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Full Length Research Paper

Poly-β-hydroxybutyrate accumulation and releasing by hydrogen producing bacteria, *Rhodobacter sphaeroides* O.U.001. A transmission electron microscopic study

DEMET ÇETİN[1]*, UFUK GÜNDÜZ[2], İNCİ EROĞLU[3], MERAL YÜCEL[2] and LEMİ TÜRKER[4]

[1]Department of Biology, Ankara University, 06100, Ankara, Turkey.
[2]Department of Biology, Middle East Technical University, 06531, Ankara, Turkey.
[3]Department of Chemical Engineering, Middle East Technical University, 06531, Ankara, Turkey.
[4]Department of Chemistry, Middle East Technical University, 06531, Ankara, Turkey.

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Photosynthetic bacterium *Rhodobacter sphaeroides* O.U.001 that is used for photobiohydrogen production can also accumulate poly-β-hydroxybutyrate (PHB) as a by-product when cultivated anaerobically with minimal medium containing L-malic acid, sodium glutamate and some vitamins under illumination. Transmission electron microscopy studies revealed that PHB granules are made of two distinct components: a homogenous and electron lucent core is covered with a more electron dense coat. PHB granules were observed in the cytoplasm, outside of the cells, in the center of cotton like aggregates in the cells, or while they were being released from the cell. In this study, two kinds of releasing were revealed; with lysis and without lysis of cell wall. Release of intact polymer outside the cells could be economically feasible way to obtain PHB for industrial applications.

Key words: Poly-β-hydroxybutyrate, PHB, *Rhodobacter sphaeroides*, transmission electron microscopy.

INTRODUCTION

Conventional petrochemical plastics have become an integral part of contemporary life, because of their desirable properties like durability and resistance to degradation. In response to problems and harmful effects of plastic wastes on the environment, there has been considerable interest in the development of biodegradeble plastic materials. Polyhydroxyalkanoates (PHA) due to their biodegradability, biocompatibility, termoplasticity, nontoxicity properties attracted attention as substitutes of conventional plastics.

Polyhydroxybutyrate (PHB), which is the most abundantly occurring PHA, is a storage polyester occurring as insoluble inclusion bodies in the cytoplasm (Steinbüchel and Füchtenbusch, 1998). Sudanophilic, lipid like inclusion, which is soluble in chloroform, was first identified as PHB in *Bacillus megaterium* by Lemoigne (1926). In following years, it became clear that it functions as an intracellular reserve material for carbon and energy in *B. megaterium* and in several Gram-negative bacteria.

PHB can be synthesized during unfavorable growth conditions like nitrogen, phosphorus, sulphur or oxygen deficiency in the presence of excess carbon source. PHB inclusions appear as transparent bodies when thin sections of PHB-containing bacteria are examined by transmission and scanning electron microscopy (TEM, SEM) (Lauzier et al., 1992; Lee, 1996; Sudesh et al., 2000).

The photosynthetic bacterium *Rhodobacter sphaeroides* can produce hydrogen anaerobically in a photobioreactor (Barbosa et al., 2001; Eroğlu et al., 1999; Sasikala et al., 1995; Yetiş et al., 2000). Poly-β-hydroxybutyrate (PHB), is among the byproducts of *R. sphaeroides* (Hustede et al., 1993; Khatipov et al., 1998; Yiğit et al., 1999).

*Corresponding Author's E-mail: dcetin@science.ankara.edu.tr, Phone: +90 312 212 67 20. Fax: +90 312 223 23 95.

In the present study, PHB production of *R. sphaeroides* in hydrogen production medium containing L-malic acid, sodium glutamate and some vitamins is analyzed with TEM. Time dependent changes on morphological futures of PHB in *R. sphaeroides* O.U. 001 and methods of release were investigated.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Rhodobacter sphaeroides O.U.001 (DSM 5864) was grown in 50 ml anaerobic jars under argon atmosphere at 36°C under illumination at 200 W/m^2. The standard growth medium containing L-malic acid and sodium glutamate in 7.5/10 molar ratio was used (Yiğit et al., 1999). For medium and electron microscopic preparations, chemicals were obtained from Sigma or Merck and were of the highest grade available. *R. sphaeroides* cells were harvested after 12 h, 3 days, 5 days and 10 days for TEM preparations.

Preparation of cells for transmission electron microscopy

For electron microscopy, prefixation was performed with addition of 1 ml of 25% glutaraldehyde to 50 ml of 12 h grown bacterial culture. After one hour incubation at +4°C, samples were centrifuged using Hettich Universal 16R Centrifuge at 2000 g, +4°C for 15 min. Then supernatant was removed, pellet was resuspended in phosphate buffer (0.2 M, pH 7). After that, centrifugation was carried out again. Supernatant was removed. For fixation step, 5 ml of 5% glutaraldehyde buffered with 0.2 M sodium phosphate (pH 7) was added on pellet. After 1 h incubation at +4°C, centrifugation was carried out. Next, the supernatant was decanted and pellet was suspended in phosphate buffer and centrifuged. Washing with buffer was repeated three times. Meanwhile 10 ml of 2 % low melting agarose was prepared in buffer and kept at 45°C. Later, agarose was poured onto pellet (agarose: pellet, v/v 1:1). It was cooled and solidified in refrigerator. Afterwards mixture of bacteria and agarose was taken out from centrifuge tube and cut into small cubes ($\approx$ 1 mm^3) in buffer. Samples were preserved at +4°C and processed like solid sample in following steps.

