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Staining and Quantification of Poly-3-hydroxybutyrate in *Saccharomyces cerevisiae* and *Cupriavidus necator* Cell Populations Using Automated Flow Cytometry

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Background: Poly [(*R*)-3-hydroxybutyric acid] (PHB) is a prokaryote storage material for carbon and energy that accumulates in cells under unbalanced growth conditions. Because this class of biopolymers has plastic-like properties, it has attracted considerable interest for biomedical applications and as a biodegradable commodity plastic. Current flow cytometric techniques to quantify intracellular PHB are based on Nile red. Here, an improved cytometric technique for cellular PHB quantification utilizing BODIPY 493/503 staining was developed. This technique was then automated using an automated flow cytometry system.

Materials: Using flow cytometry, the fluorescence of *Saccharomyces cerevisiae* and *Cupriavidus necator* with varying PHB content after staining with BODIPY 493/503 and Nile red was compared, and automated staining techniques were developed for both cultures.

Results: BODIPY 493/503 staining had less background staining, higher sensitivity and specificity to PHB, and higher saturation values than did Nile red staining. The developed automated staining procedure was capable of analyzing the PHB content of a bioreactor sample every 25 min and measured the average PHB content with accuracy comparable to offline GC analysis.

Conclusion: BODIPY 493/503 produced an overall better staining for PHB than did Nile red. When combined with the automated system, this technique provides a new method for the online monitoring and control of bioreactors. © 2005 International Society for Analytical Cytology

Key terms: Nile red; BODIPY 493/503; automated bioreactor monitoring; polyhydroxyalkanoates; *Ralstonia eutropha*

Polyhydroxyalkanoates are polyesters that are used by bacteria as a carbon and energy storage material during times of nutrient limitation and have the favorable characteristics of being biodegradable and produced from renewable resources (1–3). The preferred current methods for analytical poly-3-hydroxybutyrate (PHB) detection are limited to gas chromatography (4), NMR (5), and mass-spectrometric analysis (6). However, these methods do not allow real-time monitoring of the PHB content in a culture when this polymer is produced.

Flow cytometry was used to measure the PHB content in *Cupriavidus necator* (formerly *Ralstonia eutropha* (7)) by measuring the change in the cellular light scattering properties caused by the formation of PHB granules (8). A more sensitive method consists of staining the cellular PHB with Nile red followed by analysis with either fluorescence spectroscopy (9) or flow cytometry (10–12). These optical methods allow for almost real-time on-line measurement of PHB for bioreactor monitoring, control, and optimization. Furthermore, flow cytometric methods provide information on the single cell distribution of PHB content, which cannot be obtained in conventional physiological studies. However, the sample preparation for flow cytometry is time consuming, and background staining of intracellular lipids reduces the sensitivity of the method. To solve these problems, the staining steps were automated using a recently developed robotic system that can handle the preparation steps for flow cytometry (13). In addition, a method was developed using a new stain, BODIPY 493/503, which has less nonspecific staining than does Nile red when staining cells (14). Here, a staining technique using BODIPY 493/503 for *Saccharomyces cerevisiae* and *C. necator* was optimized and compared

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with Nile red staining. This technique provides a method to investigate cellular PHB distributions and has implications for bioreactor monitoring and control during PHB production. It is also useful for the isolation of mutants in molecular evolution experiments, particularly of yeast cells, since the PHB stain does not compromise cell viability.

MATERIALS AND METHODS

Strains and Growth Conditions

The recombinant *S. cerevisiae* strain D603 expressing the *C. necator* PHB pathway has been described in detail by Carlson et al. (15). Briefly, the cells contain two plasmids expressing the PHB synthesis pathway under the control of a GAL promoter. The cells were cultivated in shake flasks for 4 days at 30°C in 50 ml of medium, at a shaking rate of 250 rpm. The cells were cultivated on an SD minimal medium containing yeast nitrogen base with amino acids (6.7 g/l), adenine (100 mg/l), methionine (100 mg/l), and lysine (150 mg/l). The glucose concentration of the medium was 1%, and the galactose concentration in the medium was varied from 0 to 5%, producing cultures with PHB contents varying between 0 and 5% of the cell dry weight.

Inoculum cultures of *C. necator* H-16 (ATCC 17699) were cultivated overnight on minimal medium (16) containing 5 g/l of fructose (ICN Biomedicals, Inc. Aurora, OH) and 1.3 g/l of $(NH_4)_2SO_4$ at 30°C, at a shaking rate of 250 rpm. For the batch bioreactor studies, a 5-l bioreactor (3-l working volume) from B. Braun Biotech Inc. (Allentown, PA) that contained minimal medium with 15 g/l of fructose and 1.3 g/l of $(NH_4)_2SO_4$ was inoculated with 50 ml of cells and aerated at a rate of 1 vvm. An impeller speed of 600 rpm was used to maintain the dissolved oxygen above 80% of air saturation. The pH was maintained at 7 using 2 M NaOH. As a negative control, *C. necator* H16/PHB$^-$4, which is not capable of producing PHB, was used. This culture was grown in shake flasks under the same conditions as above.

Polyhydroxybutyrate Analysis

The cellular PHB content was determined by gas chromatography after propanolysis of dried cells by the method given in Riis and Mai (4). The resulting esters were subsequently analyzed by gas chromatography, using a model GC – 17A gas chromatograph (Shimadzu, Columbia, MD) with a flame ionization detector and a DB-Wax column (30 m, 0.32-mm inner diameter, 0.5-μm film; J&W Scientific, Folsom, CA).

Cell Counts

Cell counts for *S. cerevisiae* were obtained using an Elzone particle counter (Particle Data, Inc. Elmhurst, IL) with a 48-μm orifice. The cell samples were diluted to concentrations between 20,000 and 50,000 cells/ml. 100 μl of the diluted sample was counted.

