5. Julani, H. R. (Jr), Koroch, A. R., Julani, H. R. and Trippi, V. S., *Plant Cell Tissue Org. Cult.*, 1999, **59**, 175–179.
6. Koroch, A. R., Juliani, H. R. (Jr), Juliani H. R. and Trippi, V. S., *Plant Cell Tissue Org. Cult.*, 1997, **48**, 213–217.
7. Santos, M. C., Esquibel, M. A. and dos Santos, *Plant Cell Tissue Org. Cult.*, 1990, **21**, 75–78.
8. Taylor, P. W. J. and Dukic S., *Plant Cell Tissue Org. Cult.*, 1993, **34**, 217–222.
9. Agrawal, D. C., Morwal, G. C. and Mascranhas, A. F., *Lindleyana*, 1992, **7**, 95–99.
10. Agrawal, D. C. *et al.*, *Plant Cell Rep.*, 1997, **16**, 647–652.

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Studies of poly(3-hydroxybutyric acid) inclusions in whole cells of *Azotobacter chroococcum* MAL-201

Soma Pal and A. K. Paul*

Microbiology Laboratory, Department of Botany, Calcutta University, Kolkata 700 019, India

Accumulation of poly(3-hydroxybutyric acid) inclusions in whole cells of *Azotobacter chroococcum* MAL-201 has been studied. Cells harvested at the late exponential phase of growth gave bright orange fluorescence when stained with Nile blue A. Electron microscopy of ultra-thin sections of such cells revealed 4–5 granules cell^{-1}, each measuring 0.1–0.44 μm in diameter. The infrared absorption spectra of lyophilized cells confirmed the chemical nature of the polymer, while in *in vivo* conditions the inclusions appeared to be amorphous as observed under wide angle X-ray scattering.

POLY(3-hydroxybutyric acid), [P(3HB)], is the most well-known representative of polyhydroxyalkanoic acids [PHAs] which represent a complex class of bacterial storage polyesters. As reserve material it is synthesized by a wide variety of bacteria grown under conditions of nutrient limitations, but with excess of carbon[1–3]. P(3HB) and copolymers of 3-hydroxybutyric acid and 3-hydroxyvaleric acid [P(3HB-co-3HV)] derived from bacterial sources have been identified as substitutes for petrochemical-based plastics mainly due to their thermoplastic properties, biocompatibility and complete degradation in nature[4,5].

The polymer being insoluble in water is produced *in vivo* in the form of inclusion bodies or granules. The number and size of granules vary considerably depending on the nature of the organism, growth condition, substrate used and the degree of polymer accumulation[1]. Earlier studies have suggested that the native P(3HB) granules are crystalline[6,7]. However, the concept of crystalline polymer existing within native granules has been revised during 1980s. Studies of granules using ^{13}C-NMR spectroscopy, X-ray diffraction and electron microscopy revealed that the polymer in *Methylobacterium* and *Alcaligenes eutrophus* is actually amorphous, at 30°C is mobile and elastomeric[8–11]. The question why the granules remained amorphous has been debated extensively in literature and several hypotheses have been advanced, mostly involving auxillary factors or 'plasticizers'[11–13].

Azotobacter chroococcum MAL-201, a mucoid, free-living, nitrogen-fixing strain isolated from a soil sample of Malda, West Bengal is reported to accumulate P(3HB), accounting more than 68% of its cell dry weight when grown in nitrogen-free medium under laboratory conditions[14,15]. In this communication, an attempt has been made to detect and determine the characteristic feature of the P(3HB) inclusions in whole cells of *A. chroococcum* MAL-201 grown in nitrogen-free medium under batch culture.

