing gel electrophoresis

SDS-PAGE was carried out in a vertical slab gel apparatus (Bangalore Genei, India). Stacking gel of 5% and resolving gel of 10% acrylamide concentration (1mm thickness) contained 0.1% (w/v) SDS. The samples were pre-incubated in boiling water bath for 3 min with sample buffer containing 8 M Urea, 2% (w/v) SDS, 20% (v/v) glycerol, 50 mM DTT, 0.05% (w/v) bromophenol blue, in the ratio of 2:1 (sample: sample buffer). Electrophoresis was carried out at room temperature using Laemmli buffer system (Laemmli, 1970). Protein bands were visualized by silver staining. PMWH (Bangalore Genei, India) used as a protein molecular weight standard contained Phosphorylase B (Mr 97,400 Da), Bovine serum albumin (Mr 66,000 Da), Egg albumin (Mr 43,000 Da), Carbonic anhydrase (Mr 29,000 Da), Trypsin soyabean inhibitor (Mr 22,100 Da) and Lysozyme (Mr 14,300 Da).

4.2.9 Characterization of PHB depolymerase

4.2.9.1 Effect of pH on PHB depolymerase activity

The optimum conditions for enzyme activity were determined using purified PHB depolymerase by turbidimetric PHB assay as described before. For determination of
effect of pH on PHB depolymerase activity, suspensions of 0.5 mg/ml PHB were prepared in the following buffers (100 mM) at 1.0 pH intervals: Phosphate (pH 6.0-8.0), Tris-HCl (pH 9.0) and Glycine-NaOH (pH 10.0 – 12.0).

4.2.9.2 pH stability of PHB depolymerase

The pH stability of PHB depolymerase was determined by pre-incubating the enzyme at pH 9 upto 100 min. The samples were withdrawn after every 10 min and residual activity was measured by depolymerase assay as mentioned previously.

4.2.9.3 Effect of temperature on PHB depolymerase activity

The effect of temperature on PHB depolymerase activity from *B. mcbrellneri* DP1 was determined by performing the assay at various temperature in the range of 30 - 80 °C.

4.2.9.4 Thermal stability of PHB depolymerase

For determination of thermal stability, the enzyme was pre-incubated at 40 °C temperature for 100 min and samples were withdrawn at desired time interval. The residual activity was measured under standard assay condition.

4.2.9.5 Enzyme kinetics

Aliquotes of PHB stock solution (500 µg/ml) were taken so as to get different PHB concentrations (25 µg - 100 µg) in 3 ml assay system containing 0.5 ml of 100 mM Tris-HCl buffer (pH 9.0), 0.5 ml of purified PHB depolymerase and distilled water so as to make the final volume (3 ml) of the assay system. $K_m$ and $V_{max}$ of the PHB depolymerase was obtained from the Lineweaver-Burk double reciprocal analysis and the activation energy (Ea) was determined using Arrhenius plot.
4.2.9.6 Thermodynamics of PHB hydrolysis

The thermodynamic parameters for substrate hydrolysis were calculated using the Eyring’s absolute rate equation derived from the transition state theory.

\[ K_{\text{cat}} = \left( \frac{k_b T}{h} \right) \times e^{-\left( \frac{\Delta H^*}{RT} \right)} \times e^{\left( \frac{\Delta S^*}{R} \right)} \]

Where,

- \( k_b \) Boltzmann’s constant \((\text{R}/\text{N}) = 1.38 \times 10^{-23} \text{ J K}^{-1}\)
- \( T \) Absolute temperature (K)
- \( h \) Planck’s constant = 6.626 \times 10^{-34} \text{ Js}
- \( R \) Gas constant=8.314 \text{ J K}^{-1} \text{ mol}^{-1}
- \( \Delta H^* \) Enthalpy of activation
- \( \Delta S^* \) Entropy of activation

\[ \Delta H^* = E_a - RT \]  \hspace{2cm} (2)

\[ \Delta G^* \text{(free energy of activation)} = -RT \ln \left( \frac{k_{\text{cat}} h}{k_b \times T} \right) \]  \hspace{2cm} (3)

\[ \Delta S^* = \left( \Delta H^* - \Delta G^* \right) / T \]  \hspace{2cm} (4)