Cell counts for *C. necator* were obtained by flow cytometry. A serial dilution of cells was performed, and the cells were mixed with a bead suspension whose concentration was determined using an Elzone particle counter. The number of cells relative to the number of beads could be measured using flow cytometry, and the original number of cells could then be calculated. A calibration curve was then set up to correlate cell number concentration with the PHB-free cell dry weight.

Staining of Cells for Cytometric PHB Analysis

Using the cell count data, $\sim 5 \times 10^6$ cells/ml of live *S. cerevisiae* cells were suspended in 1 ml of phosphate buffered saline (PBS) at room temperature in a 1.5-ml eppendorf tube. BODIPY 493/503 (Molecular Probes, Eugene, OR) dissolved in DMSO was added to the samples, and the samples were incubated with the stain for 5 min. The BODIPY 493/503 volume added varied between 1 and 100 μl, and the BODIPY 493/503 concentration varied between 10 and 1,000 μg/ml. The final DMSO concentration was varied between 0.1 and 10% (v/v). The optimal dye volume and concentration was found to be 10 μl of dye with a concentration of 100 μg/ml. This optimized protocol yielded a final BODIPY 493/503 concentration of 0.038 μM and a final DMSO concentration of 1% v/v. After staining, the cells were pelleted and resuspended in 1 ml of PBS at 4°C and placed on ice and in the dark before analysis. The same procedure was used to stain the cells with Nile red (Sigma, St. Louis, MO). The final Nile red concentration found to yield optimal staining was 0.032 μM. The staining was carried out in triplicate. The same technique was used to stain *C. necator*; however, the cells were made permeable to the stain by incubation for 20 min in 35% ethanol, and then pelleted and resuspended in PBS before staining for 5 min. This ethanol exposure made the cells permeable to the stain and permitted a shorter staining time, as has been discussed elsewhere (11).

Flow Cytometry

A Becton-Dickinson FACSCalibur flow cytometer (Becton-Dickinson Immunocytometry System, San Jose, CA) with a 15-mW Ar laser with a wavelength of 488 nm was utilized to measure the single cell fluorescence intensity after staining. Nile red fluorescence was measured using a 585 ± 42 nm band pass filter. BODIPY 493/503 fluorescence was measured using a 530 ± 30 nm band pass filter. Data were collected using linear amplification for *S. cerevisiae* and logarithmic amplification for *C. necator*. Logarithmic amplification was necessary to not saturate the instrument because of the higher PHB content in *C. necator* than in *S. cerevisiae*. Measurements were triggered based upon the FSC for *S. cerevisiae* and the FSC and SSC for *C. necator*. At least 30,000 cells were measured in each sample, and the average channel number of the height of each pulse was used to determine the mean fluorescence of each sample. The cytometer channels were standardized by setting the gains such that signals from 8.1-μm fluorescein beads (Bangs Laboratories, Fishers, IN) always appeared in the same channel.

On-Line Analysis

The system developed by Abu-Absi et al. (13) was utilized to stain the cells on-line. The core of the unit is a