Azotobacter chroococcum MAL-201 was grown in modified Norris nitrogen-free medium[16] containing 2% (w/v) glucose. The medium (50 ml per 250 ml Erlenmayer flask) was inoculated with 2 ml of freshly prepared inoculum and incubated at 30°C on a rotary shaker (120 rpm) for 27 h. Cells were harvested by centrifugation (10,000 *g*) for 10 min and washed thoroughly with distilled water. Intracellular accumulation of the polymer was detected following staining with Nile blue A[17]. Cell suspension was smeared on a clean grease-free slide and stained with aqueous solution of Nile blue A at 55°C for 10 min in a coplin jar. The excess stain was removed with tap water and washed with 8% (v/v) aqueous acetic acid and again rinsed with water. After blot-drying, the smear was re-moistened with tap water, covered with cover glass and examined under Leitz-Labour-Lux D microscope with an I_2 filter which provides an excitation wavelength of approximately 460 nm. For transmission electron microscopy, ultra-thin sections of cells fixed in uranyl acetate (0.5%, w/v) were stained with lead citrate and examined in Hitachi H600 electron microscope with an acceleration voltage of 75 kV and photographed using Fuji film.

The infrared spectrum of lyophilized whole cell (in KBr pellate) was recorded with a Perkin-Elmer Model

*For correspondence. (e-mail: akpaul@cal3.vsnl.net.in)

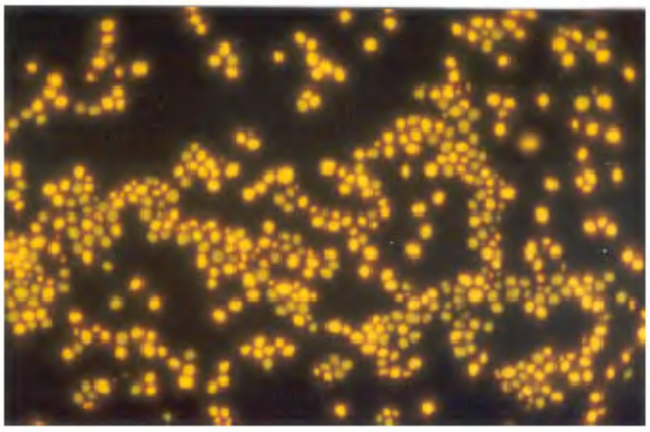

Figure 1. Photomicrograph of cells of *Azotobacter chroococcum* MAL-201 stained with Nile Blue A (× 1442). Cells from late exponential phase of growth were stained with 1% Nile Blue A and photographed in Leitz-Labour-Lux D fluorescence microscope fitted with an I_2 filter having an excitation wavelength of 460 nm.

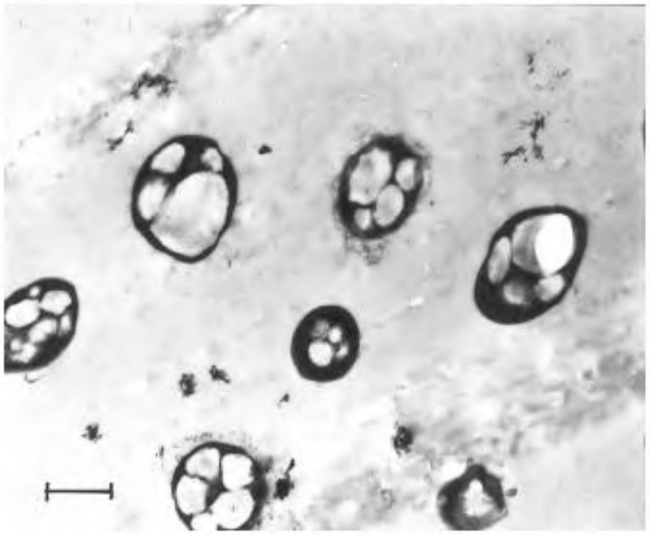

Figure 2. Transmission electron microscopy of ultra-thin section of cells of *Azotobacter chroococcum* MAL-201 showing P(3HB) granules. Cells were harvested at mid exponential phase, treated with OsO_4, fixed in uranyl acetate, ultra-thin sections (700 Å) stained with lead acetate were examined in Hitachi H600 electron microscope. Bar represents 1 µm.

