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Monitoring the in situ crystallization of native biopolyester granules in *Ralstonia eutropha* via infrared spectroscopy

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ABSTRACT

Poly(3-hydroxybutyrate) (PHB), a representative polyhydroxyalkanoate (PHA), is a naturally occurring biopolyester stored as tiny, intracellular granules in microbial cells. In vivo, native PHB granules are amorphous, stabilized by a monolayer membrane and intra-granule water. When subjected to varying environmental conditions, the native granules may become partially crystalline. The in situ crystallinity of native PHB granules in *Ralstonia eutropha* cells suspended in aqueous solution was monitored with attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). No sample preparation was required for measurement. A major measurement error could be caused by the evaporation of water. Therefore, the infrared absorption spectra should be taken after the initial settlement of cells, but before excessive dehydration. Background interference caused by water and non-PHB biomass was constant throughout the time course of measurement, regardless of granule crystallinity. The wavenumber 1184 cm^{-1} was found to be most sensitive to the in situ crystallinity of native PHB granules.

1. Introduction

Microbial biopolyesters have gained much attention as eco-friendly alternatives to petrochemical-based plastics because they are biodegradable and can be produced from renewable feedstocks. One class of biopolyester with many potential environmentally friendly applications is polyhydroxyalkanoate (PHA). The most widely studied, representative form of PHA and focus of this work is poly(3-hydroxybutyrate) (PHB).

PHB is synthesized and stored as tiny, intracellular inclusion body granules (0.2–0.5 μm in diameter) by a wide variety of bacterial species as carbon and energy reserves (Anderson and Dawes, 1990; Byrom, 1994). In vivo, PHB granules are fully amorphous, contain a small amount of intra-granule water (5–10%), and are surrounded by a monolayer membrane composed of phospholipids and proteins (Barnard and Sanders, 1989; Horowitz and Sanders, 1994; Sudesh et al., 2000). Purified PHB, on the other hand, becomes a semi-crystalline (50–70%) material (Gunaratne et al., 2004). To recover PHB for commercial applications, the granules must be separated from the cells and purified. During recovery processes the environment of the granules changes, causing PHB to undergo varying degrees of irreversible crystallization (Barnard and Sanders, 1989; Horowitz and Sanders, 1994; Sudesh et al., 2000). Little is known about the in situ crystallization of PHB granules in their native form under changing environmental conditions. This information is important to develop more efficient recovery methods and reduce the production costs of PHA.

There are several methods to measure polymer crystallinity. Popular methods include: x-ray diffraction (XRD), differential scanning calorimetry (DSC), and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) (Barham et al., 1984; Gunaratne et al., 2004; Smith, 1999). Monitoring the instantaneous crystallinity of biopolyesters in their native aqueous environment, however, is difficult via XRD and DSC because of demands on material purity and dryness. ATR-FTIR is particularly useful for in situ measurements because no such sample preparation is necessary (Smith, 1999). That is, the infrared absorption spectra of PHB-containing cells in an aqueous solution can be measured directly to determine the instantaneous crystallinity of the native PHB granules without purifying or drying the PHB.

ATR-FTIR is an efficient analytical tool used to measure the chemical composition and structural arrangement of local molecular environments within solid, liquid, gas, semi-solid, and polymeric samples (Smith, 1999). When infrared light interacts with a polymer the energy is absorbed causing molecular vibrations at wavenumbers specific to its chemical bonds. In polyesters, for instance, two spectral regions are characteristic. The carbonyl group (C═O) absorbs infrared light around 1800–1600 cm^{-1} and the ester backbone (C–O–C) absorbs infrared light around 1300–1000 cm^{-1} (Smith, 1999). Conformational changes in a sample, such as melting and crystallization, can be seen as an increase, decrease, or shift in the characteristic absorption bands (Smith, 1999). Consequently, the infrared spectrum of PHB is information rich,

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providing a quick and convenient method to monitor conformational changes in PHB, such as crystallization.

The crystallization of pure PHB describing the material transition from an amorphous melt to a semi-crystalline solid has been studied by many research groups (Barham et al., 1984; Gunaratne et al., 2004; Padermshoke et al., 2005; Sato et al., 2004; Xu et al., 2002; Zhang et al., 2005). When pure PHB is heated above its melting point (~180 °C) it exists as an amorphous entanglement of loosely packed molecules (Barham et al., 1984). Upon cooling, the PHB molecules crystallize, aligning into tightly packed helical chains, forming lamellar sheets and/or spherulites (Barham et al., 1984). The proposed crystal structure of PHB consists of two antiparallel, left-handed 2_1-helices (Cornibert and Marchessault, 1975; Padermshoke et al., 2005; Sato et al., 2004). The driving force behind the energetically favorable process is thought to be C–H···O hydrogen bonding between the C═O and CH_3 groups in PHB (Furukawa et al., 2005; Sato et al., 2004; Zhang et al., 2005). During the crystallization process, the molecular mobility of the PHB molecules becomes restricted due to chain folding and close packing into a crystalline state. Conversely, melting PHB causes the C–H···O hydrogen bonds to weaken, increasing molecular mobility.