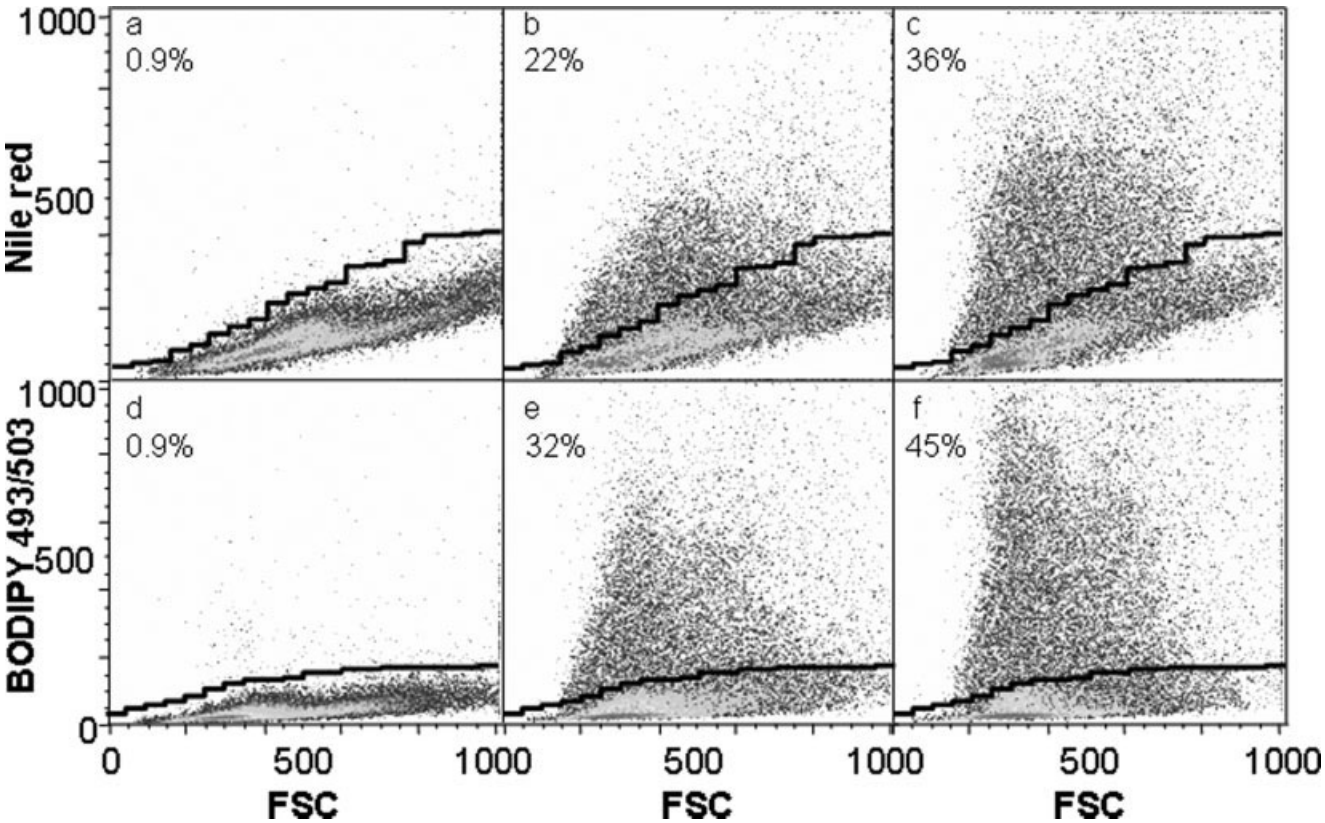

FIG. 1. Bivariate distribution of the FSC and PHB fluorescence of *S. cerevisiae* strain D603 containing the plasmids for PHB synthesis. The cultures were grown as described in the Materials and Methods section. The cells were stained with 10 μl of 100 μg/ml of either Nile red (**a–c**) or BODIPY 493/503 (**d–f**) and analyzed by flow cytometry. The bivariate distributions display data from cells containing 0% (a, d), 3.2% (b, e), and 5.2% (c, f) PHB of the total cell dry weight as determined by gas chromatography. Fluorescence data was collected on a flow cytometer using a 585 ± 42 nm band pass filter for Nile red fluorescence and a 530 ± 30 nm band pass filter for BODIPY 493/503 fluorescence. Linear signal amplification was used. A gate was fit by minimizing the difference between the model PHB content and the measured PHB content of each sample and is indicated as the black line. Cells with fluorescence above this line were considered to contain PHB (see text). The percentage of cells that were determined to contain PHB in each sample is listed in the corner of each graph.

microchamber that has one inlet port and two outlet ports. One of the outlet ports is separated by a membrane so that the liquid in the microchamber can be exchanged while the cells are retained in the microchamber. For the testing of the automated system, samples were manually diluted to 5 million cells/ml and then pumped into the microchamber of the staining apparatus. *S. cerevisiae* were stained with Nile red using the automated system. *S. cerevisiae* was stained by inserting a 22-μl Nile red plug at a concentration of 100 μg/ml into the tubing, and this plug was washed into the microchamber using 250 μl of PBS using the outlet port separated from the microchamber by a membrane. The cells and staining solution were incubated for 1 min in the microchamber and then the cells were washed with 1.2 ml of PBS before cytometric analysis. *C. necator* cells were stained with BODIPY 493/503 using the automated system. The cells were first made permeable to the stain by adding 40% ethanol until the final concentration in the microchamber was 35% ethanol. The cells were incubated for between 1 and 15 min in the ethanol solution. Then the cells were stained by adding a 44-μl BODIPY 493/503 plug at a concentration of 50 μg/ml. The BODIPY 493/503 was transported to the microchamber using 40% ethanol, and the cells were incubated in the BODIPY 493/503 for 1 min before being washed with PBS. The entire staining operation, starting from sampling of the cells, was completed in 12 min. The cells were then injected into the flow cytometer for analysis.

RESULTS

PHB Staining in Yeast

S. cerevisiae cells were grown under conditions that yielded average intracellular PHB contents between 0 and 5.2% of the cell dry weight. The Nile red and BODIPY 493/503 stains were optimized with regard to stain concentration, DMSO concentration, cell number, and time. The bivariate cytograms of cultures containing different PHB concentrations after staining with Nile red or BODIPY 493/503 indicate that the cell size contributed to the background fluorescence as the background fluorescence increased with light scattering intensity in the cells without PHB (Fig. 1). This background fluorescence was significantly lower in the samples stained with BODIPY 493/503 than the samples stained with Nile red, indicating that

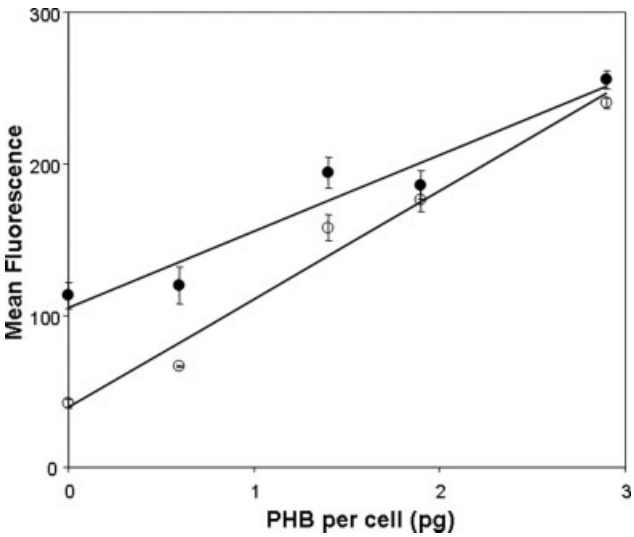

FIG. 2. Calibration curve for the manual staining of *S. cerevisiae*. *S. cerevisiae* cells were stained with Nile red (●) and BODIPY 493/503 (○) as described in Figure 1. The average mass of PHB per cell was calculated by dividing the mass of cellular PHB determined by gas chromatography by the cell number determined by the Elzone particle counter. The regression lines are plotted, and the regression statistics are shown in Table 1. The error bars reflect the standard deviation of three samples.

the BODIPY dye had a better ratio of PHB fluorescence to background fluorescence.