297 IR spectrophotometer following the method of Stockdale *et al.*[16]. The sample was scanned between 600 and 4000 wave number (cm^{-1}) at a speed of 1 µm/min and with a programmed slit opening 2X and air as reference.

Wide angle X-ray scattering (WAXS) of the lyophilized whole cell was recorded in a computerized X-ray provided with Philips PW-1730/00 X-ray generator and PW-1965-60C proportional counter. The nickel filtered CuK_α radiation ($\lambda = 0.1542$ nm, 40 kv; 30 mA) was used at room temperature. The sample was packed in an

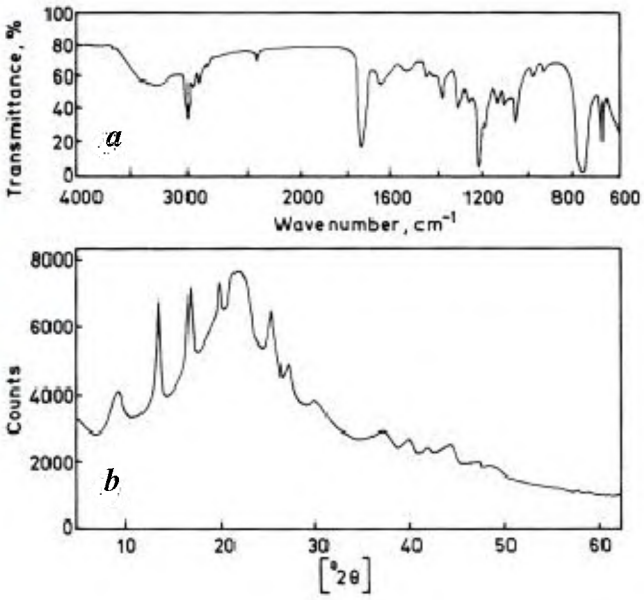

Figure 3. Infrared (IR) absorption spectra (*a*) and wide angle X-ray scattering (WAXS) pattern (*b*) of freeze-dried cells of *Azotobacter chroococcum* MAL-201. The IR spectra of whole cells were recorded as KBr pellate. The pellates were scanned between 600 to 4000 wave number (cm^{-1}) at a speed of 1 µm min^{-1}. For WAXS the dried cell mass was packed in an aluminium sample holder and the diffractometer was run from 5° 2θ to 60° 2θ with a scan speed of 5° 2θ/min. The nickel filtered Cuk_α radiation was used.

aluminium sample holder. The diffractometer was run from 5° 2θ to 60° 2θ with a scan speed of 5° 2θ min^{-1}.

When cells of *A. chroococcum* MAL-201 harvested at late exponential phase of growth were examined under optical fluorescence microscopy using the dye Nile Blue A, a selective histological stain for P(3HB) gave a bright orange fluorescence at an excitation wavelength of 460 nm (Figure 1). This confirmed the accumulation of P(3HB) inclusions in cells of *A. chroococcum* and the results are consistent with the finding that Nile Blue A not only confirms the P(3HB), but also distinguishes it from other accumulated fatty materials[17]. Nile Red, a related dye has also been reported to be very specific for P(3HB)[18].

Electron microscopy of ultra-thin sections of such cells revealed 4–5 granules per cell filling the cell almost entirely (Figure 2). The granules were spherical to oval in shape and varied from 0.1 to 0.44 µm in diameter. These results conform well with the findings of earlier workers[1,7,19], although both needle-type and mushroom-type polyhydroxyalkanoate granules have also been reported recently in *Pseudomonas* sp.[20].

The infrared spectrum of lyophilized whole cells (Figure 3 *a*) showed a major peak at 1720 cm^{-1} corresponding to ester carbonyl (C=O) stretching. A distinct broad O–H stretching absorption peak at 3440 cm^{-1} rep-

resented the free O–H stretch of the polymer end groups. The peaks at wave number 2980–3000 cm^{-1} and 1210–1370 cm^{-1} represented the typical C–H bending of aliphatic compounds. The additional peaks represent other cellular components. The IR-spectrum of lyophilized cells of *A. chroococcum*, therefore, compared well with those of P(3HB)-containing cells of *A. beijerinckii*[16].

