Quantified High-Throughput Screening of Escherichia coli *Producing Poly(3-hydroxybutyrate) Based on FACS*

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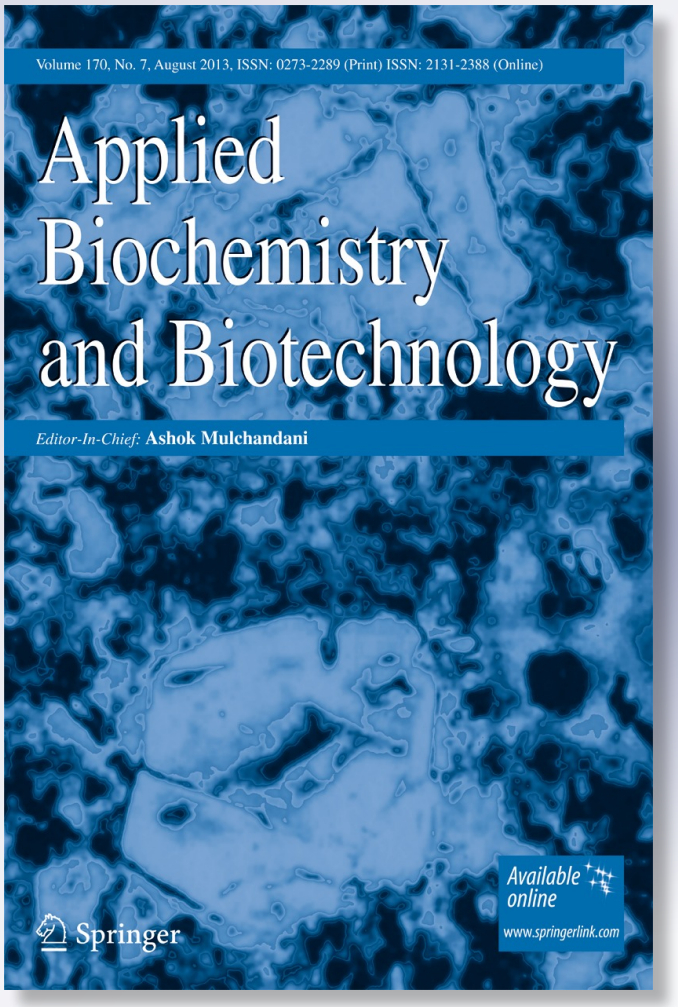

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Quantified High-Throughput Screening of *Escherichia coli* Producing Poly(3-hydroxybutyrate) Based on FACS

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Abstract Here, we report on a highly sensitive method for the detection of P(3HB) accumulation in *Escherichia coli* cells based on the automated flow cytometry system using fluorescent dyes. *E. coli* containing P(3HB) were stained with either BODIPY or Nile red fluorescent dye, and their staining properties were analyzed under a variety of conditions. Compared with Nile red, BODIPY was much more sensitive in staining P(3HB) and overall demonstrated a more rapid staining of cells, a greater resistance to photobleaching, and greater cell viability. In addition, we also successfully monitored heterogeneity in P(3HB) accumulation within a cell population using BODIPY staining and flow cytometry. We believe this optimized staining method using BODIPY in combination with screening by

Jae Hyung Lee and Seung Hwan Lee equally contributed to this work.

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J. H. Lee · S. S. Yim · K. J. Jeong (✉)
Department of Chemical and Biomolecular Engineering (BK21 Program), KAIST,
291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea
e-mail: kjjeong@kaict.ac.kr

S. H. Lee · K.-H. Kang
Industrial Biochemicals Research Group, Research Center for Biobased Chemistry,
Division of Convergence Chemistry, Korea Research Institute of Chemical Technology,
P.O. Box 107, 141 Gajeong-ro, Yuseong-gu, Daejeon 305-600, Republic of Korea

S. Y. Lee (✉)
Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical and Biomolecular Engineering (BK21 Program), Center for Systems and Synthetic Biotechnology, and Institute for the BioCentury, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea
e-mail: leesy@kaist.ac.kr

K.-H. Kang · S. J. Park (✉)
Department of Environmental Engineering and Energy and Department of Energy and Biotechnology,
Myongji University, San 38-2, Nam-dong, Cheoin-gu, Yongin-si, Gyeonggido 449-728, Republic of Korea
e-mail: parksj93@mju.ac.kr

high-speed flow cytometer will be helpful in the engineering of host cells toward an enhanced production of bioplastics.