The measured fluorescence intensity was compared with the cellular PHB content obtained from gas chromatography, and a linear regression was performed to quantify the relationship (Fig. 2). The calibration curve with the BODIPY 493/503 stain had a lower intercept, indicating less background staining, and a higher slope, indicating increased specificity to PHB granules (Table 1). Also, the correlation coefficient was higher for BODIPY than for Nile red, which may be due to increased reproducibility seen for BODIPY 493/503 (error bars in Fig. 2 and Table 1). When cells grouped by their FSC values were plotted on calibration curves, the slope and intercept were proportional to the cell size (data not shown). Using these results, a "PHB content map" was constructed that shows the single cell PHB content as a function of both BODIPY fluorescence and FSC (Fig. 3). Therefore, the calibration curve in Figure 2, which averages the effect of the cell size, will only be accurate when cells with the same size distribution are present, such as in asynchronous cultures used here.

Flow cytometry techniques are useful because they permit the measurement of the single cell PHB content and not just the average value. Of particular interest are cells with PHB content much higher than the mean cellular PHB content as they indicate the potential of cells to synthesize and store this polymer. To confirm the validity of the PHB content map and the calibration curve for cells with higher PHB content, two possibilities exist. First, the cells with high fluorescence could be sorted and then analyzed using GC. However, the low sensitivity of the GC would require sorting cells for a significant time to accumulate a sufficient number of cells. An alternative is to analyze only the cells containing PHB to increase the range over which the calibration curve is valid, as is illustrated here. This analysis assumes that continuous density functions of the single cell PHB content (x) and fluorescence (y), $f(x)$ and $g(y)$, can be used to describe the PHB and fluorescence distributions of the culture. Then, the linear proportionality between the means can be expressed as

$$\int_0^\infty x\, f(x)\, dx \propto \int_0^\infty y\, g(y)\, dy \qquad (1)$$

Breaking Eq. (1) into the fraction of cells that contain PHB, and using the definition that the mean PHB of PHB-free cells is zero,

$$\int_{>0}^\infty x\, f(x)\, dx + 0 \propto \int_0^b y\, g(y)\, dy + \int_b^\infty y\, g(y)\, dy \qquad (2)$$

where b is the fluorescence level above which cells contain PHB. Assuming that the cellular forward scatter distribution is the same from sample to sample, the PHB cells have a constant mean background fluorescence, z:

$$\int_{>0}^\infty x\, f(x)\, dx \propto z + \int_b^\infty y\, g(y)\, dy \qquad (3)$$

The term for the background fluorescence, z, is equal to the intercept of the calibration curve (Fig. 2) and is a function of cell size as seen in the PHB map (Fig. 3). A mass balance on the total cellular PHB can be made by equating the average PHB content of the entire culture to PHB in the PHB-containing fraction of cells a, and the PHB-free cells:

Table 1
Staining Characteristics of Nile Red and BODIPY 493/503 for S. cerevisiae *and* C. necator

Organism	Method	Stain	Slope	Intercept	Correlation (R)	Sample-to-sample % variation
S. cerevisiae	Manual	BODIPY 493/503	71 ± 6	39 ± 11	0.988	3.8
	Manual	Nile red	50 ± 8	105 ± 13	0.967	9.0
	Automated	Nile red	26 ± 2	52 ± 4	0.990	6.3
C. necator	Manual	BODIPY 493/503	44 ± 2	9 ± 30	0.987	12.5
	Manual	Nile red	49 ± 2	64 ± 30	0.990	15.7
	Automated	Bodipy 493/503	16.8 ± 0.4	7 ± 7	0.998	8.3

The linear regression statistics are provided for the average single cell fluorescence vs. the average single cell PHB content. The sample-to-sample variation is the coefficient of variation of the samples.

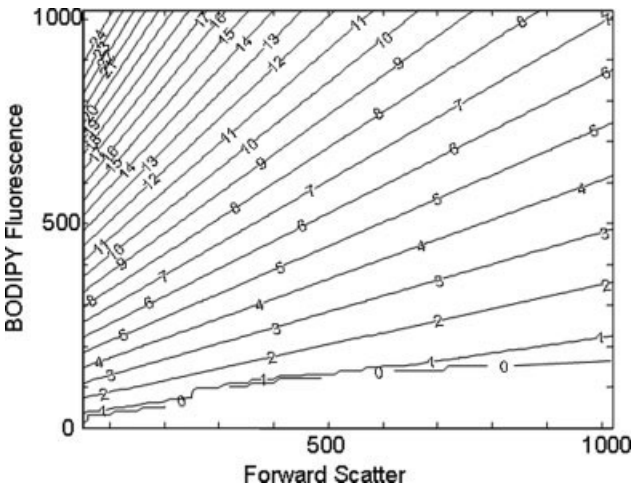

FIG. 3. PHB map for *S. cerevisiae* stained with BODIPY 493/503. This map indicates the single cell PHB contours for cells stained by BODIPY 493/503 as a function of the BODIPY 493/503 fluorescence and forward scatter. The staining was performed as described in Figure 1. The lines are single cell PHB contours in picograms/cell (see text for more explanation).

$$\int_0^\infty x\,f(x)\,dx = (1-a)*0 + a\int_{>0}^\infty x\,f(x)\,dx \qquad (4)$$

Dividing through by a and combining with Eq. (3) yields

$$\int_{>0}^\infty x\,f(x)\,dx = \frac{1}{a}\int_0^\infty x\,f(x)\,dx \propto \int_b^\infty y\,g(y)\,dy \qquad (5)$$

However, to use the relationship in Eq. (5), the PHB-containing cell fraction a, and the background fluorescence level b, must first be determined. These values were determined by fitting a threshold under which all cells had zero PHB over all samples. The threshold level was adjusted to minimize the residual between the calculated PHB and the PHB measured by GC for the individual samples (gate in Fig. 1). A few cells in the control still were found to contain PHB, indicating possible false positives, errors in the threshold setting, and/or cells with abnormally high neutral lipid content.