The free energy of substrate binding and transition state formation was calculated using the following derivations:

\[ \Delta G^* \text{E-S (free energy of substrate binding)} = -RT \ln Ka \]  \hspace{2cm} (5)

where \( Ka = 1/K_m \)

\[ \Delta G^* \text{E-T (free energy for transition state formation)} = - RT \ln(k_{\text{cat}}/K_m) \]  \hspace{2cm} (6)
4.2.9.7 Effect of metal ions and inhibitors on PHB depolymerase activity

Effect of different metal ions (5 mM) namely; Zn$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, Cu$^{2+}$, Fe$^{3+}$, and Hg$^{2+}$ and different inhibitors (5 mM) namely; phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), ethylenediaminetetraacetate (EDTA) and β-mercaptoethanol (β-ME) on PHB depolymerase activity was checked. The metal ions and inhibitors were pre-incubated with the purified PHB depolymerase in 100 mM Tris-HCl buffer (pH 9.0) for 15 min at 37 °C. Enzyme activity was determined by the standard PHB turbidimetric assay.

4.2.10 Biodegradation studies of natural and synthetic polymer

For degradation studies the pre-weighed natural polymer i.e. nitrile rubber and synthetic polymer weighing 0.50 g and 10 mg, respectively, as per the availability of the individual polymer, were kept in a 100 g autoclaved soil. PHB depolymerase enzyme containing culture supernatant of 4 days grown culture was inoculated to the soil to achieve 50 % moisture. Culture supernatant was inoculated at regular time interval to maintain its moisture level. Percent weight loss of the polymer was calculated after 60 days considering the respective initial weight of the polymer taken for the analysis (Ramsay et al., 1993). Scanning electron microscopy (Philips XL30 ESEM TMP + EDAX) of the polymers before and after degradation was carried out in order to observe the changes in the surface morphology of the polymer due to degradation.


4.3. RESULTS AND DISCUSSION

4.3.1 Isolation and screening of PHB degrading organisms

All the different types of synthetic and natural plastic based waste products are frequently disposed in soil, water or sewage and hence soil was chosen to isolate PHB degrading microorganism. After the enrichment process in BHM medium having 0.1% w/v PHB as sole carbon source, the organisms were isolated onto agar plate containing PHB. PHB degrading organisms were isolated by means of clear halos surrounding the colonies. Screening of cultures on the basis of their PHB depolymerase production yielded four potent cultures; DP1, DP2, DP3, and DP4.

4.3.2 PHB depolymerase production and extracellular protein content of PHB degrading bacterial isolates

The studies on production of PHB depolymerase were carried out in shaking as well as static conditions which provide different oxygen availability to organism during the growth. Four isolates having the PHB degradation capability were studied for PHB depolymerase production under shaking and static condition. From the Fig. 4.4 and 4.6, it can be observed that DP1, DP2, DP3 and DP4 showed maximum PHB depolymerase production between 4th to 6th day in shaking condition. As compared to static condition, maximum PHB depolymerase was obtained in shaking condition.
Figure 4.4: PHB depolymerase production by different bacterial isolates under shaking condition

Figure 4.5: Extracellular protein content of different bacterial isolates under shaking condition
For the survival in the presence of only PHB, organisms might be producing enzymes other than PHB depolymerase, hence extracellular protein concentration in the medium was also measured. Higher extracellular protein content was obtained in
PHB depolymerase and biodegradation

shaking condition, compared to static condition (Fig. 4.5 and 4.7). Among four isolates; the DP1 was found to be the most potential PHB degrader producing PHB depolymerase of 6.42 U/ml on 4th day, hence it was selected for further studies for PHB depolymerase production. Many organisms that degrade PHB have been isolated and characterized (Jendrossek et al., 1993; Briese et al., 1994; Mergaert et al., 1993).