Keywords FACS · BODIPY · Nile red · Poly(3-hydroxybutyrate) · *Escherichia coli*

Introduction

Polyhydroxyalkanoates (PHAs) are biomass-derived polyesters consisting of a variety of hydroxycarboxylic acids, which are synthesized and accumulate in the cytoplasm of bacteria [1, 2]. As polymer materials, PHAs are attractive polyesters because they are biocompatible and biodegradable plastics; the material properties of which can be designed by modulating the types and compositions of monomer constituents of PHAs [3–5]. For example, poly(3-hydroxybutyrate) [P(3HB)], the representative member of PHAs, has thermoplastic properties similar to polypropylene [2]. Some PHAs such as P(3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] are now produced on a large scale for their commercialization [6]. However, the high production costs of PHAs are hindering their commercialization [7, 8]. Optimization of the fermentation process in the production of PHAs is required to reduce the associated production costs. Factors affecting the production costs of PHAs include the PHA content in bacteria, PHA yield on carbon sources, and PHA productivity [7, 8]. A critical factor in the production of PHAs is the performance of the host microorganism to produce the desired polymer at reduced cost; consequently, there is a strong need to develop microorganisms that can efficiently produce PHAs from renewable carbon sources.

Flow cytometers equipped with a sorting system can be used to analyze and distinguish the fluorescent signals of a range of biomolecules (from bacteria to animals) and to isolate the desired biomolecules with a high specificity and reliability [9]. Given its unique capability to analyze a large number of individual cells across several parameters simultaneously, this technique has been used for the rapid analysis of a number of biomolecule interactions (protein–protein, protein–cells, etc.) and biomolecule formations (lipid, inclusion body, etc.), as well as for high-throughput screening of engineered proteins and host cells [10]. For fluorescent activated cell sorting (FACS) to be successful, the use of an appropriate fluorescent dye for the selective labeling of target molecules with a high sensitivity is critical. Previously, screening protocols were developed for the direct detection of accumulated PHAs in vivo in cells stained with lipophilic dyes [11]. Lipophilic dyes such as Nile blue A [12] and Sudan black B [13] provided researchers with fluorescence-based detection methods that were relatively sensitive to PHA accumulating cells. However, the application of these dyes in high-throughput screening of PHA accumulating microorganisms has not been successful because the dyes are dissolved in organic solvents such as ethanol and acetone; both of which significantly inhibit the growth of microorganisms. Master plates should be prepared to select PHA accumulating cells because microorganisms stained by these dyes lose viability. Recently, a highly sensitive viable colony staining method used for the detection of PHA accumulating cells was developed by employing Nile red [9-(diethyl amino) benzo[a]phenoxazin-5(5H)-one] dissolved in dimethyl sulfoxide (DMSO) as the staining dye [11]. The Nile red staining method has been used successfully in screening natural PHA-producing bacteria and for a PHA synthase engineered to have enhanced activity and different substrate specificity [14, 15]. However, these colony-based staining methods were based on time- and labor-intensive screening of colonies, which cannot provide us with optimized high-throughput screening of PHA synthases.

In this study, we developed a highly sensitive viable screening method using BODIPY (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) for the detection of P(3HB) accumulating cells. BODIPY is a highly lipophilic green fluorescent dye, and because of its exceptional spectroscopic properties, it has been widely used in the labeling of a variety of biomolecules such as phospholipids, cholesterol, etc. [16–18]. In this work, we optimized the conditions for BODIDPY staining of P(3HB) granules in *Escherichia coli*, and the staining properties of this dye were compared with those of Nile red.

Materials and Methods

Bacterial Strains, Plasmids, and Genes

E. coli strain XL1-Blue (Stratagene Cloning Systems, La Jolla, CA, USA) was used for cloning and production of P(3HB). Plasmid pCnCAB, which expresses the *Ralstonia eutropha* PHA biosynthesis genes, has been previously described [19].

Culture Conditions

Recombinant *E. coli* XL1-Blue transformed with pCnCAB was initially cultured at 37 °C in Luria-Bertani (LB) medium (containing 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl). After overnight cultivation in 5 mL of LB broth, the *E. coli* cells were inoculated into 25 mL of LB medium supplemented with 20 g/L of glucose in a 100 mL flask and cultivated at 37 °C in a rotary shaker at 250 rpm for 48 h. In all flask cultivations, ampicillin (Amp, 50 μg/mL) was added to the medium.

Pre-treatment of Cell for Staining with Fluorescent Dyes

For flow cytometric analysis, cells were stained with either Nile red or BODIPY fluorescent dyes. After cultivation, the cultures were cooled on ice for 10 min, and the cells harvested by centrifugation (5 min, 1,000×*g*, 4 °C) to an optical density at 600 nm (OD_{600}) of 0.4. The cell pellets were first resuspended (the first suspension) in either 1 mL of (1) ice-cold TSE buffer (10 % (*w/v*) sucrose, 10 mM Tris–HCl (pH 7.5), 2.5 mM Na–EDTA) or (2) distilled deionized water (DDW) and incubated on ice for an additional 10 min. After centrifugation (5 min, 3,000×*g*, 4 °C), cells were resuspended (the second suspension) in either 1 mL of (1) ice-cold 1 mM $MgCl_2$ or (2) DDW. These four different conditions for pretreatment are summarized in Table 1. The cells were mixed with 12.5 μl of Nile red (Sigma-Aldrich Co.,