In contrast to purified PHB, the amorphousness of native PHB granules in vivo is stabilized by proteins, lipids, and water (Barnard and Sanders, 1989; Horowitz and Sanders, 1994; Sudesh et al., 2000). Structural proteins, known as phasins, and lipids contained in the granule membrane may play a role in regulating or stabilizing the amorphous granules (Grage et al., 2009; Stuart et al., 1998; Sudesh et al., 2000). More importantly, the 5–10% of intra-granule water present in the granules is thought to act as a plasticizer, forming hydrogen bonds (C–H···OH_2···O) with the carbonyl groups of the polyester backbones in PHB (Sudesh et al., 2000). This phenomenon prevents strong dipole–dipole interactions from occurring between the C═O and CH_3 groups in PHB (Sudesh et al., 2000). Removal of the intra-granule, PHB-bound water promotes in situ crystallization due to increased C–H···O hydrogen bonding, analogous to the cooling crystallization process in pure PHB (Sudesh et al., 2000). When monitoring the infrared absorption spectra of PHB-containing cells in an aqueous solution, these impurities (i.e. non-PHB biomass and water) may generate considerable background noise and spectral interference. Moreover, evaporation of water during the measurement may cause the granules to crystallize. Hence, the infrared absorption spectra may not reflect the true, instantaneous crystallinity of PHB in vivo.

In this work, we developed the first known method to monitor the instantaneous crystallinity of native PHB granules in *Ralstonia eutropha*, a representative bacterial microorganism containing PHA, suspended in an aqueous solution. Spectral interference from background absorptions caused by water and other non-PHB cellular components were observed and accounted for in the measurements at specific wavenumbers. This paper reports the details of the in situ method and provides a quantitative description of the true, instantaneous crystallinity of PHB granules by correlating ATR-FTIR spectral measurements with DSC crystallinity measurements. Although this paper is specific to PHB granules contained in *R. eutropha* cells, the method outlined here may prove useful as a framework to monitor the in situ crystallization of biopolyesters in microorganisms similar to *R. eutropha*.

2. Material and methods

2.1. Materials

In this work, *R. eutropha* cells containing 60 wt.% PHB were stored at room temperature (~23 °C) in a solution of 0.2 M H_2SO_4 (pH 2) as a slurry of 250 g cell mass/L. The acidic condition (pH 2) was used to stop possible microbial activity in the aqueous solution, a standard method used in water sampling and preservation. In the acidic solution, the cells exhibited no biological activity and the PHB granules remained mostly intact in their native amorphous state. A slurry density of 250 g cell mass/L provided the most optimal working conditions for the in situ measurements that were dependent on water evaporation and cell sedimentation rates. Three aqueous solutions of the PHB-containing cells were prepared from the slurry for controlled samples of different crystallinity: (1) *native PHB granules*, suspended in acidic solution as described above; (2) *centrifuged PHB granules*, where the solution of native granules was spun at 10,000 g for 1 h and resuspended in the supernatants solution; and (3) *heated PHB granules*, where the solution of native granules was heated at 140 °C for 2 h and set at room temperature (~23 °C) for 6–9 months to reach a stable crystallinity.

For preparation of pure PHB and non-PHB biomass, the microbial cells were freeze dried, dissolved in hot chloroform, and filtered to separate the polymer solution from the residual non-PHB biomass components. Pure PHB was precipitated from the chloroform solution by adding hexane, filtered and dried. The non-PHB biomass leftover from solvent extraction was oven dried at 60 °C and saved for later use.

Pure PHA samples (PHB melt, PHB film, PHB powder, and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxyvalerate) (PHBVV) film) were prepared from various cell slurries (not discussed in this work), and used as generic standards of different crystallinity to calibrate ATR-FTIR spectral measurements with DSC crystallinity measurements. The PHB melt was prepared by melting pure PHB at 180 °C. The PHB film was prepared by dissolving pure PHB in hot chloroform and casting the solution on a clean glass surface as a thin film (~0.2 mm). The PHB powder and the PHBVV film were extracted and purified from different cell slurries using proprietary methods not important to the details of this work. All the pure PHA samples prepared by our research group were determined to be at least 99% pure via HPLC analysis. Biopolyesters were also purchased from Sigma-Aldrich for comparison.

2.2. Chemical analysis

The PHB content of the microbial cells was determined via acid-catalyzed methanolysis of the biopolyester in methanol (3 wt.% H_2SO_4) at 100 °C for 8–10 h (Hesselmann et al., 1999). The resulting 3-hydroxybutyric methyl ester was hydrolyzed into 3-hydroxybutyric acid at pH 11 with 10 N NaOH. The liquid samples were analyzed at 210 nm using an HPLC equipped with a UV detector (Shimazu, Japan) and an organic acid column (OA-1000, Alltech, Deerfield, IL) maintained at 65 °C and eluted with a sulfuric acid solution (pH 2) at 0.8 mL/min.

2.3. ATR-FTIR spectroscopy

The infrared absorption spectra of the pure PHA samples, PHB-containing cells, non-PHB biomass, and water were recorded with a Nicolet Avatar 370 FTIR spectrometer (Thermo Electron Co., Madison, WI). All measurements were taken in ambient conditions on a germanium crystal window of micro-horizontal attenuated total reflectance (ATR). A total of 32 scans were averaged for the measurement of a single sample over 1 min.

For measurements of the PHB film, PHB powder, and PHBVV film, the solid samples were pressed directly onto the ATR window and the absorption spectra were recorded. For measurements of the PHB melt, pure PHB was melted at 180 °C, then removed from the heat source and immediately placed on the ATR window to record its absorption spectra before significant cooling occurred. For accuracy, each of these measurements were repeated and averaged over 18 samples.

For in situ measurements of the PHB-containing cells, a small drop (~2 μL) of aqueous solution was placed directly on the ATR window and allowed to evaporate. During water evaporation, the PHB-containing cells deposited onto the ATR window via sedimentation. The total