By comparing the threshold level with the PHB map, the minimum single cell PHB content that measures above zero was estimated. The stain sensitivity was much better for cells stained with BODIPY at 1.0 pg/cell than that for cells stained with Nile red (3.0 pg/cell). Based on Eq. (5), additional points using only the average fluorescence and cellular PHB content of PHB-containing cells were added to the calibration curve and fell exactly upon the previous best fit line (Fig. 4). For example, in Figure 1f, the 45% of cells that contained PHB have an average fluorescence and PHB content of 470 fluorescence units and 5.8 pg/cell. Assuming that the curve in Figure 4 could be further extended to all fluorescent channels measured, cells with fluorescence in channel 1000 have PHB contents of 13.3 pg/cell or about 30% of the cell dry weight of an average cell.

Analysis of the PHB Content of *C. necator*

To compare the BODIPY stain to the Nile red stain on smaller cells with much higher PHB content, a similar staining was performed on *C. necator*. The cellular PHB distributions are shown as bivariate distributions in Figure 5. As a negative control (0% PHB), *C. necator* H16/PHB$^-$4, which is not capable of producing PHB, was grown and stained under similar conditions as the PHB-producing cultures. These cytograms indicate that in *C. necator* the PHB accumulation was more uniform than in *S. cerevisiae* as nearly all the cells contain some PHB. During the PHB production phase (middle column of Fig. 5), there is a noticeable widening of the cellular PHB distribution. This effect was likely due to individual cells entering the PHB synthesis phase at different times. Even at high PHB content, a large distribution in the single cell PHB content was seen as evidenced by the gates in Figure 5f illustrating the 1% of cells with the highest and lowest fluorescence.

At cellular PHB concentrations below 25 pg/cell, a linear relationship between the cellular PHB and the mean fluorescence was observed for both BODIPY 493/503 and Nile red (Fig. 6). In this region, the slope and correlation coefficient were comparable for both stains (Table 1). However, at cellular PHB concentrations above 25 pg/cell the Nile red stain saturated, while the BODIPY 493/503 stain did not. This saturation effect can be observed in Figure 5c by the appearance of a subpopulation of cells, which is not as fluorescent as the rest of the cell population (see arrow).

Automated Optimization of Staining Protocol on *C. necator*

The automated cell preparation instrumentation was programmed to optimize the staining conditions by sys-

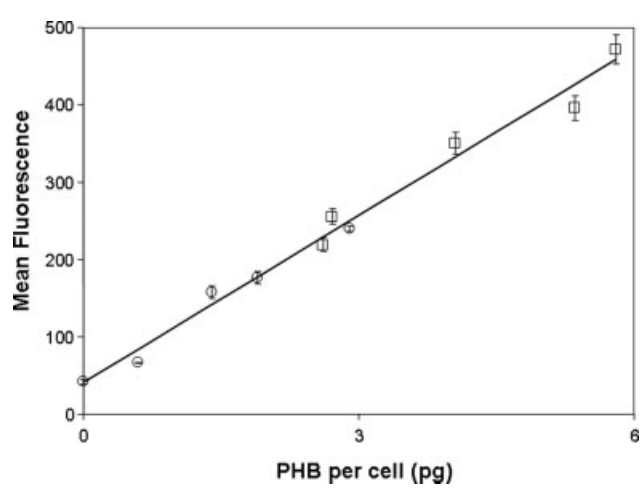

FIG. 4. Extended calibration curve for *S. cerevisiae* stained with BODIPY 493/503. The average PHB content and average fluorescence based on all cells (○), and based on only PHB containing cells (□) are plotted. These data points for PHB-containing cells were determined using the average fluorescence and average PHB per cell for the cells that produced fluorescence above the background fluorescence as indicated by the gate in Figure 1 (see text). The error bars reflect the standard deviation of three samples. The line is an extension of the calibration curve from Figure 2.