4.3.3 Identification of isolate DP1

Gram’s staining of DP1 revealed its gram positive natures. The culture was characterized using Biolog Phenotypic Microarray kit on account of its biochemical properties. It showed 97% similarity with *Brevibacterium mcbrellneri*. So it was identified and is referred further as *Brevibacterium mcbrellneri* DP1 in this study.

![Figure 4.8: Brevibacterium mcbrellneri DP1 showing zone of clearance on PHB agar plate](image)

4.3.4 Optimization of cultural conditions for maximum PHB depolymerase production by *B. mcbrellneri* DP1

The conditions of the disposal sites for the PHB/PHA based products may vary with respect to pH and temperature. It is therefore essential to investigate the influence of
these parameters on depolymerase production and PHB degradation. Only few reports are available for PHB depolymerase production by bacterial culture.

4.3.4.1 Optimization of pH

pH of contaminated site influenced the degradation process so it is necessary to study effect of initial pH of medium. Effect of initial medium pH on PHB depolymerase production is shown in Fig. 4.9.

![Figure 4.9: Effect of initial medium pH on PHB depolymerase production by B. mcbrellneri DP1](image)

When *B. mcbrellneri* DP1 was inoculated in BHM having different initial pH ranging from 4 – 9 to study the effect of pH on PHB depolymerase production it was observed that medium pH 4.0 - 9.0 supported the production of PHB depolymerase by *B. mcbrellneri* DP1 and maximum PHB depolymerase activity of 9.55 U/ml was obtained at 7 pH (Fig. 4.9). A thermophilic *Streptomyces* sp. is reported to have maximum PHB depolymerase production at pH 7.5 (Calabia and Tokiwa, 2006).
4.3.4.2 Optimization of temperature

Temperature is the very important factor which affects the degradation of PHB. Thus effect of temperature on PHB depolymerase production was studied.

![Graph showing the effect of temperature on PHB depolymerase production](image)

**Figure 4.10: Effect of temperature on PHB depolymerase production by *B. mcbrellneri* DP1**

PHB depolymerase production at different temperatures was examined which showed maximum production of 8.98 U/ml at 37 °C (Fig. 4.10). Most of the reports suggested PHB depolymerase production between 30 - 40 °C.

Both the pH and temperature studies have shown results which are highly significant as far as possible applications of the isolated culture for PHB/PHA degradation under natural conditions is concerned. The soil, water and other ecological niches where disposed plastics accumulate might have similar prevailing conditions.
4.3.4.3 Optimization of substrate concentration

PHB was provided at the concentration of 0.05, 0.1, 0.15 and 0.2 (% w/v) for PHB depolymerase production. Maximum PHB depolymerase production 9.3 U/ml was achieved in presence of 0.1% w/v PHB concentration by *B. mcbrellneri* DP1 (Fig. 4.11).

![Graph showing the effect of substrate concentration on PHB depolymerase production by B. mcbrellneri DP1](image)