Table 1 Four different conditions for pretreatment of cells

Conditions	Reagent in the first suspension	Reagent in the second suspension
Pretreatment I	TSE buffer[a]	DDW
Pretreatment II	TSE buffer[a]	1 mM $MgCl_2$
Pretreatment III	DDW	DDW
Pretreatment IV	DDW	1 mM $MgCl_2$

[a] 10 % (*w/v*) sucrose, 10 mM Tris–HCl (pH 7.5), 2.5 mM Na–EDTA

St. Louis, MO, USA) dissolved in DMSO (0.4 μg/μl) or 5 μl of BODIPY (Invitrogen, Eugene, OR, USA) dissolved in DMSO (1 μg/μl). After vigorous mixing by vortex, both mixtures were incubated in the dark for 5 min at room temperature. Finally, the cells were centrifuged (5 min, 3,000×*g*, 4 °C) and washed with ice-cold DDW twice and immediately analyzed using flow cytometer and confocal fluorescence microscope.

Flow Cytometric Analysis

After staining, the cellular fluorescence was measured using a Moflo XDP flow cytometer (Beckman Coulter, Brea, CA, USA). Cell excitation was achieved using blue light (488 nm) provided by an air-cooled argon ion laser. The emission signals of Nile red and BODIPY were measured on different channels: FL1 channel (530/40 nm) for BODIPY-stained cells and FL2 channel (580/30 nm) for Nile red-stained cells. For the flow cytometric analysis of each dye, a total of 5×10^5 cells were analyzed, and all FSC (forward scatter) and SSC (side scatter) image and mean fluorescence intensity values were recorded using the MoFlo^{TM} XDP SUMMIT Software version 5.2 (Beckman Coulter).

Microscopic Analysis of P(3HB) Granules

The cells were harvested, washed twice with DDW, and visualized using light microscope (Nikon optiphot-2, Nikon, Tokyo, Japan). The images were captured using Nikon ACT-2U Imaging Software version 1.62. Stained cells were washed three times with DDW to remove any residual fluorescent dye prior to visualization by confocal fluorescence microscope (Carl Zeiss LSM510 META, Jena, Germany). Cell excitation was accomplished using a 488 nm argon laser, and images were filtered by a band pass 500 ~ 550 nm (BODIPY) and long pass 560 nm (Nile red) filter. Photographs were captured with a Carl Zeiss LSM software (version 4.2.rk).

Cell Viability Analysis

During the flow cytometric analysis of the stained cells, the highly fluorescent cells were sorted at "single cell" mode, and each cell was collected in a 96 deep-well plate (Bioneer, Daejeon, Republic of Korea) containing 1 mL LB with ampicillin (50 μg/mL). After the sorting of 48 cells (half plate for each sample), the plates were incubated at 37 °C with vigorous shaking (200 rpm). At the completion of the 48 h incubation, the number of wells with viable cells was counted.

Analytical Methods

P(3HB) concentration was determined by gas chromatography using the Agilent 6890 N GC System (Agilent Technologies, Palo Alto, CA, USA) equipped with a fused silica capillary column (SPB^{TM}-5, 30 m × 0.32 mm ID, 0.25 μm film; Supelco, Bellefonte, PA, USA) and benzoic acid as an internal standard [20]. Cell concentration, defined as dry cell weight per liter of culture broth, was determined as previously described [21]. The residual cell concentration was defined as the cell concentration minus P(3HB) concentration. The P(3HB) content (wt%) was defined as the percentile ratio of P(3HB) concentration to cell concentration.

Results and Discussion

Production of P(3HB) in *E. coli*

After cultivation of *E. coli* XL1-Blue harboring pCnCAB for 24 h, the accumulation of P(3HB) granules in cells were first observed by light microscope. The accumulation of P(3HB) granules was clearly observed in the culture of recombinant cells, while no granules were observed for the control, wild-type *E. coli* XL1-Blue (Supplementary material Fig. S1). The cells were stained with either BODIPY or Nile red, and the resultant staining of P(3HB) granules was analyzed by confocal fluorescence microscope. The results obtained confirmed the specificity of both dyes for cellular P(3HB) granules (Supplementary material Fig. S1). In contrast, the negative control sample failed to stain with either of the dyes because of the absence of P(3HB) producing cells (data not shown). The conditions for the staining of P(3HB) granules with fluorescent dyes were further optimized using the recombinant P(3HB) producing *E. coli* culture.