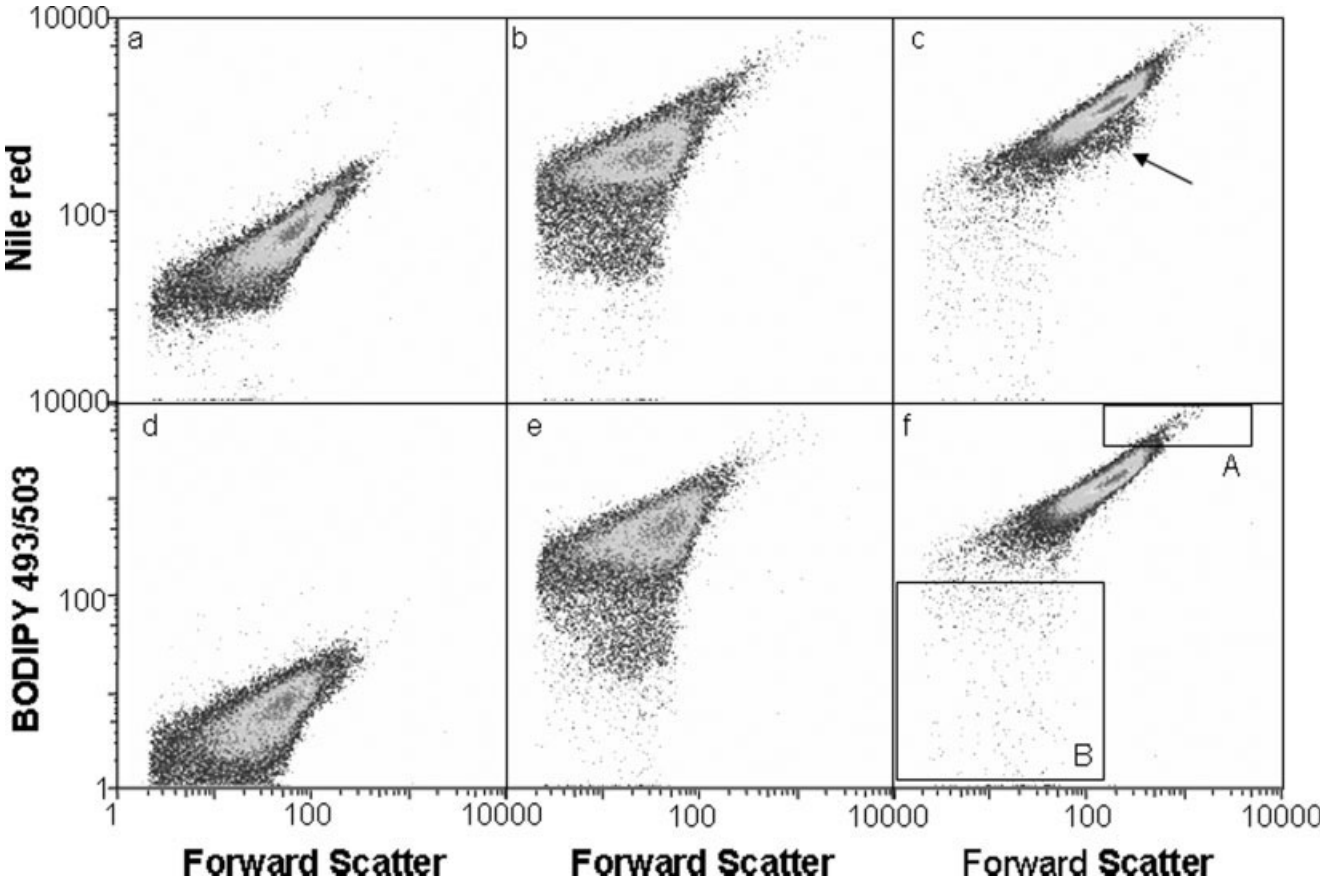

FIG. 5. Bivariate distribution of the forward scatter and PHB fluorescence of *C. necator*. The cells were stained with 10 μl of 100 μg/ml of Nile red (**a–c**) or BODIPY 493/503 (**d–f**) and analyzed using flow cytometry. The bivariate distributions indicate data from cells containing 0% (a, d), 38% (b, e), and 76% (c, f) PHB of the total cell dry weight as determined by gas chromatography. The fluorescence data was collected on a flow cytometer, using a 585 ± 42 nm band pass filter for Nile red fluorescence and a 530 ± 30 nm band pass filter for BODIPY 493/503 fluorescence. Logarithmic signal amplification was used. The boxes labeled A and B indicate the 1% of cells with the highest and lowest BODIPY 493/503 fluorescence, respectively. The arrow indicates a subpopulation of cells, which is not as fluorescent as the rest of the cell population.

tematically changing the staining variables without any external input. To illustrate this process, the optimal ethanol concentration and ethanol treatment time of cells in the microchamber were determined for *C. necator* cells stained using BODIPY 493/503 (Fig. 7). First, the automated system was programmed to vary the ethanol concentration in the microchamber between 0 and 75% and measure the cellular fluorescence distributions after staining (Fig. 7a). The goal was to determine the ethanol concentration that would cause all the cells in the microchamber to be permeable to the stain. When the cells were not treated with ethanol prior to staining, the fluorescence was lower and the distribution coefficient of variation (CV) was high, indicating that not all cells were stained. Under these conditions, the bivariate distributions indicated two subpopulations (data not shown). One subpopulation was at high fluorescence and contained stained cells, while the other subpopulation was at the background fluorescence level and contained cells that were not stained. Once the ethanol concentration reached 25%, the fluorescence and CV remained constant because all the cells were permeable to the stain. Under these conditions, the bivariate distributions showed only the high fluorescence population (data not shown). Therefore, an ethanol concentration of 35% was determined to be sufficient to make the cells permeable to the stain. Additionally, by using a lower ethanol concentration than the typical 70%, problems with cells sticking together were minimized.

The ethanol concentration was determined using a 10-min ethanol exposure time. Because rapid sampling is useful for bioreactor monitoring, a shorter time would be ideal. The optimal ethanol exposure time was determined by programming the system to vary the time from 1 to 15 min in increments of 1 or 5 min and to measure the cellular fluorescence distributions after staining (Fig. 7b). The optimization goal was to find the minimum time that allows all the cells in the sample to be made permeable to the stain. For times less than 5 min, the cellular mean fluorescence increased while the CV decreased with increasing exposure time. After 5 min of staining, the mean fluorescence and CV remained constant as the exposure time increased. Therefore, at exposure times less than 5 min not all the cells in the microchamber were

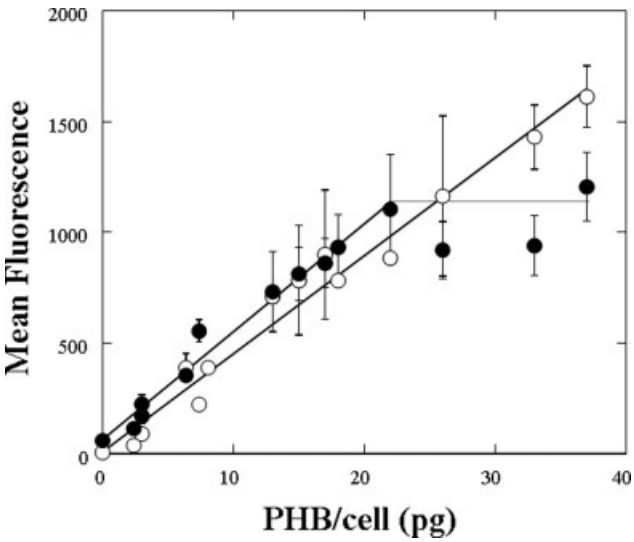

FIG. 6. Calibration curves for the manual staining of *C. necator*. *C. necator* cells were stained with Nile red (○) and BODIPY 493/503 (●) as described in Figure 5. The average mass of PHB per cell was calculated by dividing the mass of PHB in a sample of cells, determined by gas chromatography, by the number of cells in the sample. The solid lines indicate the regression lines. The regression statistics are shown in Table 1. The dotted line indicates the saturation point of the staining procedure using Nile red. The error bars reflect the standard deviation of three samples.

completely permeable. The complete set of data shown in Fig. 7b has been automatically acquired in ~2.5 h. To maximize the sampling frequency, 5 min was selected as the best exposure time. The optimal staining conditions have been determined in a similar fashion for the dye incubation time (0–10 min) and added dye volume (0–88 μl) (data not shown).

