CHAPTER 4
PHYSICAL CHARACTERIZATION OF PHA FROM
BACILLUS THURINGIENSIS R1
4.1 INTRODUCTION

PHB and its co-polymers have been industrially produced since 1982 as substitutes for petroleum based plastics. Global environment concerns and solid waste management problems have generated much interest in the development of biodegradable plastics that retain the desired physical and chemical properties of the conventional synthetic plastics. Non-degradability and persistence in the environment of the fossil fuel based plastics has prompted the scientific community to look for degradable alternatives with comparable properties. The discovery of naturally occurring biodegradable polymers like polyhydroxyalkanoates (PHAs), polylactides, polyglycolic acids, polysaccharides and their blends has opened avenues for their utilization (Leaversuch 1987; Dawes 1990; Chum 1991; Steinbüchel 1991; Graham 1993; Chang 1994).

A better understanding of the physical and thermal properties of biopolymers will help us design these with improved properties for commercial purpose. Poly(3-hydroxybutyrate)P(3HB), the most widely spread PHA belonging to the short-chain-length (scl) family, is highly crystalline, hard and brittle (Holmes 1988; Doi 1990; Hocking and Marchessault 1994). Medium-chain-length (mcl) PHAs on the other hand have a low degree of crystallinity and are soft and sticky (Gross et al. 1989; Marchessault et al. 1990; Preusting et al. 1990). These properties limit the applications of mcl-PHAs as thermoplastic materials (Hammond and Liggat 1995; Amass et al. 1998). In addition to this medium chain length PHAs have been found unsuitable for applications due to their low melting and decomposition temperature and low glass transition temperatures (Gross et al. 1989; He et al. 1999). It has been shown that copolymers of various PHAs possess superior thermal and mechanical properties than the homopolymers (Steinbüchel 1991). Analytical techniques such as GC, NMR, FTIR and improving upon the protocols from time to time has helped to identify, characterize and quantitate PHAs in relatively quick time (Riis and Mai 1988; Rozsa et al. 1996; Hong et al. 1999; Misra et al. 2000). Though, many organisms produce PHB, it’s properties differ depending upon the organism producing it (Van der Walle et al. 2001). This necessitates physical characterization of the polymer from *Bacillus thuringiensis* R1.
4.2 EXPERIMENTAL PROCEDURES

4.2.1 Organisms and growth

*Bacillus thuringiensis* R1 grown and maintained as enumerated in Chapter 2.

4.2.2 Recovery of the Biopolymer from the cells

This is enumerated in section 3.2.5 of chapter 3.

4.2.3 Scanning electron microscopy

PHA sample purified from *B. thuringiensis* R1 cells was sonicated in water at 20 KHz for 3 cycles of 5 min. each. A drop of the suspension was dried on a brass stub under an IR lamp and later coated with gold using Polaron sputter unit. SEM photographs were taken with a LEICA Stereoscan 440 Scanning electron microscope equipped with a Phoenix EDAX attachment.

4.2.4 $^1$H NMR spectroscopy

The $^1$H NMR of the extracted polymer was carried out using Bruker Ac200 at 24°C (Rozsa *et al.* 1996). The polymer was solubilized in CDCl$_3$. The samples were analysed in 5mm sample tubes in chloroform-d. The spectra were referenced to internal tetramethylsilane.

4.2.5 FTIR analysis

The polymer sample (2 mg) was thoroughly mixed with KBr (Spectroscopic grade). KBr treated pellet (15 mg) was dried at 100°C for 4 h (Misra *et al.* 2000). FTIR spectrum was taken using a Perkin Elmer (USA) model 1720 Fourier Transform IR spectrometer.

4.2.6 Determination of molecular weight and molecular weight distribution by GPC

The molecular weight and molecular weight distribution of the polymer was determined by gel-permeation chromatography (GPC) using a Waters 515 pump with four Stryagel HR columns. Monodisperse polystyrene and chloroform were used as
the molecular weight standard and the mobile phase respectively. For each analysis 250 μl of 0.1% (w/v) polymer solution in CHCl₃ was injected.