Optimization of Pre-treatment Conditions

The internalization of dyes into host cells may require a carrier such as acetone [22], glycerol [23], DMSO [23, 24], or ethanol [17]. Tyo et al. [25] demonstrated that sucrose shock was the most effective method for staining *E. coli* cells with Nile red. Based on this finding, we investigated four different conditions for the pre-treatment of *E. coli* cells (Table 1). After pre-treatment with one of the four aforementioned conditions, cells were stained with either Nile red or BODIPY for 5 min, and their fluorescence signal intensities were measured by flow cytometer. As expected, the intensity of the fluorescence signals for the P(3HB) non-producing cells (negative control) was very low and was independent of the pre-treatment conditions and fluorescent dyes used (data not shown). In contrast, all cells producing P(3HB) granules recorded much higher and very different signal intensities, and this was dependent on both the fluorescent dye and pre-treatment conditions used. The intensities of the fluorescence signals for the BODIPY-stained cells differed for each pre-treatment condition. Of the four pre-treatments, 'Pretreatment I' yielded the highest intensity followed by 'Pretreatment II' (Fig. 1a). The use of DDW for the first suspension resulted in considerably lower signal intensities compared with the corresponding sucrose treatments. In addition, the use of DDW in the first suspension (Pretreatment III and IV) yielded twin-peak histograms, which indicated heterogeneity in cell staining. The use of DDW in the second suspension (Pretreatment I) gave a slightly higher signal, but there was no substantial difference to that for $MgCl_2$ (Pretreatment II). The results for Nile red-stained cells were similar to those observed for BODIPY staining in that the use of sucrose for the first suspension (Pretreatment I and II) produced higher signal intensities than those for DDW (Pretreatment III and IV), and the use of DDW or $MgCl_2$ for the second suspension did not give any appreciable difference in signal intensities (Fig. 1b). These results suggest that sucrose treatment might help fluorescent dyes permeate the cell wall and allow cells to be stained more effectively. For all conditions investigated, BODIPY staining gave much higher fluorescent intensities when compared with Nile red (Fig. 1a and b). This was most evident for cells pre-treated under the Pretreatment I condition. The mean fluorescent intensities of BODIPY-stained cells were as high as 14.8 times those of the corresponding Nile red-stained cells. These results suggest that BODIPY is more effective than Nile red in staining P(3HB), and its superiority in staining was confirmed further in the proceeding experiments.

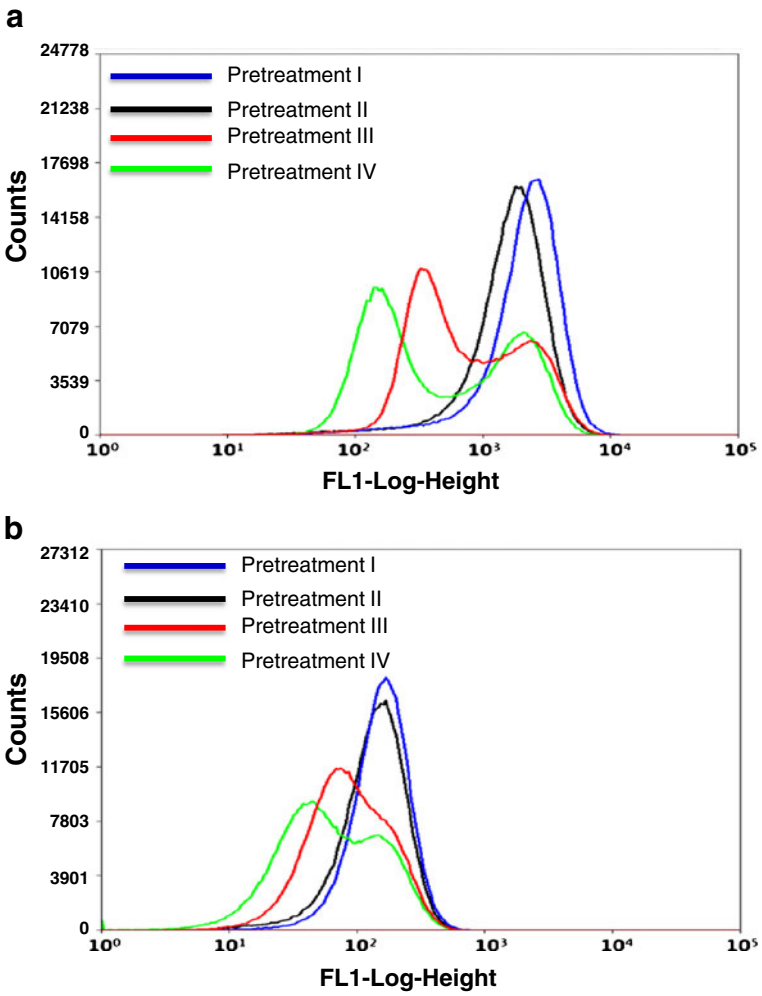

Fig. 1 The effect of pre-treatments on cell staining with fluorescent dyes. After cell pre-treatment with four different conditions, P(3HB)-producing cells were stained with **a** BODIPY or **b** Nile red, and their fluorescence signal intensities analyzed by flow cytometry with excitation wavelength at 488, 530, and 560 nm for emission wavelength