For postfixation with osmium tetroxide (OsO_4), cells in agarose cubes were placed in 10 ml, 1% OsO_4 solution buffered with 0.2 M sodium phosphate (pH 7) and incubated at +4°C for 2 h. Then OsO_4 was removed and samples rinsed three times with phosphate buffer. Then the samples were dehydrated in ascending series of acetone and were embedded in Araldite (Glauert and Glauert 1958). Reichert OM U 3 Ultramicrotome was used for sectioning of araldite block. The ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963). The samples were viewed in Jeol 100CX II Transmission Electron Microscope at an accelerating voltage of 80 KV and transmission electron micrographs were taken with Agfa Scientia Electron Microscope films.

RESULTS

In *R. sphaeroides* cells, PHB granules always have two distinct components: a homogenous and electron lucent core is covered by a more electron dense coat. Both of the components of PHB granules have lower electron density from the cytoplasm (Figure 1A, B). In the *R. sphaeroides* cells, one or more PHB granules in different sizes exist in the cytoplasm after 12 h of growth. Most of the PHB granules are spherical. However, ovoid and elongated shaped granules can be also seen in the same cell. Electron lucent core of PHB granule is round or ovoid in shape and its boundary is clearly seen. On the contrary to central part of PHB, the boundary of electron dense coat is not clearly distinguished and thickness of this coat varies on the granule (Figure 1A, B). When the sections pass through only coat of the PHB, the central core is not seen (Figure 1A).

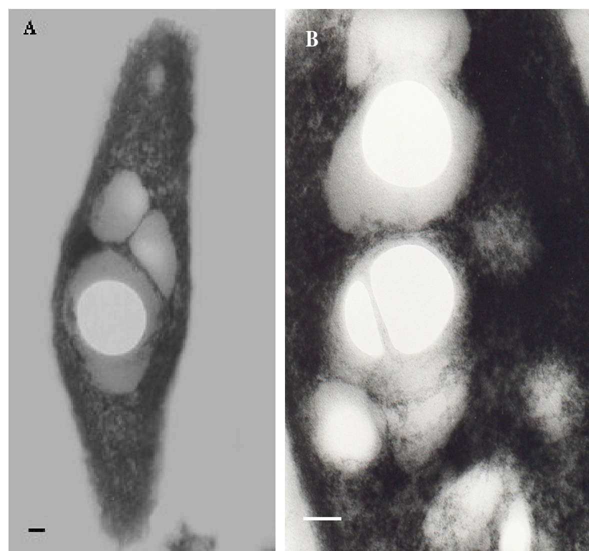

Figure 1. Transmission electron micrograph of poly-ß-hydroxybutyrate (PHB) granules in R. sphaeroides at 12 hours of the growth (A, B). Scale bars = 0,3 µm

In *R. sphaeroides* cells, PHB in various forms were observed after 12 h, 3 days, 5 days, and 10 days of growth. After 3 days of growth, some cells fill with up to 70-80% white, electron transparent and cotton-like aggregates. Some of the cells have PHB granules in the center of these aggregates or sometimes PHB granules alone were observed in the cytoplasm. PHB granule was also observed in spherical form in outside of the cells (Figure 2A). After 5 days, the similar granules and aggregate structures are seen in the cells. The wall of the cells, which were filled with cotton like white, electron transparent aggregates, was thin or lysed in some places (Figure 2B). In a few cells, PHB was released from very large holes in the cell where the cell wall had been peeled back (Figure 2C).

After 10 days, one or more PHB granules were observed in the cells, but the white cotton like, electron lucent aggregates were observed to a lesser extent. PHB releasing from the cells was also observed at 10 days of growth (Figure 2D). Released polymer is just only the electron lucent (center) part of the PHB and has a globular appearance. The electron dense part of PHB does not