The structure of P(3HB) both in *in vivo* and *in vitro* conditions has been studied by WAXS[11,21]. The diffraction pattern (Figure 3 *b*) of freeze-dried cells showed weak crystalline bands on the amorphous background scattering and was identical with that of *A. eutrophus*[21]. The X-ray diffraction pattern of P(3HB) containing whole cells differs significantly from that of isolated P(3HB) granules. Such a difference has been explained by the compositional analysis of native granules and isolated polymer[22,23]. The present findings suggest the amorphous nature of the inclusions, which might be due to the presence of phospholipid covering delimiting the polymer from the cytoplasm. However, more detailed analysis is necessary to confirm the proposition.

1. Anderson, A. J. and Dawes, E. A., *Microbiol. Rev.*, 1990, **54**, 450–472.
2. Dawes, E. A. and Senior, P. J., *Adv. Microbiol. Phys.*, 1973, **10**, 135–266.
3. Steinbuchel, A., *Biomaterials: Novel Materials from Biological Sources* (ed. Byrom, D.), Macmillan Publishers Ltd, Basingstoke, 1991, pp. 123–213.
4. Byrom, D., *TIBTECH*, 1987, **5**, 246–250.
5. Lee, S. Y. and Chang, H. N., *Advances in Biochemical Engineering/Biotechnology* (ed. Fiechter, A.), Springer-Verlag, Berlin, 1995, pp. 27–58.
6. Eller, D., Lundgren, D. G., Okamma, K. and Marchessault, R. H., *J. Mol. Biol.*, 1968, **35**, 489–502.
7. Lundgren, D. G., Pfister, R. M. and Merrick, J. M., *J. Gen. Microbiol.*, 1964, **34**, 441–446.
8. Barnard, G. N. and Sanders, J. K. M., *FEBS Lett.*, 1988, **231**, 16–18.
9. Barnard, G. N. and Sanders, J. K. M., *J. Biol. Chem.*, 1989, **264**, 3286–3291.
10. Horwitz, D. M. and Sanders, J. K. M., *Can. J. Microbiol.*, 1995, **41**, 115–123.
11. Kawaguchi, Y. and Doi, Y., *FEMS Microbiol. Lett.*, 1990, **79**, 151–156.
12. Barham, P. J., Keller, A., Otum, E. J. and Holmes, P. A., *J. Mater. Sci.*, 1984, **19**, 2781–2794.
13. Nobes, G. A. R., Holden, D. A. and Marchessault, R. H., Polymer, 1994, **35**, 435–437.
14. Pal, S. and Paul, A. K., *Curr. Microbiol.*, 1997, **35**, 327–330.
15. Pal, S. and Paul, A. K., *Folia Microbiol.*, 1998, **43**, 177–181.
16. Stockdale, H., Ribbons, D. W. and Dawes, E. A., *J. Bacteriol.*, 1968, **95**, 1798–1803.
17. Ostle, A. G. and Holt, J. G., *Appl. Environ. Microbiol.*, 1982, **44**, 238–241.
18. Greenspan, P., Mayer, E. P. and Flower, S. D., *J. Cell Biol.*, 1985, **100**, 965–973.
19. Dunlop, W. F. and Robards, A. W., *J. Bacteriol.*, 1973, **114**, 1271–1280.
20. Fukui, T., Kato, M., Matsusaki, H., Iwata, T. and Doi, Y., *FEMS Microbiol. Lett.*, 1998, **164**, 219–225.
21. Cornibert, J. and Marchessault, R. H., *J. Mol. Biol.*, 1972, **71**, 735–756.
22. Griebel, R., Smith, Z. and Merrick, J. M., *Biochemistry*, 1968, **7**, 3676–3681.
23. Steinbuchel, A. *et al.*, *Can. J. Microbiol.*, 1995, **41**, 94–105.

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