Effect of Staining Time and Comparison of Photobleaching

To optimize time for staining with fluorescent dyes, the effect of staining time on the individual cells was investigated. After cultivation, the cells were pre-treated under Pretreatment I condition prior to the addition of either Nile red or BODIPY. The cells were incubated in the presence of the dye for periods of 1, 5, 10, or 20 min, and the fluorescent intensities analyzed by flow cytometer. The fluorescent intensities of the sample changed very little over time irrespective of the dye used (Fig. 2a). However, as reported previously in this study, BODIPY staining produced greater fluorescence intensity compared with Nile red staining and was consistent across all time periods investigated. The results obtained from

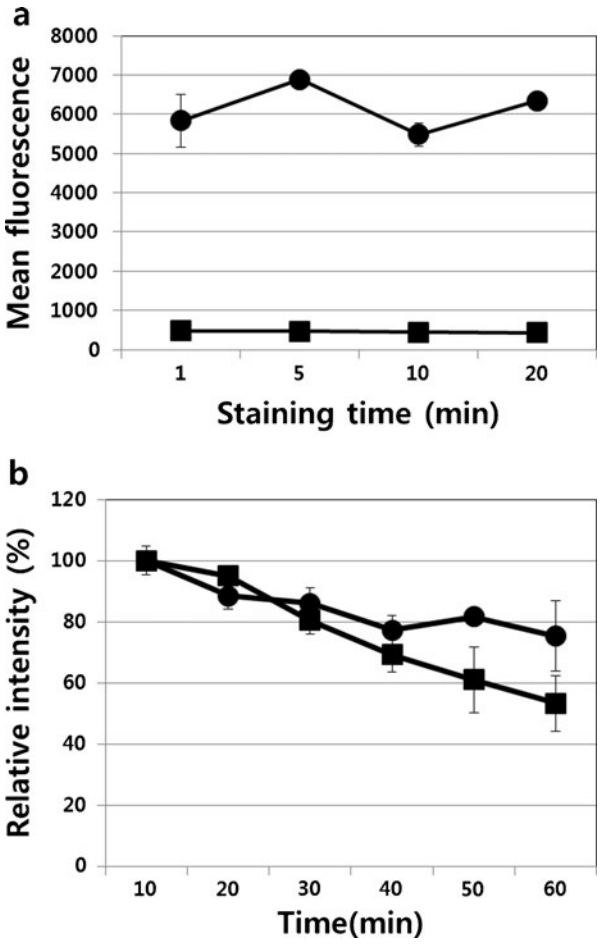

Fig. 2 Effect of staining time on cell staining and analysis of photobleaching. **a** Cells were mixed with fluorescent dye for different time intervals to address the effects on staining. **b** After staining cell with fluorescent dyes for 5 min, the fluorescent intensities of cells were analyzed at different time intervals. The *closed circle* and *square* symbols represent the BODIPY and Nile red-stained cells, respectively

this analysis demonstrated that 5 min was the optimum time required for BODIPY staining of *E. coli* cells.

A loss in cellular fluorescence or photobleaching is of concern when undertaking fluorescence studies, particularly when the screening is performed over a prolonged period of time. After staining cells with either dye for 5 min, the samples were photobleached under fluorescent light for various durations (up to 1 h), after which the fluorescent intensities were measured as previously described. Cells stained with Nile red demonstrated a gradual loss in fluorescence with time, and only 52 % of initial intensities remained after 1 h (Fig. 2b). Cells stained with BODIPY also showed a gradual loss in fluorescence with time, but the loss rate was relatively slower than Nile red (Fig. 2b). A relatively higher intensity (75 % of initial intensities) was kept until 1 h after staining. These results indicate that BODIPY is superior to Nile red for the long-term screening of cells.

Effect of Fluorescent Dye Concentration on Cell Staining

To determine the optimum dye concentration required for cell staining, *E. coli* cells were stained with Nile red or BODIPY at three different concentrations (0.1, 1, or 5 μg/mL) for 5 min. The cells were harvested from culture at four different time points (12, 24, 36, and 48 h). The content of P(3HB) would be expected to be quite variable for each sample culture. After cell staining, the fluorescence intensities were measured and compared with the actual contents of P(3HB), which were determined by gas chromatography (GC). In the early stages of cell growth (12–24 h), the cells accumulated P(3HB) to a greater extent (up to approx. 45 % of total dry cell weight), compared with the later stage (36–48 h), for which there was a gradual decrease in P(3HB) content. When staining with BODIPY, greater fluorescent signal intensities were obtained when the higher concentration (5 μg/mL) of dye was used (Fig. 3a). Also, the mean