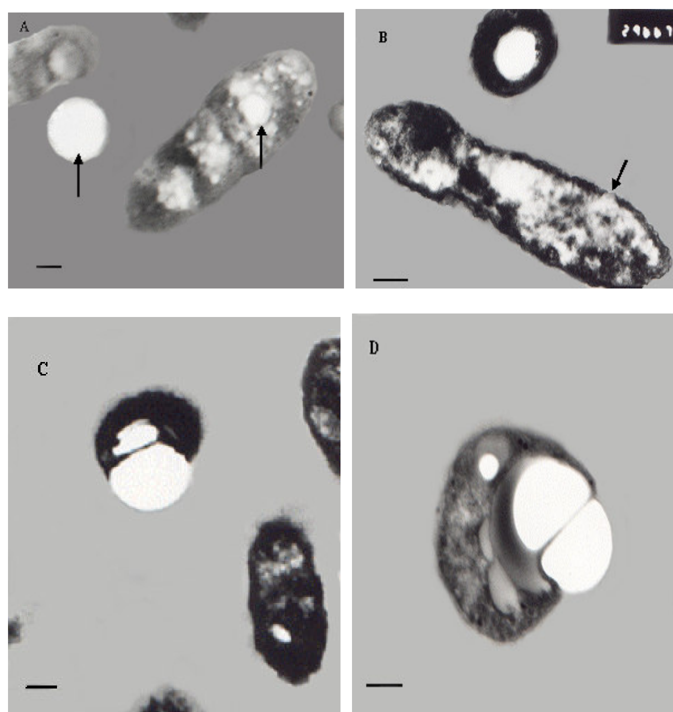

Figure 2. Transmission electron micrograph of *R. sphaeroides* cells with PHB granules. A) 3 days of the growth. Arrows indicate the PHB granules inside and outside of the cell. B) 5 days of the growth. Arrow indicates lysis of cell wall C) 5 days of the growth. PHB releasing from the cell. D) 10 days of the growth. The core part of the PHB releasing from the cell. Scale bars = 0.3 µm

leave the cell and this remainder part of the PHB is clearly seen within the cell (Figure 2D). The smaller intact PHB granules can be seen in the cytoplasm of the same cell.

DISCUSSION

The morphologies of the PHB inclusions observed in *R. sphaeroides* are in good agreement with those of previous electron microscopic observations in *Bacillus cereus, Alcaligenes eutrophus*, etc, and with the general concept of electron lucent core and outer coat (Dunlop and Robards, 1973; Lauzier et al., 1992). In *R. sphaeroides* cells, one or more PHB granules exist in the cytoplasm. Most of the PHB granules are spherical. However, ovoid and elongated shaped granules can be also seen in the same cell. The number and size of PHB granules found in cells is depending on the bacterial species, cultural and environmental conditions (Quillaguamán et al., 2006). The cells can be filled up PHA granules without disrupting cell integrity (Loo et al., 2005; Quillaguamán et al., 2006). Phasins, which are non-catalytic proteins, are thought to be regulating the size, number and surface to volume ratio of PHB inclusions as well as involved in stabilization of hydrophobic PHA inclusion in hydrophilic cell cytoplasm (Luengo et al., 2003; Rehm and Steinbüchel, 1999; Sudesh et al., 2000; Tian et al., 2005).

According to recent findings, phospholipid monolayer membrane with phasins, proteins for biosynthesis (PHA synthase), proteins for degradation/mobilization (intracellular PHA depolymerase) are located on the surface of PHA granules (Luengo et al., 2003; Rehm and Steinbüchel, 1999; Sudesh et al., 2000). Since PHA is a storage compound for excess carbon its natural that microorganisms are equipped with a depolymerizing system to recover the stored carbon. Researches showed that intracellular degradation of PHB is a very slow process. The rate of PHB degradation is about ten times slower than the rate of synthesis (Sudesh et al., 2000). The cotton-like appearance of PHB in *R. sphaeroides* cells (Figure 2A, B) may be related to intracellular PHB degradation. In addition it seems that lysis on the cell wall of these cells cause breakdown of cell integrity and release of degraded polymer to the environment.

Some microorganisms also produce an extracellular PHA depolymerase which is secreted to degrade crystalline PHA material in the environment (Luengo et al., 2003). During hydrogen production studies in the glass column photobioreactor, we observed white precipitates (which are most probably released PHB granules) on the wall of photobioreactor (not shown here) after 48 h of growth. These precipitates on the wall of photobioreactor were later lost. Released polymer might be depolymerized extracellularly by the same microbial culture.

In industrial applications, it is economically feasibly to obtain PHB by lowering cost for purification and recovery as well as producing high amount of PHB (Choi and Lee, 1999). For this purpose many researches have been conducting to produce and release polymer outside the cells. Genetic studies revealed that release of PHB is possible by cloning of lysis gene on recombinant PHB producing *E. coli* (pTZ18U-PHB, pSH2) (Resch et al., 1998). Jung et al. (2005) also produced high amounts of PHB and enabled the cell to release the polymer through autolysis by trying various initial inoculum size and initial medium composition in recombinant *E. coli* (MG1655/pTZ18-UPHB). Page et al. (1995) showed that pleomorphic cells of *Azotobacter vinelandii* UWD appear to leak or extrude fluid PHB into surrounding medium.

In the reported studies, PHB releasing is partially different from that of *R. sphaeroides*. In this study two kinds of releasing were revealed. One of them is via lysis of cell wall. The other one is releasing of only the core of PHB in intact and solid form without cell wall lysis. The remainder of the cell has similar appearance to cells that contain PHB. Besides, the core of PHB in spherical form is also found outside the cells. In the 12 h grown culture, PHB granules are mostly observed in the cells. Two ways of PHB releasing are generally observed in aged culture.

It is not thought that as an artifact generated during electron microscopic preparations because of observing these findings in not all cells but some cells in aged culture.

These results could also be extended to different cultivation strategies to improve polymer productivity and releasing by *R. sphaeroides* without reducing hydrogen production capacity by further work.

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