Automated Staining of *S. cerevisiae*

Five samples of *S. cerevisiae* with varying PHB content were stained in triplicate with Nile red using the automated flow cytometry system. A calibration curve as described above was constructed and compared with the previous curve constructed using manual staining (Fig. 8a). The automated technique produced a significantly better correlation coefficient and had higher reproducibility than did the manual technique, as seen by the smaller error bars for each sample (Table 1).

Automated Staining of *C. necator*

The automated staining system was calibrated for *C. necator* by automatically staining and analyzing cells with varying PHB content with BODIPY 493/503 in triplicate. The linear correlation from the automated system (Fig. 8b) was better than the correlation from the manual technique (Table 1). To determine the reproducibility of the automated staining, a concentrated cell sample was suspended in cold phosphate buffer saline (PBS) and placed on ice to arrest PHB synthesis. This sample was then diluted online in the microchamber, stained, and analyzed 15 times over 7 h. The CV of the samples was 8.3, which was significantly better than that of the manual staining (12.5%). The measured mean fluorescence was lower in the automated samples than that in the manual samples because the dye concentration in the microchamber was likely lower than that in the manual cell preparation because of dispersion in the lines.

DISCUSSION

A protocol using BODIPY 493/503 stain was developed that improved the staining characteristics for cellular PHB. This technique increased the sensitivity and decreased the background staining of non-PHB lipophilic components in the cells, which have been observed previously to impair the staining of *C. necator* (9,11). The BODIPY 493/503 staining technique resulted in better reproducibility in both cell types. Additionally, in *S. cerevisiae* it provided higher sensitivity than did Nile red. *C. necator* cells

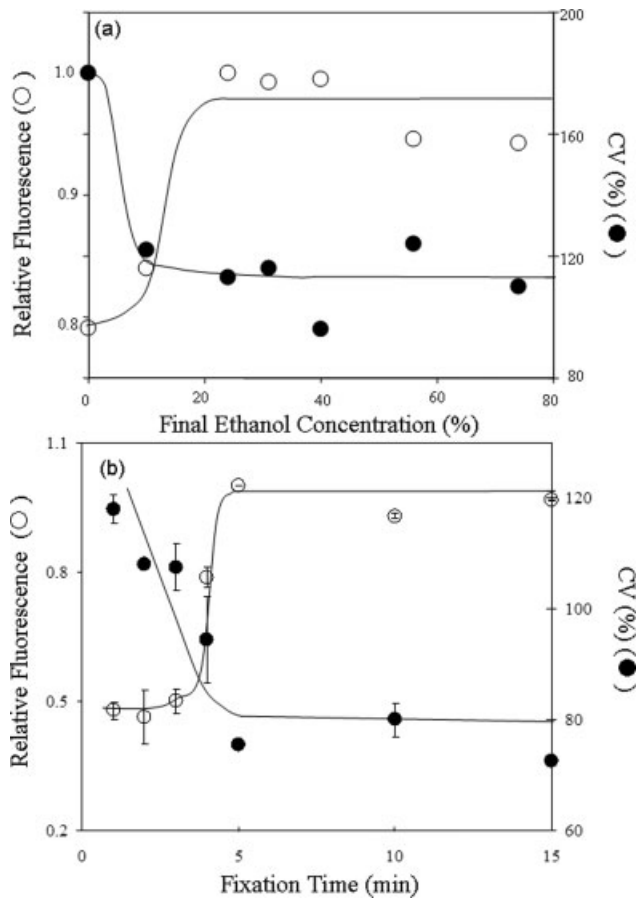

FIG. 7. Determination of the optimal ethanol concentration and staining time. (**a**) The automated system was programmed to vary the ethanol concentration in the microchamber before staining of *C. necator* with BODIPY 493/503. The ethanol concentration was varied between 0 and 75% and the normalized mean fluorescence (○) and coefficient of variation (CV) (●) of the cell population were measured. The fluorescence was normalized to the maximum fluorescence measured. (**b**) The automated staining was programmed to systematically vary the ethanol exposure time before staining *C. necator* with BODIPY 493/503. The time was changed between 1 and 15 min, and the normalized mean fluorescence (○) and CV (●) of the cell population were measured. The error bars reflect the standard deviation of three samples.