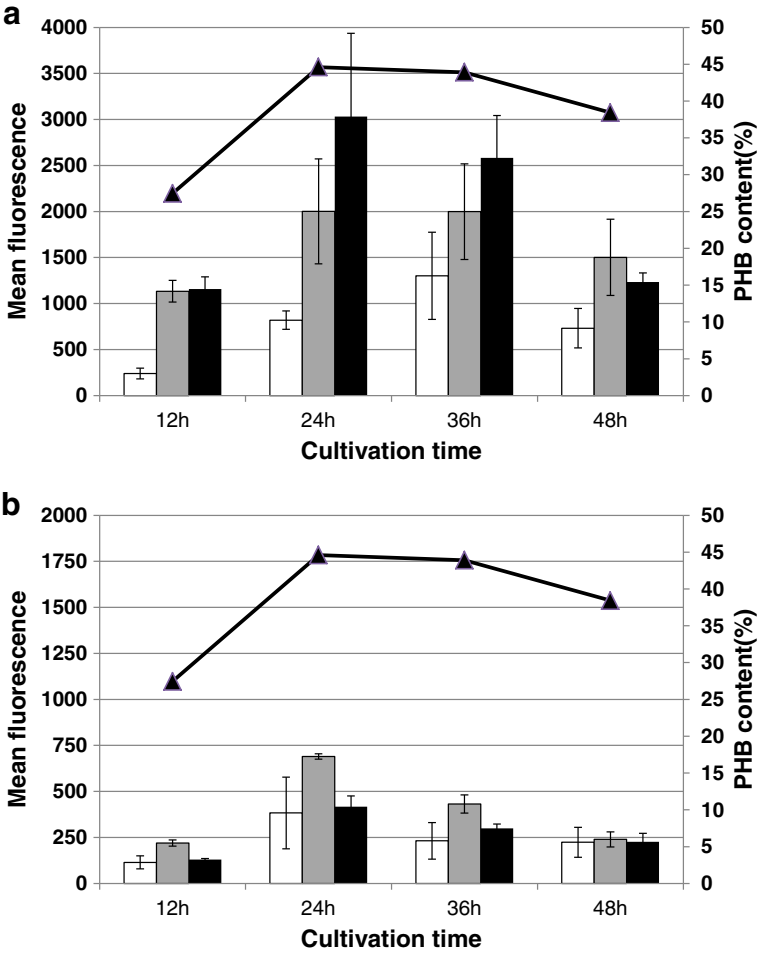

Fig. 3 The effect of fluorescent dye concentration on BODIPY and Nile red staining. Cells containing different amounts of P(3HB) were stained with fluorescent dyes at different concentrations: **a** 0.1, **b** 1 or **c** 5 μg/mL of BODIPY or Nile red. The *closed triangle symbols* indicate the content of P(3(HB) determined by GC analysis

fluorescence highly correlated with P(3HB) contents, except when the low concentration of dye (0.1 μg/mL) was used. In the case of Nile red staining, the fluorescent signal intensities correlated well with P(3HB) content (Fig. 3b). Of the three concentrations investigated, the intermediate concentration (1 μg/mL) of Nile red yielded the highest signal intensities, but overall the intensities were very low when compared with those for BODIPY staining. We conclude that BODIPY is more effective at staining P(3HB) than Nile red and that the optimum conditions for the staining of cells producing P(3HB) is the pre-treatment of cells under Pretreatment I condition prior to staining with 5 μg/mL of BODIPY for 5 min.

Correlation of Fluorescence Intensity with P(3HB) Content

Figure 4 indicates a good correlation between fluorescence signal intensity and P(3HB) content (%wt). To verify this correlation, additional cells with variable amounts of P(3HB) were examined. Each cell was stained with BODIPY under optimum staining conditions, and the fluorescent intensities were analyzed by flow cytometer. P(3HB) content of each cell was