4.2.7 Differential Scanning Calorimetry

To determine the morphological state of the polymer, the melt temperature and the enthalpy of fusion were measured using a Perkin Elmer Differential Scanning Calorimeter (DSC-7). Samples (10-15 mg) were encapsulated in aluminium pans and heated from 0 to 200°C at a rate of 20°C/min. The melting temperature (T_m) was taken at the peak of the melting endotherm.

4.2.8 Thermogravimetric analysis of PHA

Thermogravimetric analysis of the polymer sample was done using Rheometric TG analyser (Rheometric Scientific, USA). The analysis was carried out under nitrogen flow rate of 15 ml/min. with scanning rate of 10°C/min. The temperature was increased until weight loss was 20%. After cooling rapidly to room temperature (100°C/min), the residue was recovered. This residue was completely soluble in chloroform.

4.2.9 Purification of inclusion bodies from Bacillus thuringiensis R1

Purification of the inclusion bodies from the cell lysate of Bacillus thuringiensis R1 was done with modifications of the extraction procedure described by McCool et al. (1996). Briefly, cells were pelleted and resuspended in a minimum volume of 10mM Tris-HCl pH 8.0, 1 mM EDTA, 20 mM MgSO₄, 0.25 M sucrose at 4°C. Lysozyme was added to a final concentration of 1 mg/ml followed by incubation at 37°C for 1 hour. To ensure proper lysis, cells were treated with NaOCl (30%). Aliquots of 1.8 ml of cell lysate were loaded on sucrose step gradient in centrifuge tubes (Beckman) and consisted of 1.5 ml each of the following sucrose concentrations 2.00 M, 1.66 M, 1.33 M, 1.00 M and 1.0 ml of 0.66 M in TE (10mM Tris-HCl pH 8, 1 mM EDTA). The tubes were centrifuged at 30,000 rpm for 2 hours. The inclusion bodies, which banded about mid tube were collected, washed in 20 volumes of TE and stored at 4°C.
4.2.10 Separation of polypeptides associated with PHA inclusion bodies

Inclusion bodies were purified as described above followed by suspension in 1x TE buffer with 2% SDS. An equal volume of sample buffer was added prior to boiling for 5 min., and samples were centrifuged for 3 min. to pellet PHA, the supernatant was loaded on an SDS 10% polyacrylamide gel (as described in Section 2.9) and run at 20 mA for 2 h. The gel was stained by Coomassie blue staining procedure.

4.3 RESULTS AND DISCUSSION

PHB granules isolated from the B. thuringiensis cells observed under scanning electron microscope showed stable spherical configuration with an average diameter of 5 microns (Fig. 4.1).

Fig. 4.1 Scanning electron microscopy (SEM) of the gold coated PHB granules obtained from Bacillus thuringiensis R1. PHB granules of uniform spherical shape with a stable configuration are seen. The size of the granule is 5 µm. Bar represents 50 µm.
The NMR spectra identified the polymer as an isotactic homopolymer (Fig. 4.2). The spectrum revealed the presence of three groups of signals characteristic of PHB homopolymer. The doublet at 1.3 ppm was attributed to the methyl group coupled to one proton; the doublet of the quadruplet at 2.57 ppm to the methylene group adjacent to an asymmetric carbon atom bearing a single proton and the multiplet at 5.28 ppm to the methyne group. Chloroform-d gave a chemical shift signal at 7.25 ppm. The NMR signal multiplicity by a proton as a quadruplet or octet in case of protons of CH$_2$ group was obtained due to proton coupling in isotactic form unlike in syndiotactic where duplet signal is obtained due to coupling (Fig.3). Similar spectral signatures have been reported for PHB isolated from *Bacillus cereus* (Labuzek *et al.* 1994) and *Bacillus circulans* (Rozsa *et al.* 1996).