**Figure 4.11: Effect of substrate concentration on PHB depolymerase production by *B. mcbrellneri* DP1**

4.3.5 Purification of PHB depolymerase produced by *B. mcbrellneri* DP1

A three step protein purification protocol was followed. These three steps included; total protein precipitation using ammonium sulphate, dialysis and the gel permeation chromatography of the protein using Sephadex G-100 as the final step. The PHB depolymerase from *B. mcbrellneri* DP1 was effectively purified with an overall yield of 83.40% at the end of the three steps as compared to initial culture filtrate activity. Ammonium sulphate was added to the filtered broth which reduced the water activity around the proteins and led to precipitation of proteins. A fold purification of 1.48
(Table 4.3) was achieved at the end of ammonium sulphate precipitation. This concentrated protein was loaded on the column filled with Sephadex G-100. Fractions of 1 ml were collected and the enzyme was finally purified to 2.73 fold with yield of 83.40%. The purification results of PHB depolymerase from *B. mcbrellneri* DP1 are summarized in Table 4.3. The homogeneity of purified enzyme was checked using SDS-PAGE analysis as shown in figure 4.12. A single band of about 39 kDa was obtained on denaturing polyacrylamide gel. PHB depolymerase was purified from different bacteria as well as from fungal cultures by other workers. The molecular weight of PHB depolymerases was found to be 48 kDa for *Paecilomyces lilacinus* D218 and *Emericellopsis minima* W2 (Oda et al., 1997; Kim et al., 2002). Iwata and Doi, (1997) purified a 60 kDa PHB depolymerase from *Pseudomonas stutzeri* YM1006 with 4.1 fold purification.

**Table 4.3: Purification of PHB depolymerase of *B. mcbrellneri* DP1**

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total Units</th>
<th>Total Protein</th>
<th>Specific activity</th>
<th>Fold Purification</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>9.10</td>
<td>62</td>
<td>0.146</td>
<td>1.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation.</td>
<td>8.28</td>
<td>38</td>
<td>0.217</td>
<td>1.48</td>
<td>90.98</td>
</tr>
<tr>
<td>Gel permeation chromatography</td>
<td>7.59</td>
<td>19</td>
<td>0.399</td>
<td><strong>2.73</strong></td>
<td><strong>83.40</strong></td>
</tr>
</tbody>
</table>
4.3.6 Characterization of PHB depolymerase

4.3.6.1 Effect of pH on PHB depolymerase activity

The optimum conditions for enzyme activity were determined using purified PHB depolymerase by turbidimetric PHB assay as described before. Optimum pH for PHB depolymerase was found to be pH 9 with maximum activity of 21.61 U/ml. PHB depolymerase from *B. mcbrillneri* DP1 showed good activity within the range of pH 7 -10 (Fig. 4.13).

**Figure 4.12:** Silver stained 10% SDS polyacrylamide gel; Lane1: protein marker, Lane2: purified PHB depolymerase
Figure 4.13: pH optima of PHB depolymerase from *B. mcbrellneri* DP1

PHB depolymerase from *Streptomyces* sp. MG has optimum pH 8.5 (Calabia and Tokiwa, 2006). The optimum pH for PHB depolymerases from other bacteria is reported to be between pH 7.5 to 9.5 (Jendrossek et al., 1993; Kita et al., 1995; Nakayama et al., 1985).

**4.3.6.2 pH stability of PHB depolymerase**

PHB depolymerase from *B. mcbrellneri* DP1 was stable upto 60 min with 18.11 U/ml activity and 19% residual activity at pH 9 (Fig. 4.14). After 60 min, with increase in time activity of PHB depolymerase decreased.
4.3.6.3 Effect of temperature on PHB depolymerase activity

PHB depolymerase from *B. mcbrellneri* DP1 showed maximum activity at 40 °C (21.73 U/ml). PHB depolymerase was also found to be active at higher temperature such as 50 °C and 60 °C with 21.44 U/ml and 19.15 U/ml activities respectively (Fig. 4.15). PHB depolymerase from *Streptomyces exfoliates* K10 is reported to have temperature optima of 40 °C (Klingbeil et al., 1996).
4.3.6.4 Thermal stability of PHB depolymerase

For checking of thermal stability, the enzyme was pre-incubated at temperature 40 °C for 100 min. As shown in Fig. 4.16, PHB depolymerase from *B. mcbrellneri* DP1 was found to be stable upto 60 min without significant loss of activity at 40 °C. Our result is in accordance with Asano and Watanabe, (2001), who reported PHB depolymerase from *Arthrobacter* sp. W6 being stable at 40 °C for 1 h.