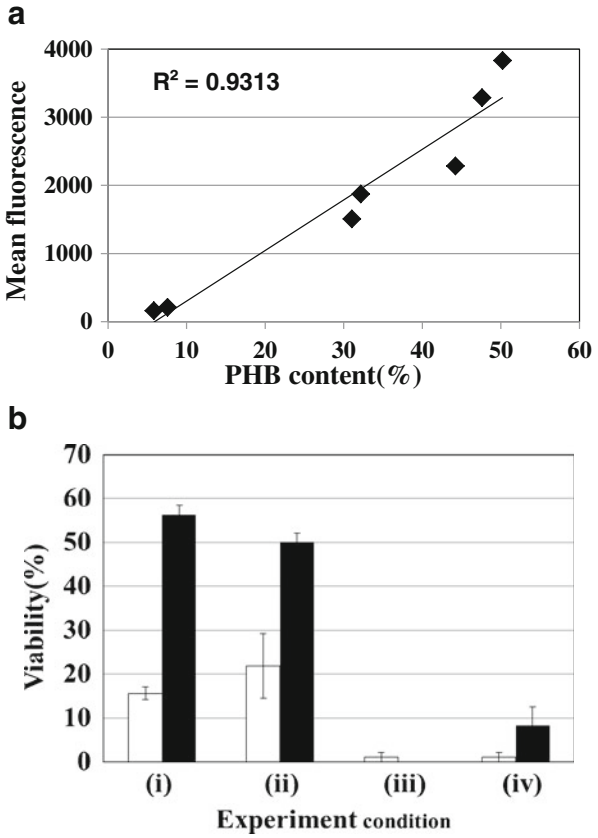

Fig. 4 **a** Correlation of P(3HB) contents (%) and mean fluorescence of BODIPY-stained cells. The solid line indicates the regression line. **b** Comparing the cell viability after staining with Nile red or BODIPY. Cells containing low P(3HB) were prepared under (*i*) Pretreatment I or (*ii*) Pretreatment II. Also, cells containing high P(3HB) were prepared under (*iii*) Pretreatment I or (*iv*) Pretreatment II. The *white* and *black bars* represent the cells stained with Nile red and BODIPY, respectively

also analyzed by GC. As illustrated in Fig. 4a, the geometric mean of the flow cytometer measurement correlated very well (R^2=0.93) with the analytical P(3HB) measurements over a wide dynamic range (from 5 % to 50 %) of P(3HB) contents. It can be concluded that FACS analysis data for BODIPY-stained cells are highly reliable and that the combination of BODIPY staining and FACS analysis can be a useful tool for P(3HB) analysis.

Viability of Cells Stained with Fluorescent Dyes

Cell viability is also an important consideration when performing cell staining and cell sorting. After staining with the fluorescent dyes (BODIPY or Nile red), the effect of cell staining on cell viability was investigated. In this work, we examined cells that had differing contents of P(3HB): low content (~4.5 %) or high content (~34.3 %). First, each cell was pretreated under Pretreatment I or Pretreatment II conditions (Table 1). Previously, Tyo et al [25] reported that the pre-treatment of cells with $MgCl_2$ prior to staining with Nile red improved cell viability. So, the effect of $MgCl_2$ pre-treatment (Pretreatment II) on cell viability was also investigated in the current study. For an accurate analysis of cell viability, only highly fluorescent cells that contained P(3HB) granules were sorted individually into 96 deep-well plates by flow cytometer. After overnight incubation, only wells exhibiting cell growth were counted and their viabilities determined. The combined techniques of single cell sorting and individual cultivation in 96 deep-well plates affords greater accuracy and more reliable data than other methods in which all stained cells are spread together on agar plates. The viability data obtained from the spreading agar plate method may inadvertently include false-positive cells that failed to stain but grew well. When the P(3HB) content was low (~4.5 %), cells stained with BODIPY showed much higher viabilities (50~55 %) than the Nile red-stained cells, independent of $MgCl_2$ treatment (Fig. 4b). As reported by Tyo et al [25], cells subjected to Nile red staining and treated with $MgCl_2$ (Pretreatment II condition) exhibited slightly higher viability (21 %) when compared with DDW-treated (Pretreatment I) cells (15 %). But, overall cell viability was much lower for Nile red-stained cells than for those stained with BODIPY. When P(3HB) content was high (~34.3 %), overall cell viability was low and was independent of the fluorescent dyes used and $MgCl_2$ treatment. It has been well documented that too high an accumulation of P(3HB) significantly inhibits cell growth [26]. Considering that P(3HB) accumulation significantly affects cell physiology [27, 28], low cell viability might result from cell damage by the high P(3HB) content and not by the fluorescent dye.

Monitoring of Heterogeneous Populations During P(3HB) Synthesis

P(3HB) synthesis requires the coordinated reactions of several enzymes, and it is highly sensitive to the culture environment [29]. In some cases, individual cells respond differently to their environment and accumulate P(3HB) to differing levels. Consequently, P(3HB)-producing and non-producing cells can reside in a single culture reactor. When P(3HB) accumulates to a high level in the cell, heterogeneity in populations becomes a serious issue. Under these circumstances, P(3HB) non-producing cells can grow more rapidly and may cause a decline in P(3HB) productivity. Sample heterogeneity cannot be detected by conventional methods such as GC analysis, which measures the total content of P(3HB) across the whole population. In contrast, the formation of P(3HB) granules in individual cells can be analyzed using a flow cytometer, which measures the change in the cellular light-scattering properties caused by the accumulation of P(3HB) granules. Two cells that either did or did not produce P(3HB) were analyzed by flow cytometer, and their light-

scattering properties were compared by FSC versus SSC channels. The negative cells that did not produce P(3HB) granules were more concentrated at a low range in FSC versus SSC channel where most wild-type *E. coli* cells appeared generally, while cells producing P(3HB) granules showed the shift of populations to right-up position in SSC versus FSC channels (Supplementary materials Fig. S2).

