Note

Rapid differentiation between short-chain-length and medium-chain-length polyhydroxyalkanoate-accumulating bacteria with spectrofluorometry

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Abstract

An approach for rapid differentiation between short-chain-length (scl) and medium-chain-length (mcl) polyhydroxyalkanoate (PHA) producers was developed. Polyhydroxyalkanoate-accumulated bacterial cells stained with Nile red were suspended in water and subjected to fluorescence spectroscopy at a fixed excitation wavelength of 488 nm. The scl-PHA-accumulated bacteria revealed a maximum emission wavelength at 590 nm, and for mcl-PHA producers were seen at a wavelength of 575 nm. Combining Nile red staining and fluorescence spectroscopy, the accumulated PHA granules could be rapidly differentiated into scl-PHA and mcl-PHA from the intact cells.

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Polyhydroxyalkanoates (PHAs), a sort of biological polyester (Fig. 1), function as carbon and energy reserves in prokaryotic cells (Anderson and Dawes, 1990); a wide range of bacteria synthesize them when a carbon source is provided in excess and one essential growth nutrient is limited (Ramsay et al., 1990). PHA polyesters have been studied intensively by academia and industry in recent decades. Since their physical characteristics are similar to those of petrochemical polyesters, such as polypropylene, and possess biocompatible and biodegradable features, they

are considered good candidates for biodegradable plastics. Generally, PHAs are categorized into two groups based on the number of carbon atoms in the monomer units: short-chain-length (scl) PHAs consist of 3–5 carbon atoms, and medium-chain-length (mcl) PHAs consist of 6–14 carbon atoms (Anderson and Dawes, 1990). Short-chain-length PHAs possess higher melting temperatures and are stiffer than those of mcl-PHAs, while mcl-PHAs own properties of an elastomer with poor tensile strength and high extension to breakage (Poirier et al., 1995).

Nile red is an oxazone form of Nile blue A, formed by the spontaneous oxidation of Nile blue A in aqueous solution or by refluxing Nile blue A with dilute sulfuric acid. It is poorly soluble in water but dissolves in a wide variety of organic solvents, such as

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n = 1	R = hydrogen	poly(3-hydroxypropionate)	P(3HP)
	R = methyl	poly(3-hydroxybutyrate)	P(3HB)
	R = ethyl	poly(3-hydroxyvalerate)	P(3HV)
	R = propyl	poly(3-hydroxycaproate)	P(3HC)
	R = butyl	poly(3-hydroxyheptanoate)	P(3HH)
	R = pentyl	poly(3-hydroxyoctanoate)	P(3HO)
	R = hexyl	poly(3-hydroxynonanoate)	P(3HN)
	R = heptyl	poly(3-hydroxydecanoate)	P(3HD)
	R = octyl	poly(3-hydroxyundecanoate)	P(3HUD)
	R = nonyl	poly(3-hydroxydodecanoate)	P(3HDD)
n = 2	R = hydrogen	poly(4-hydroxybutyrate)	P(4HB)
n = 3	R = hydrogen	poly(5-hydroxyvalerate)	P(5HV)

Fig. 1. Chemical structure of the polyhydroxyalkanoates produced in bacteria (figure adapted from Poirier et al., 1995).

acetone. The dye is strongly fluorescent in all organic solvents, but is quenched in water (Greenspan et al., 1985). Many methods have been developed for detecting or quantifying intracellular PHA granules based on Nile red staining (Ostle and Holt, 1982; Degelau et al., 1995; Fouchet et al., 1995; Gorenflo et al., 1999; Kranz et al., 1997; Spiekermann et al., 1999). Nonetheless, these methods are not capable of analyzing or differentiating PHA compositions.

In this study, a rapid approach based on Nile red staining was developed for differentiating between scl- and mcl-PHAs from intact cells. Four strains of well-described scl-PHA producers—*Ralstonia eutropha* H16 ATCC 17699, *Comamonas acidovorans* ATCC 15668, *Pseudomonas glathei* ATCC 29195, and *Paracoccus versutus* (environmental isolated strain), three strains of mcl-PHA producers—*Pseudomonas putida* ATCC 12633, *P. putida* GPo1 [formerly known as *Pseudomonas oleovorans* GPo1 (van Beilen et al., 2001)] ATCC 29347, and *Pseudomonas citro-*

nellolis ATCC 13674, and two PHA[−] mutants, *P. putida* GPp104 PHA[−] and *R. eutropha* PHB[−]4 were used for evaluating this approach. Bacteria were cultured in a 250-ml baffled flask containing 50 ml of mineral salt medium (MSM) as a nitrogen limitation condition (Ramsay et al., 1990) and an appropriate carbon source, with a final concentration of 1.5% gluconate (Sigma) for scl-PHA producers and PHA[−] mutant and 0.5% sodium octanoate (Sigma) for mcl-PHA producers. These bacteria were cultured in a reciprocal shaker at 30 °C for 2 days to accumulate polyesters. The PHA-accumulated cells were diluted with water until the absorbance measured at 600 nm was 1.0 or less. One milliliter of diluted cells was collected by centrifugation and discarded the supernatant as clear as possible. Ten microliters of Nile red stock solution (1 mg/ml of Nile red in acetone) was slowly added to the cell pellet and mixed with the pipette tip. After incubating at room temperature for 5 min, the stained cells were dried with Speed Vac®