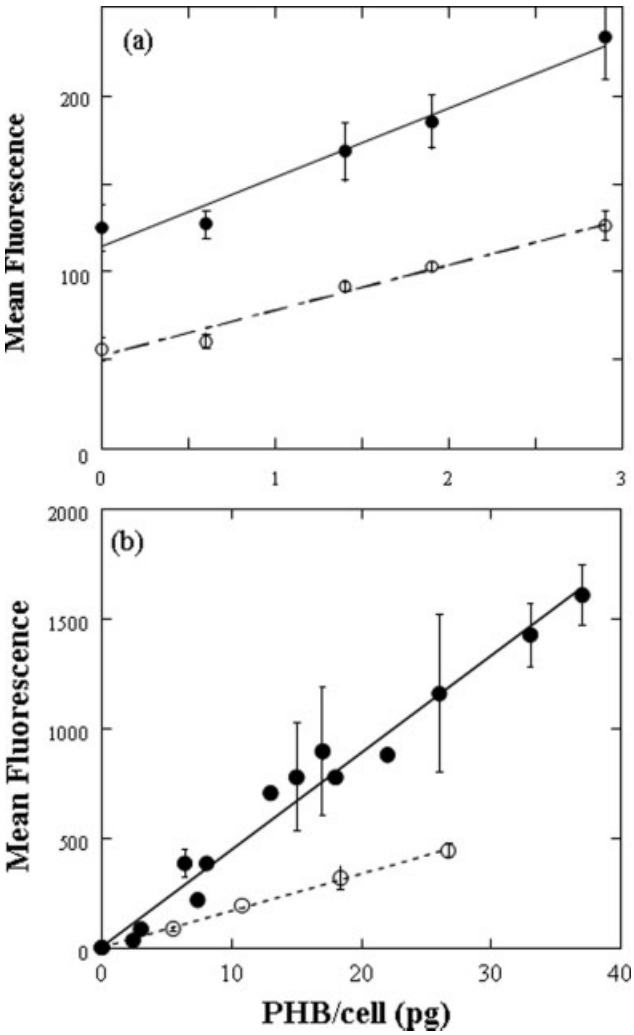

FIG. 8. Comparison of the calibration curves from manual and automated sample preparation. The automated sample preparation system was used to stain (**a**) *S. cerevisiae* cells with Nile red and (**b**) *C. necator* cells with BODIPY 493/503. Before staining, *C. necator* cells were made permeable to the stain by exposure to 35% ethanol for 5 min in the microchamber using the automated system. The average single cell fluorescence was plotted as a function of the single cell PHB content (○). All samples were stained and analyzed in triplicate using the procedure detailed in the Materials and Methods section. For comparison, the calibration curve of (a) *S. cerevisiae* stained manually with Nile red from Figure 2 and (b) *C. necator* stained manually with BODIPY 493/503 from Figure 6 are also shown (●). The regression statistics for both manual and automated sample preparation are shown in Table 1.

stained with BODIPY 493/503 saturated at a higher PHB quantity than cells stained with Nile red. Therefore, the staining characteristics of BODIPY 493/503 are better than those of Nile red for PHB analysis. Additionally, because the emission spectrum of BODIPY 493/503 is at a lower wavelength than that of Nile red, the use of this dye allows additional flexibility for multiparameter analysis using multiple stains.

Of particular interest is single cell PHB data obtained using the flow cytometer, as displayed in the bivariate distributions in Figure 1. First, in the *S. cerevisiae* cultures that contained 0% PHB, there were still a few cells that had high fluorescence. These few cells may have very high neutral lipid contents, which would stain with the BODIPY 495/503 or Nile red. Because only a very small percentage of cells (<1%) were "false positives," these few high fluorescent cell would have little effect on the average fluorescence measured for the calibration curves in Figures 2 and 4. However, this variation in the background must be accounted for if the growth conditions are changed such that it results in a change in the lipids produced in the culture.

In the recombinant yeast cultures with the highest PHB content between 50 and 60% of cells did not contain any PHB. Because the two plasmid system has both high copy number and also unstable, a large variation in the single cell enzyme expression is expected because of plasmid recombination and loss. Therefore, the single cell PHB production is expected to vary from very high, in cells that maintain a high copy number of both of the plasmids, to medium, in cells with low copy numbers of both plasmids, to zero, in cells that have recombined plasmids or have lost the plasmid containing the PHB synthase gene.

In Figure 1, the yeast cells with the highest PHB content tended to be smaller cells. Smaller cells have a higher surface area for nutrient transport into the cell per unit cell volume than that of larger cells. Therefore small cells may have maintained higher intracellular nutrient concentrations, which resulted in a higher PHB production rate. If the fraction of smaller cells could be maximized, for example by decreasing the size of daughter cells, the PHB production of the culture could be optimized.

Because the *S. cerevisiae* cells remain viable after staining with BODIPY 493/503, cells with high PHB content can be isolated and regrown for further analysis. In this way, mutant cell lines that produce high quantities of PHB could be isolated. The distribution seen in Figure 1 indicated that some cells may contain PHB in excess of 30% of the cell dry weight. If these cells are isolated and a stable cell line is developed, bioreactor productivity could be greatly improved. Changes to the staining protocol for *C. necator* in which the ethanol step is removed could result in viable staining of the bacteria, but the time for this method would be much longer (11), making it less effective for on-line culture monitoring. The addition of an ethanol step in the staining protocol for *S. cerevisiae* was not necessary because the cells were already permeable to the stain. This was also shown by the fact that the staining time for yeast was not reduced after the addition of an ethanol fixation step.

An on-line staining method was developed using the automated flow cytometer setup, allowing the automated quantification of PHB content approximately every 25 min. The consistency of the on-line data with the off-line data illustrates that the on-line system is capable of accurately and reproducibly staining cells containing PHB. If higher sampling frequency was desired, additional microchambers for cell staining could be added, permitting more frequent sampling. Although this frequent sampling could be useful in specific cases, the higher sampling capacity could also be used to monitor multiple bioreactors by

appropriately multiplexing the inputs into the cell staining device. Because the total time necessary to process each sample using the automated system is 25 min, four bioreactors could be monitored with a sampling frequency of each bioreactor of about 2 h, which is still less than the doubling time for either *S. cerevisiae* or *C. necator*. Although this sampling frequency may not be high enough to allow for efficient control of the bioreactors, it would still provide a detailed description of what is happening in each reactor. Because of the speed of analysis, precision of measurement, and ability to measure the single cell distribution, this method could have important implications for the monitoring and control of cultures producing PHB.

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