Can cyanobacteria be a potential PHA producer?

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Polyhydroxyalkanoates (PHAs) are microbial polyesters produced by various microorganisms. Many of these bacteria when fed with suitable carbon sources can produce PHAs up to about 30 wt% of the cell dry weight (CDW) and a few such as *Ralstonia eutropha* can accumulate PHAs up to almost 90 wt% of the CDW. In cyanobacteria (blue-green algae), the PHA content is usually about 5 wt% of the CDW. In this study, we have investigated various aspects of PHA biosynthesis in the cyanobacterium *Synechocystis* PCC6803. PHA biosynthesis can be improved by introducing multicopies of heterologous PHA synthase gene. Nile blue A staining and freeze-fracture electron microscopy revealed the presence of many PHA inclusions in the cell cytoplasm. Based on the sizes and number of these inclusions, the amount of PHAs produced by cyanobacteria is comparable to that produced by most bacteria. The relatively low wt% of PHA in cyanobacteria when compared to other bacteria is probably due to the larger size and mass of the former. Our investigations show that the PHA synthesizing ability of cyanobacteria may in fact be quite similar to that shown by most bacteria in nature.

Introduction

Polyhydroxyalkanoates (PHAs) are among the most investigated biodegradable polymers in recent years.[1)] PHAs are superior to other biodegradable polymers because of the large number of different monomer constituents that can be incorporated. At present, about 150 different hydroxyalkanoate units have been identified. The most common is 3-hydroxybutyrate. By introducing other monomers, copolymers with differing physical properties can be produced.[2)] The ratio of the monomers can also be easily adjusted by regulating the type and concentration of carbon sources fed to the microorganisms. Various types of PHAs with interesting physical properties have been designed and synthesized in this manner.[3)]

In addition to studies of heterotrophic bacteria as PHA producers, PHA production using photosynthetic organisms as production hosts are also being explored by various research groups. This research has resulted in the development of transgenic plants carrying the PHA biosynthetic genes of bacteria. Like higher plants, cyanobacteria are also oxygen-evolving photoautotrophs with the added advantage that some of them naturally possess the key enzyme in PHA biosynthesis, i.e, PHA synthase.[4)] In addition, due to their minimal nutrient requirements, cyanobacteria are viewed as attractive hosts for the production of PHA.[5)] However, in comparison with other heterotrophic bacterial strains, it is generally thought that cyanobacteria can only produce very small amounts of PHAs. We will provide evidence to show that such comparisons can be misleading.

Materials and methods

Synechocystis sp. PCC6803 was cultured in 250 ml flasks containing 50 ml of BG11 medium at 30°C under continuous illumination with cool white fluorescent light ($\sim 150\,\mu$E m^{-2} s^{-1}). The cultures were shaken at 100 rpm on a rotary shaker placed inside an incubator saturated with 1% CO_2. For nitrogen-limited conditions BG11 medium without sodium nitrate was prepared. A recombinant *Synechocystis* sp. PCC6803 strain was constructed by introducing the *Ralstonia eutropha* PHA biosynthetic operon, pJRDCAB by using the spot-mating technique.

Freeze-fracture electron microscopy

Preparation of cells for freeze-fracture analysis was according to the method described previously.[6)] The replicas were observed using a JEM-2000FX II electron microscope operated at an acceleration voltage of 120 kV.

Enzyme activity analysis

Crude cell extracts were immediately used to estimate the PHA synthase activity towards (*R*)-3-hydroxybutyryl-CoA by spectroscopic assay using 5, 5′-dithiobis(2-nitrobenzoic acid) (DTNB) according to the established method.

Results and discussion

Growth and phenotype of both the wild-type and recombinant *Synechocystis* sp. PCC6803 cells are shown in Fig. 1. The PHA synthase activity can be detected in growing cells of both wild-type and recombinant. The PHA synthase ac-

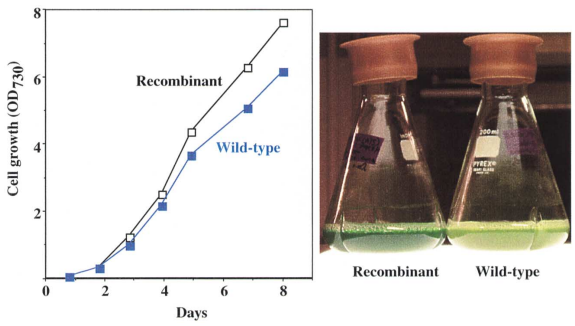

Fig. 1. Growth of wild-type (■) and recombinant (□) *Synechocystis* sp. PCC 6803. PHA synthase activity was measured on the 8th day of cultivation. At the end of 28 days cultivation, the recombinant strain remained greener than the wild-type.

tivity in the recombinant cells harbouring heterologous PHA synthase gene was twice of that in the wild-type. It was also observed that upon nitrogen limitation, the recombinant cells remained green for a longer period of time compared to the wild-type. Poly(3-hydroxybutyrate) [P(3HB)] accumulation was detected in both the recombinant and wild-type cells even in the absence of reduced carbon source. The P(3HB) content can be significantly increased by the addition of 10 mM of sodium acetate to the growth medium. This resulted in the accumulation of 11 wt% P(3HB) of the CDW in the recombinant while the wild-type produced about 7 wt% of the CDW.