Fig. 4.2 $^1$H NMR spectra of the PHB recovered from *Bacillus thuringiensis* R1 showing a doublet, doublet of quadruplet and a multiplet at 1.3, 2.57 and 5.28 ppm which are due to proton coupling. The signal at 7.25 ppm is characteristic of chloroform-d.
Fig. 4.3 Arrangement of molecules and pendant groups in an isotactic and syndiotactic PHB are shown. In isotactic form the pendant group is always away from the main chain and are arranged towards one side of the plain unlike in syndiotactic wherein the pendant groups are alternately arranged.
Hong et al. (1999) communicated a rapid method to identify and differentiate cells producing scl- or mcl- PHA using FTIR. FTIR analysis of PHA extracted from *Bacillus thuringiensis* R1 cells revealed two absorption bands at 1280 cm\(^{-1}\) and 1735 cm\(^{-1}\) corresponding to C=O and C-O stretching groups respectively (Fig. 4.4). Rozsa et al. (1996) have shown that the FTIR absorption band at about 1730 cm\(^{-1}\) is a characteristic of carbonyl group and a band at about 1280-1053 cm\(^{-1}\) characterizes the valence vibration of the carboxyl group. These are characteristics of the polyhydroxybutyrate.

Fig. 4.4 FTIR spectra of polymer extracted from *Bacillus thuringiensis* R1. The absorption bands at 1280 and 1735 cm\(^{-1}\) correspond to C=O and C-O of PHB, respectively.
The thermodynamic properties of a polymer are dependent on its number-average molecular mass and the bulk properties connected with large deformations are largely determined by weight-average molecular mass (Van Krevelen 1972). Molecular weight of PHB is dependent on the physiological background or on the abundance of PHA synthase present within the cell (Rehm and Steinbüchel 1999; Madison and Huisman 1999). The GPC analysis revealed that the polydispersity index (Q) (defined as Mn/Mw), number average molecular weight (Mn), weight average molecular weight (Mw) were 1.77, $5.8526 \times 10^4$ and $1.0385 \times 10^5$ respectively (Fig. 4.5).

![Gel permeation chromatography of the purified PHB sample from Bacillus thuringiensis R1](image-url)

**Fig. 4.5** Gel permeation chromatography of the purified PHB sample from *Bacillus thuringiensis* R1
The melting temperature and the enthalpy of fusion of the polymer were 165.6°C and 84.1 J/g respectively. The polymer degraded rapidly between 225°C and 270°C with a peak at 261°C (Fig. 4.6). The high enthalpy of fusion (84.1 J/g) suggests high crystalline nature of the recovered PHB that was calculated to be of 60 - 65 % assuming the enthalpy of fusion of 100% crystalline sample to be 146 J/g. The melting temperature of PHB (165.6°C) in the present study was slightly lower than reported for PHB from *B. cereus* (170°C) (Labuzek *et al.* 1994) and *B. circulans* (173°C) (Rozsa *et al.* 1996). The difference between the melting temperature and the decomposition temperature (261°C) was high enough to facilitate processing of the polymer.

**Fig. 4.6** Differential Scanning Colorimetry of the polymer purified from *Bacillus thuringiensis* R1.
PHA inclusion bodies from *Bacillus megaterium* are known to contain 97.7% PHA, 1.87% protein and 0.46% lipid with protein and lipid forming an outer layer (Griebel et al. 1968). PhaC and PhaP are found to be the two most abundant proteins that copurify with inclusion bodies and essential for PHB accumulation in *Bacillus megaterium* (McCool and Cannon 2001). The SDS gel when stained with Coomassie blue showed two bands (~45 kDa and ~40 kDa) that may correspond to the proteins associated with inclusion bodies (Fig. 4.7).

![SDS gel](image)

**Fig. 4.7** Inclusion body associated proteins purified from *Bacillus thuringiensis* R1 cell lysate.

### 4.4 CONCLUSIONS

- SEM of the recovered polymer revealed a uniform spherical shape with a stable configuration with a granule size of 5 microns.
- NMR and FTIR studies have shown the accumulated polymer to be isotactic homopolymer of PHB when *Bacillus thuringiensis* R1 was grown on a single carbon source.
- Molecular weight of the PHB synthesized and accumulated by *Bacillus thuringiensis* R1 was found to be around $1.0385 \times 10^5$ by GPC.
- DSC and TGA results show a shift in the thermal degradation temperature pattern of the polymer, which is found to be at 261°C.
- Inclusion bodies associated proteins of ~45 kDa and ~40 kDa were purified from *Bacillus thuringiensis* R1 cell lysate.