![Graph showing thermal stability of PHB depolymerase from B. mcbrellneri DP1](image)

**Figure 4.16: Temperature stability of PHB depolymerase from *B. mcbrellneri* DP1**

The enzyme in present study showed pH optima of 9.0 and temperature optima of 40 °C, indicating that it is an alkali tolerant as well as thermo tolerant enzyme. The purified PHB depolymerase from *E. minima* possess the pH optima of 9.0 with temperature optima at 55 °C (Kim et al., 2002). Generally all the reported fungal PHB depolymerases possess the temperature optima between 40 ° to 60 °C (Kim and Rhee, 2003). The purified PHB depolymerase showed stability in range of pH and temperature thereby making *B. mcbrellneri* DP1 a potent organism to be used for
PHB degradation in various natural environments.

**4.3.6.5 Enzyme kinetics**

Estimated Km is 0.5 mg/ml and Vmax is 0.038 µM/min (Fig. 4.17). Higher Km value than Vmax indicates that more substrate is required. The Kcat (turnover number) of PHB depolymerase was 0.12 s⁻¹ (Table 4.4). PHB depolymerase from *B. mcbrellneri* DP1 can produce up to 0.12 molecule of product per second. The specificity constant Kcat/Km for PHB depolymerase was 0.24. The value of specificity constant indicates the specificity of enzyme for substrate. Lower value of specificity constant indicates that specificity of enzyme to PHB is low. The catalytic efficiency value (Vmax/Km) of PHB depolymerase was 0.076 µmoles. Enzyme reduce 0.076 µmoles product per sec per mg substrate. The catalytic efficiency value provides a useful model for selecting the most efficient enzyme.

![Figure 4.17: Lineweaver-Burk plot for PHB depolymerase](image_url)
PHB depolymerase and biodegradation

Figure 4.18: Arrhenius plot for PHB depolymerase

The effect of temperature on an enzymatic reaction can be analyzed through the Arrhenius equation by plotting the velocity (log$_{10} V$) against the inverse of temperature (1/T expressed in K$^{-1}$). The activation energy (Ea) was determined as 1.703 kcal/mol (Fig. 4.18).

Table 4.4: Kinetic constants of PHB depolymerase

<table>
<thead>
<tr>
<th>Km (mg/ml)</th>
<th>Vmax (µM/ml/min)</th>
<th>Kcat(s$^{-1}$)</th>
<th>Kcat/Km</th>
<th>Vmax/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.038</td>
<td>0.12</td>
<td>0.24</td>
<td>0.076</td>
</tr>
</tbody>
</table>

4.3.6.6 Thermodynamic parameters of PHB hydrolysis using *B. mcbrellneri* DP1

**PHB depolymerase**

The enthalpy of activation ($\Delta H^*$), Gibbs free energy ($\Delta G^*$) and entropy of activation ($\Delta S^*$) for PHB hydrolysis by PHB depolymerase of *B. mcbrellneri* DP1 were calculated as 4.523 KJmol$^{-1}$, -416.619 KJmol$^{-1}$ and 3.193 KJmol$^{-1}$K$^{-1}$, respectively (Table 4.5). The lower enthalpy value of PHB depolymerase indicated that the
formation of transition state or activated complex between enzyme-substrate was very efficient. Negative ΔG* value suggested that the conversion of its transition complex into products was spontaneous. Positive ΔS* indicated the disorderliness of the reaction. The feasibility and extent of a chemical reaction is best determined by measuring change in Gibbs free energy (ΔG*) for substrate hydrolysis, i.e. the conversion of E-S complex into products (Muhammad et al., 2007). The free energy for activation of substrate binding (ΔG*_{E-S}) and the free energy for the formation of activation complex (ΔG*_{E-T}) were 1.804 KJmol⁻¹ and 3.715 KJmol⁻¹, respectively.

