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Analysis of bacterial polyhydroxybutyrate production by multimodal nanoimaging

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ABSTRACT

In this paper, we will employ two microscopy techniques, transmission electron microscopy and infrared nanospectromicroscopy, to study the production of polyhydroxybutyrate in *Rhodobacter capsulatus* and to evaluate the influence of glucose and acetone on the production yield. The results overlap which leads us to a consistent conclusion, highlighting that each technique brings specific and complementary information. By using electron microscopy and infrared nanospectromicroscopy we have proved that both glucose and acetone had a positive effect on the biopolymer production, although the first study done by Fourier transform infrared spectroscopy only identified the effect of acetone. In conclusion, we have now established a method to be able to perform fast diagnostic for PHB production.

1. Introduction

Polyhydroxyalcanoates (PHAs) are known as natural polyesters produced by bacteria with a great potential in biodegradable plastic applications (Doi, 1990). The scientific interest of the production optimization has come from the knowledge of the PHA biosynthetic pathways, the numerous promising bacterial species and finally by the genetic engineering and biochemical techniques efficiencies (Hahn et al., 1998; Kranz et al., 1997; Wong and Lee, 1998; Zhao and Cheng, 2004). Polyhydroxybutyrates (PHB) are part of this PHAs family. Produced by bacterial fermentation from carbon sources such as sugars or fatty acids, they are then degraded in soil, sludge, or seawater, under optimal conditions, at an extremely fast speed. Furthermore PHB has mechanical properties similar to those of thermoplastic synthetic polymers derived from petroleum, such as polypropylene or polyethylene. Nevertheless, the PHB bacterial production was labeled by industrials as only a prospective project because the biotechnological processes (fermentation, extraction) had too high costs compared to petroleum products.

Recently, many scientists have refocused their studies on those polyesters by integrating the industrial constraints and have proposed attractive solutions to optimize PHB production yield in different strains of *Alcaligenes*, *Azotobacter*, *Pseudomonas* or recombinant *Escherichia coli* (Hahn et al., 1998; Naik et al., 2008; Wong and Lee, 1998). In this context, the study of PHB at the single cell level is crucial for the PHB biosynthesis efficiency to be evaluated.

Usually, the PHB yield is first evaluated by biochemical analysis and then confirmed by transmission electron microscopy (TEM). But these techniques are time-consuming, and real-time diagnosis for PHB production is not possible.

Consequently, we propose a combination of infrared spectroscopy and microscopy, to estimate the production of bio-polymer to rapidly diagnose the efficiency of a bacterial strain as well as a culture condition.

The ester carbonyl group of the PHB shows a specific mid-infrared response at 1740 cm^{-1} which differs from the proteins carbonyl group (Amide I at 1650 cm^{-1}) (Misra et al., 2000; Naumann, 1998), making it possible to determine, by classical Fourier transformed infrared spectroscopy (FTIR), the mean production of PHB and to analyze the general behavior of a cell population. In order for this study to be performed at the sub-cellular level, a second technique is required with a nanometric resolution. We used an original set-up called AFMIR that couples an atomic force microscope (AFM) and a pulsed IR laser (Dazzi et al., 2005). This technique allows spectromicroscopy at the 10 nm scale with representative local spectra (Dazzi et al., 2008, 2010; Mayet et al., 2010).

Our study was performed on *Rhodobacter capsulatus* whose PHB production is efficient (Madigan, 1990; Madigan et al., 2001; Pantazopoulous and Madigan, 2000). This study was done for different culture conditions that may increase the production yield. The study of PHB is difficult as PHBs are stored in the form of insoluble vesicles inside the bacteria, so it involves high-resolution microscopy such as TEM or our IR nanospectromicroscopy AFMIR. As we need to evaluate the influence of glucose and acetone on the production yield, the PHB vesicle size will be determined by TEM and AFMIR microscopy. Finally the advantages and the limitations of each technique will be discussed.

2. Materials and methods

2.1. Rhodobacter capsulatus cultures

2.1.1. Standard culture

Wild type *Rhodobacter capsulatus* bacteria were grown in malate yeast medium supplemented with kanamycin (20 mg/ml) and tetracycline

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(4 mg/ml) at 50% of the total volume. Cultures were cultivated darkness at 30 °C and kept for 48 h on a gyratory shaker (140 rpm).

2.1.2. Glucose culture

The preparation of the culture was similar to the standard one, but 80% of the malate volume was substituted by glucose.

2.1.3. Acetone culture

In this case all the malate was substituted by acetone.

In order to be studied in infrared, the cell suspension of each culture was centrifuged at 1000 rpm for 15 min. The supernatant was removed and the cell pellet diluted in distilled water. This process was repeated three times in order to obtain suitably clean samples. Finally, a droplet of the cellular suspension was deposited on ZnSe (transparent in mid-infrared) coverglass for FTIR spectroscopy or ZnSe prisms for AFMIR studies and dried at room temperature.