In the course of this study we found that significantly large number of P(3HB) inclusions were present in the *Synechocystis* cell cytoplasm (Fig. 2a). Nevertheless, GC analysis revealed a polymer content of only 11 wt% of the CDW. To further investigate this matter, freeze-fracture electron microscopy was carried out. The deformation behaviour of the P(3HB) inclusions accumulated by the *Synechocystis* cells were essentially the same as that of P(3HB) inclusions found in other microorganisms.[6] This suggests that the internal microstructure of the P(3HB) inclusions in all microorganisms are similar. The average density of the P(3HB) inclusions can also be assumed to be identical because the isolated inclusions aggregate at the same density when subjected to sucrose density gradient ultracentrifugation. The average number of P(3HB) inclusions in a recombinant *Synechocystis* cell was

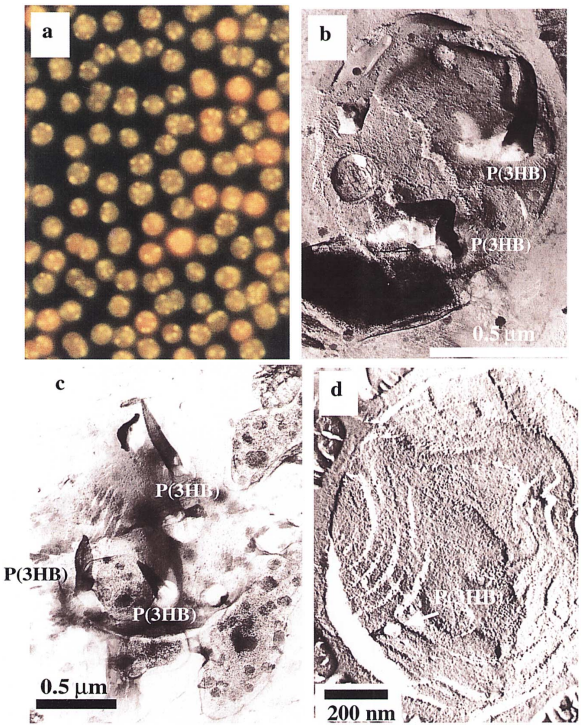

Fig. 2. Observation of the P(3HB) inclusions by microscopy. a) Nile blue A stained recombinant *Synechocystis* containing 11 wt% P(3HB) of the CDW. The inclusions can be seen fluorescing bright yellow in color. On average there are about six inclusions in each cell. b, c) Freeze-fracture electron microscopy of recombinant *Synechocystis* cells showing needle-like deformation of the P(3HB) inclusions. The fracture plane runs only through some of the inclusions. d) Freeze-fracture electron microscopy of wild-type *Synechocystis* cell from growing culture, containing less than 1 wt% P(3HB) of the CDW. The inclusion diameter is 70 nm and shows needle-like deformation.

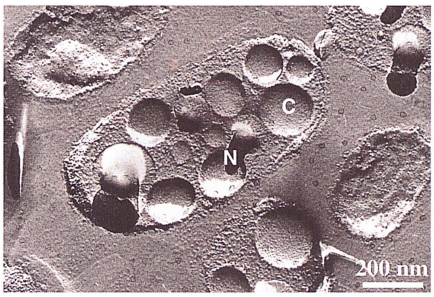

Fig. 3. Freeze-fracture electron microscopy of recombinant *R. eutropha* containing 50 wt% P(3HB) of the CDW. The P(3HB) inclusions have either been deformed into a needle-like protrusion (N) or completely scooped out leaving a crater-like hollow (C) mark in the cell cytoplasm.

six (Fig. 2a). Their average sizes were 70–250 nm (Fig. 3b,c). Inclusions smaller than 100 nm (Fig. 3d) were not readily visible by Nile blue A staining, indicating that the inclusions observed in Fig. 3a all measure more than 100 nm in size.

For comparison purposes we investigated the sizes and numbers of P(3HB) inclusions in a recombinant *R. eutropha* bacterium containing about 50 wt % P(3HB) of the CDW. Figure 3 shows a representative freeze-fracture electron micrograph of the *R. eutropha* bacterium containing an average of about 12 P(3HB) inclusions with sizes in the range of 80–200 nm. By carrying out a modest comparison with the quantity of P(3HB) inclusions in the recombinant *Synechocystis*, one would expect the polymer content to be at least half of that in the recombinant *R. eutropha*, which is about 25 wt% of the CDW. The apparently low P(3HB) content as determined by GC for the cyanobacterium cells maybe because of; i) the larger size and mass of the *Synechocystis* cells compared to bacterial cells; ii) thicker cell wall in cyanobacteria that may prevent the efficient removal of water by freeze-drying.[7] Both these reasons can contribute to a much larger mass for cyanobacteria as compared to bacteria. Therefore, the P(3HB) content in wt% of the CDW as determined by GC would be lower in cyanobacteria than in bacteria.

Conclusion

We have shown that the PHA-producing ability of *Synechocystis* sp. PCC6803 matches that of most bacteria. The wt% content of P(3HB) in cyanobacteria can not be directly compared with that in bacteria because of the significant difference in the cell mass of both systems. Cyanobacteria therefore do have potential to produce significant amounts of P(3HB) from a mixture of acetate and CO_2. The challenge would be to achieve similar PHA contents from CO_2 as the sole carbon source.

References

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