To demonstrate the monitoring of heterogeneity during P(3HB) synthesis by FACS, cells were cultivated and harvested at four different culture times (12, 24, 36, and 48 h), and after staining under optimal conditions, cells were analyzed using a flow cytometer. In the early stages of cell growth (12 and 24 h), microscope analysis confirmed that cells were homogeneous in P(3HB) formation (Fig. 5a). This was consistent with the data obtained from the flow cytometric analysis whereby single major populations in the FSC-SSC channel and a single peak in the FL1 histogram were observed (Fig. 5b and c). However, as cells progressed to a late phase of cell growth (36 and 48 h), P(3HB) non-producing cells

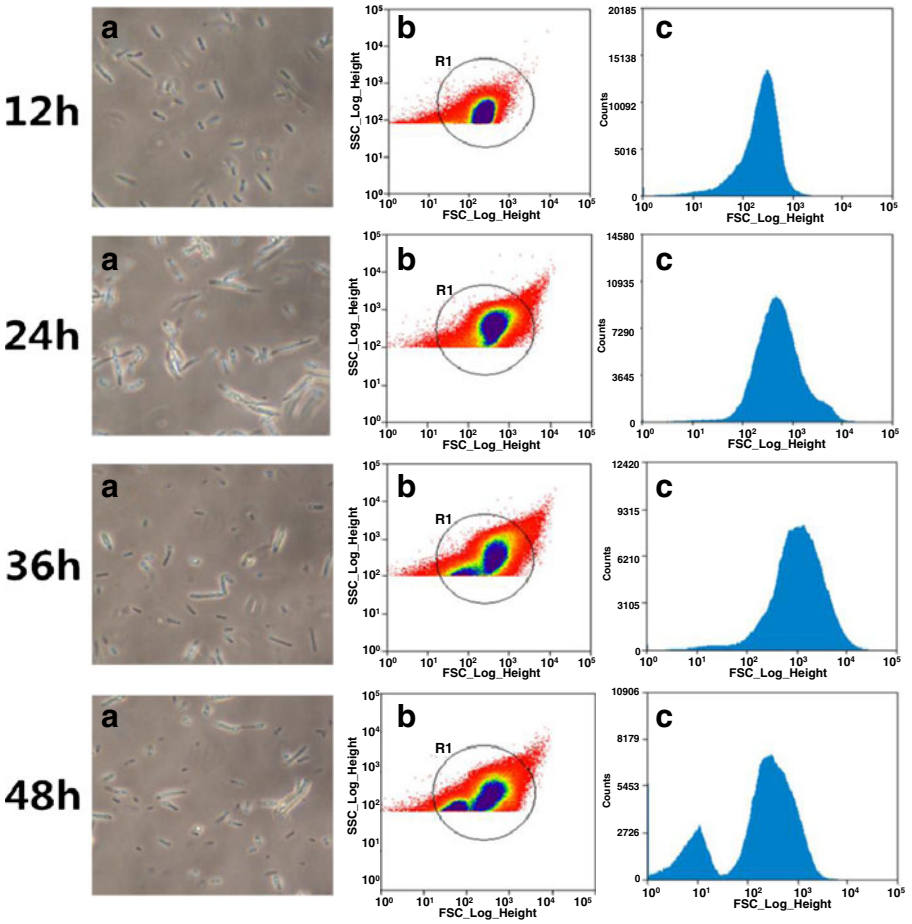

Fig. 5 Monitoring of population heterogeneity in P(3HB) production. **a** After cultivation, cells were analyzed by optical microscope. After staining with BODIPY, **b** the light-scattering image (FSC versus SSC) and **c** fluorescent signal intensities were analyzed. The *numbers* in the *left margin* represent the cultivation time. Region '*R1*' in (**b**) was used to set a gate in each histogram (**c**)

emerged, and two populations co-existed in the same culture (Fig. 5a). This heterogeneity was correctly detected by flow cytometer. In the FSC-SSC channel, a new dense area came out from the original positions of P(3HB) producing cells, and in the last stage, two populations were detected in the same channel (Fig. 5b). The FL1 histogram contained two peaks; one peak had a high mean fluorescence signal, while the other peak had a relatively low mean fluorescence signal, which was indicative of fewer P(3HB) granules (Fig. 5c).

Conclusions

In this study, we optimized the conditions for the staining of cellular P(3HB) using the fluorescent dye BODIPY. BODIPY stain had a high specificity for P(3HB) granules accumulated within *E. coli* host cells, and the resultant fluorescence signal intensities strongly correlated with real P(3HB) contents. Compared with Nile red staining, BODIPY was the superior dye, having a greater sensitivity, longer maintenance in the individual host cell, and greater cell viability. In addition, flow cytometer can provide more reliable information on the distribution of P(3HB) granules at a single cell level, which cannot be obtained by conventional analysis. The use of non-toxic BODIPY dye and high-speed FACS sorter will be a powerful tool in the engineering of a PHA synthase host cell and ultimately toward the enhanced production of bioplastics.

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