**Table 4.5: Thermodynamic parameters of PHB hydrolysis**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔH*(Enthalpy)</td>
<td>4.523 KJmol⁻¹</td>
</tr>
<tr>
<td>ΔS(Entropy)</td>
<td>3.193 Jmol⁻¹K⁻¹</td>
</tr>
<tr>
<td>ΔG* (Free Energy for Activation)</td>
<td>-416.619 KJmol⁻¹</td>
</tr>
<tr>
<td>ΔG*_{E-S} (Free Energy for substrate binding)</td>
<td>1.804 KJmol⁻¹</td>
</tr>
<tr>
<td>ΔG*_{E-T} (Free energy for transition state formation)</td>
<td>3.715 KJmol⁻¹</td>
</tr>
</tbody>
</table>

### 4.3.6.7 Effect of metal ions on PHB depolymerase activity

The activity of PHB depolymerase was markedly inhibited by Hg²⁺, Fe³⁺, and Cu²⁺ (Fig. 4.19). On the other hand Ca²⁺ and Mg²⁺ stimulated the enzyme activity by 10 to 15 % (Fig. 4.19).
Figure 4.19: Effect of metal ions on PHB depolymerase activity

Oda et al., (1997) also obtained similar effect on PHB depolymerase from *Paecilomyces lilacinus* D218. Many PHB depolymerases are reported to be activated by Ca²⁺ (Jendrossek et al., 1996).

**4.3.6.8 Effect of inhibitors on PHB depolymerase activity**

Inhibition by phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), β-mercaptoethanol (β-ME) (Fig. 4.20) showed the dependence of sulphydryl and disulfide bonds on PHB depolymerase activity from *B. mchrellneri* DP1. Inhibition by PMSF indicated that PHB depolymerase probably has serine residues at the active site.
PHB depolymerase from *E. minima* W2 and *Streptomyces* sp. was also inhibited by PMSF (Kim et al., 2002; Calabia and Tokiwa, 2006).

### 4.3.7 Biodegradation studies of natural and synthetic

Biodegradation of natural and synthetic polymer by *B. mcbrellneri* DP1 has been studied. Scanning electron micrographs of natural and synthetic polymer both for control and after degradation studies are shown in Fig. 4.21 a, b, c and d.

### Table 4.6: Degradation of polymers in terms of % weight loss

<table>
<thead>
<tr>
<th>Natural Polymer (experimental)</th>
<th>Synthetic polymer (experimental)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.769%</td>
<td>0%</td>
</tr>
</tbody>
</table>
It is clearly seen from SEM that the smooth surface of polymer is modified after 60 days of incubation. The slightly smooth appearance of natural polymer (Fig. 4.21a) is modified to the clustered irregular structure (Fig. 4.21b). Similar observation for synthetic polymer has been made; smooth structure (Fig. 4.21c) of synthetic polymer was converted to flaky structure (Fig. 4.21d). In case of synthetic polymer weight loss was not observed but scanning electron micrograph confirmed the microbial action and possible biodegradation of natural and synthetic polymers by *B. mcbrellneri* DP1.
after 60 days. In a similar study scanning electron micrographs of the PHBV film buried in soil mixed with sewage sludge for 120 days showed clear evidences of degradation with pits, surface roughening, grooves, cavities and disintegration of film (Shah, 2007). The biodegradation behavior of polyhydroxybutyrate film in the presence of *Pseudomonas mendocina* DS04-T was studied by Wang et al. (2013).

**4.4 CONCLUSION**

Though many microorganisms act in consortia for the degradation of biodegradable plastics in natural environment, bacteria seem to play an important role in PHA-based plastics degradation under environmental condition. Results of production and biochemical characterization of PHB depolymerase from *B. mchrellneri* DP1, showed that this culture and enzyme produced possessed a number of distinct properties which make their application possible in PHA degradation in natural condition. It was also able to degrade natural polymer and synthetic polymer up to some extent. They have not only acted on bacterial polyesters, i.e. poly(3-hydroxybutyric acid) but also on natural and synthetic polymers in natural environment.