(Savant) apparatus for 5 min to remove trace amounts of acetone, resuspended in 1 ml of water, and subjected to scanning for maximal emission wavelength from 530 to 650 nm at a fixed excited wavelength of 488 nm (model F-4500, Hitachi, Tokyo, Japan) (Greenspan et al., 1985). To enhance the screening speed, bacteria could also be grown on mineral salt medium plates containing an appropriate carbon source instead of liquid culture. An approximately 2-mm-diameter colony was picked up with a toothpick, stained with 10 μ l of Nile red solution, and resuspended in 1 ml of water. Wavelength scanning was performed as described above. Seven PHA-accumulating bacteria exhibited significant fluorescence maxima except for two PHA⁻ mutants (Fig. 2). Furthermore, the fluorescence behaviour among PHA-accumulating bacteria could also be clearly classified into two groups, those with a fluorescence maxima around at 575 nm and those at 590 nm with *P. glathei* slightly shifted to 595 nm (Fig. 2, line 6). The bacteria strains of the 575 nm group, which included *P. putida* ATCC 12633, *P. putida* GPo1 ATCC 29347, and *P. citronellolis* ATCC 13674, are all mcl-PHAs producers according to previous reports (Timm and Steinbüchel, 1990). In addition, the strains of the 590 nm group, including *R. eutropha* H16 ATCC 17699,

C. acidovorans ATCC 15668, *P. versutus*, and *P. glathei* ATCC 29195, are all scl-PHAs producers as previous reports have described (Timm and Steinbüchel, 1990; Lee, 1996). To further confirm the PHA compositions and intracellular polyester content, these bacterial cells were collected and lyophilized (Brandl et al., 1988); approximately 10 mg of lyophilized cells was subjected to methanolysis with a solution consisting of 0.15 ml 98% sulfuric acid, 0.85 ml methanol, 2 mg benzoic acid as an internal standard, and 1 ml chloroform at 100 °C for 140 min to convert the constituents into methyl esters. After extracting the reaction mixture with 1 ml water, the organic phase was dried over anhydrous sodium sulfate and analyzed with gas chromatography (GC) on a Shimadzu GC-17A equipped with a J&W DB-5 capillary column (30 m $\times$ 0.25 mm; 1 μ m film thickness) and a flame-ionization detector operating in a split mode (split ratio, 1:50) with temperature programming (Ramsay et al., 1990). The PHA compositions of the 575 nm group strains and 590 nm group strains were mcl-PHAs and scl-PHAs, respectively, elucidated from the GC results (Table 1). The GC results strongly supported the idea that mcl-PHAs producers and scl-PHAs producers can be differentiated based on fluorescence maxima at 575 and 590 nm, respectively.

Fig. 2. Fluorescence spectra of bacteria grown on gluconate or octanoate as carbon sources and stained with Nile red. 1, *P. citronellolis* ATCC13674; 2, *P. putida* ATCC 12633; 3, *P. putida* GPo1 ATCC 29347; 4, *C. acidovorans* ATCC 15668; 5, *P. versutus*; 6, *P. glathei* ATCC 29195; 7, *R. eutropha* H16 ATCC 17699; 8, *R. eutropha* PHB⁻4; 9, *P. putida* GPp104 PHA⁻.

Table 1
Composition analysis of PHA accumulated from different bacterial strains

Strains	Wavelength at maximum (nm)	Carbon source	PHA content (% wt/wt)	Polymer composition (mol%)			
				3-HB	3-HHx	3-HO	3-HD
<i>Ralstonia eutropha</i> H16	590	gluconate	83.5	100	–	–	–
<i>Pseudomonas glathei</i>	595	gluconate	80.9	100	–	–	–
<i>Comamonas acidovorans</i>	590	gluconate	31.8	100	–	–	–
<i>Paracoccus versutus</i>	590	gluconate	46.9	100	–	–	–
<i>Pseudomonas citronellolis</i>	575	octanoate	11.0	7.4	10.2	75.1	7.3
<i>Pseudomonas putida</i> GPo1	577	octanoate	36.5	3.2	10.1	82.9	3.8
<i>Pseudomonas putida</i>	575	octanoate	22.6	3.5	5.7	88.0	2.8
<i>Ralstonia eutropha</i> PHB [−] 4	–	gluconate	0.6	100	–	–	–
<i>Pseudomonas putida</i> PHA [−]	–	gluconate	–	–	–	–	–

–: not detected; 3-HB, 3-hydroxybutyrate; 3-HHx, 3-hydroxyhexanoate; 3-HO, 3-hydroxyoctanoate; 3-HD, 3-hydroxydecanoate.

Depending upon the relative hydrophobicity of the environment, the excitation and emission maxima of Nile red fluorescence can vary over a range of 60 nm; the fluorescence colors range from golden yellow (emission wavelength, >528 nm) to deep red (emission wavelength, >590 nm) (Greenspan et al., 1985; Dutta et al., 1996). The scl-PHAs and mcl-PHAs show the chemical structure difference on the carbon side chain length and also reflect the inherent hydrophobicity, longer carbon side chain stronger hydrophobicity inherent. This may explain why Nile red staining elucidates different maximum emission wavelengths in scl-PHAs and mcl-PHAs. This approach was excellent for detecting PHAs; however, there was a disadvantage that occurred in differentiating between lipids and PHAs. To overcome this drawback, a genotype-detecting approach was required to further confirm Nile red staining results (Sheu et al., 2000).

It can be concluded that this method combining grown bacteria on a mineral salt medium plate is capable of being applied to rapid differentiating scl- and mcl-PHA producers isolated from environment.

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