2.2. FTIR analysis

The infrared spectra was acquired with a Bruker Vertex 70 FTIR spectrometer between 4000 and 400 cm^{-1} with a spectral resolution of 4 cm^{-1} using an MCT detector with a liquid nitrogen cooling system.

The biomass can be directly characterized by the amplitude of the Amide I absorption band; hence we have normalized all FTIR spectra by this band so that we can compare the amount of PHB production for the different culture condition.

2.3. Principle of the nanospectromicroscopy AFMIR

The AFMIR instrument combines an Atomic Force Microscope (AFM) with an infrared pulsed tunable laser (Fig. 1). A sample is placed on a ZnSe prism and then irradiated with the laser. When the laser wavelength is tuned on an absorption band of the sample, the absorbed laser light causes a photothermal effect and a temperature rise. This increase in temperature creates a local thermal expansion monitored by the tip of the AFM. The expansion of the sample generates a rapid impulse that leads to the oscillation of the cantilever on its eigenmodes. The oscillations amplitudes, on the 4-quadrant detector, are directly proportional to the energy absorbed. Hence, we can reconstruct the entire absorption spectrum of the sample by tuning the incident laser wavenumber, and comparing it with those obtained by classical IR spectroscopy techniques like FTIR (Dazzi et al., 2010). The main advantage of this technique is its resolution of less than 100 nm. It allows a chemical study of the samples at the subcellular level. This has already been demonstrated in microbiological (Dazzi et al., 2007, 2008; Mayet et al., 2008) and nanophotonic domains (Houel et al., 2007).

2.4. Sample preparation for TEM imaging

For all different cultures, bacteria were fixed with glutaraldehyde 2% and paraformaldehyde 2% in a buffer of sodium cacodylate 0.1 mol/l (pH 7.4) and then post-fixed in 1% buffered osmium tetroxide. The bacteria was then completely dehydrated with ethanol at room temperature and then embedded in epoxy resin (polymerization at 60 °C for 48 h). Ultrathin sections of 50 nm thickness were cut with a diamond knife, deposited on copper grids (mesh 200) and then stained with uranyl acetate aqueous solution (20 min) and lead citrate (5 min). Samples were observed at 80 kV with Philips EM208 transmission electron microscope (80 kV, mode light background) equipped with a CCD camera.

2.5. AFMIR images analysis

The AFMIR imaging analysis was performed with ImageJ software. ImageJ software is an open source software commonly used by scientist in imaging domain. This software is useful to visualize imaging data and offers the possibility of applying mathematical tools for the analysis. Each chemical mapping of PHB (already normalized by the input laser power) was renormalized by the corresponding topography (absorption signal is divided by the corresponding height), giving us the absorption density value. The signal coming from the bacteria was isolated using the threshold filter (removing the image pixels corresponding to the surface). The maximum of absorption density was then extracted for each bacterium. We assume that the maximum of these maxima corresponds to a full diameter of the vesicle also ensuring that the absorption signal is not an isolated point but is linked to a large area of absorption. As the maximum absorption density value and the volume of each bacterium are known, the theoretical maximum absorption signal if the bacterium was only full of PHB is easy to calculate. Then the real values of absorption density can be integrated (given by the chemical mapping divided by topography) on only the pixels corresponding to the surface of the bacterium to have the integral of density absorption. Finally, by dividing this last value with the theoretical maximum absorption we obtain the % of PHB absorption inside the volume of the bacterium. This process of analysis was applied for tens of bacteria for each culture condition to obtain the average PHB absorption (%). This parameter does not directly give the % of PHB volume occupation per bacterium but is a very good indicator of this value. The error comes from buried vesicles that give a smaller integrated signal than the vesicles close to the surface. So the % of PHB absorption is lower than the % of volume occupation.

3. Results

3.1. Global study by FTIR spectroscopy

For each culture (standard, glucose and acetone), an FTIR spectrum has been recorded between 1850 and 1550 cm^{-1} (Fig. 2) after

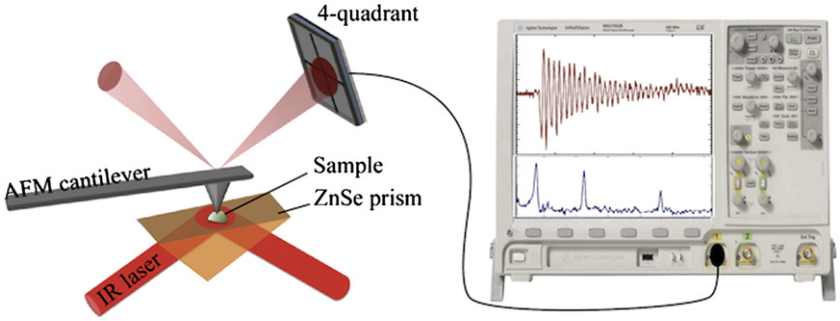

Fig. 1. Scheme of the AFMIR experimental setup. The sample is deposited on the upper side of the ZnSe prism and illuminated by the infrared pulsed laser. The resulting photothermal expansion generates a brief force under the AFM tip inducing the ringing of the AFM cantilever. The oscillation signal is recorded and stored in an oscilloscope.