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tion of horseradish peroxidase labeled probes and detection by enhanced chemiluminescence. *Mol. Biotechnol.* 6:69-73.

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Rapid Detection of *Zymomonas mobilis* Redox Activity Using 5-cyano-2,3-tolyl-tetrazolium chloride (CTC)

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As biomass pretreatment strategies are being developed for use in biomass-to-ethanol processes, it is important to evaluate the viability of ethanologenic microorganisms during fermentation of those various pretreatment hydrolyzate liquors and lignocellulosic solids. Traditional plate count or slide count is time consuming and does not provide vital information in real time (4,13). Nonfluorescent staining techniques using methylene blue or trypan blue are also used to measure cell viability (1,13). However, the presence of a biomass matrix is problematic, and staining results may not correlate well with cell viability or ethanol productivity. There is increasing interest in using fluorescent dyes for cell viability assays (6), but the presence of hydrolyzate liquor and complex solids in the biomass matrix is expected to give high background, making the fluorescent staining extremely difficult. Therefore, selecting suitable fluorescent probes and eliminating artificial background signals become important tasks. A fluorescence staining protocol has been successfully developed for hydrolyzate toxicity assays for yeast (2), although no fluorescence-based study has been reported yet for *Zymomonas mobilis*. The aim of this study is to test the feasibility of fluorescent redox dyes for *Zymomonas* cells and to develop a fluorescence microscopy protocol to monitor cellular activity during the fermentation process.

The redox activity of *Z. mobilis* was first demonstrated using nitro blue tetrazolium (NBT) salt (Sigma, St. Louis, MO, USA) on individual colonies of *Z. mobilis* 39676(pZB4L). NBT (0.2 mg/mL) was distributed evenly to *Zymomonas* colonies. Those in contact with NBT turned black after incubating at 30°C for 30 min. The demonstration of NBT reduction indicates that *Zy-*

monas cells have strong redox activity. This finding led to the development of *Zymomonas* viability assays using fluorescent staining. The redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), has been used to detect cell viability (7,14). Cells able to reduce CTC possess a high level of metabolic activity, while cells showing no apparent reduction of CTC have either low or no metabolic activity (10). However, the relationship between CTC-staining results and the actual number of (respiring) active bacteria is still not well established (12). Using flow cytometry or image analysis, the concurrent examination of both numbers and fluorescence intensity of CTC-labeled cells can be automated (3,8,11).

CTC stock solution (50 mM) was freshly prepared by dissolving CTC (Polysciences, Warrington, PA, USA) in sterile deionized water and filtering through a filter membrane (pore size 0.2 µm) to reduce nonspecific fluorescent background. CTC formazan (CTF), a reduced CTC product, is fluorescent in the solid state, with an excitation maximum at 450 nm and an emission maximum at 630 nm. In this study, a triple-band filter set (Chroma Technology, Brattleboro, VT, USA), with excitation filters covering 420 nm and an emission filter covering ranges greater than or equal to 600 nm, was used to view red CTF and the green DNA dye SYTO9 simultaneously. The epifluorescence microscope used in this study was a Nikon® LabPhoto2 equipped with a Kodak® DC120 digital camera.

In general, CTC can be used from 4–108 mM. A working solution of 1 mM CTC to 2×10^6 cells has been re-

Figure 1. CTC staining of pure *Z. mobilis*. Photo was taken with white light to visualize whole cells. All cells showed a red stain after incubating with 25 mM CTC for 1 h. (Bar = 5 µm)

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ported (9). The staining time ranges from 30 min to 5 h depending on the nature of the sample. Sugar or a culture medium is added to the CTC-cell mixture as an electron donor to maximize the number of bacteria that could actively reduce CTC. However, at the recommended concentration (5 mM) and CTC/cell ratio (1 mM: 2×10^6) with 1% glucose, only a small portion of (exponential phase) *Zymomonas* cells stained red after 1 h incubation at 28°C with agitation. Similar results were also obtained with *E. coli* or yeast cells. The number of colored cells increased as the CTC concentration increased. This implied that both active and less active cells are competing for the limited amount of CTC, and only the former will produce enough CTF to be de-

tectable. Sieracki et al. (11) suggested that some of the unstained cells actually transport CTC across their cell membranes yet do not reduce it to a detectable level.

After increasing the CTC concentration to 25 mM and changing the corresponding CTC/cell ratio to 1 mM: 2×10^5 cells, all cells stained red after 1 h incubation (Figure 1). This result indicated that the entire cell population was metabolically active and viable, and an accurate quantitation requires an excess amount of CTC. Similar results were obtained when xylose alone or xylose plus glucose was supplemented. Our CTC staining showed multiple red spots inside the cells, suggesting that *Z. mobilis* may have multiple redox active sites.

Rodriguez et al. (7) employed a fluorescent DNA dye, 4',6-diamidino-2-phenylindole, to counterstain CTC-labeled *Pseudomonas putida* cells. This technique allowed simultaneous enumeration of bacterial subpopulations within the same preparation. For this work, we used a green fluorescent DNA dye, SYTO9 or SYTO13 (Molecular Probes, Eugene, OR, USA), to stain total cells because these two dyes are more suitable for viability assessment (5). Because of its stability, SYTO9 was chosen over SYTO13 for the following experiments. We counterstained the cell-CTC preparation with SYTO9 and incubated for an additional 15 min before microscopic examination. Redox-active cells stained red on green-stained cells, while redox-inactive cells stained

green only. We noticed red spots outside the cells, probably caused either by impurity or autoreduction of the dye.

The interactions of pretreatment hydrolyzate liquor with CTC and SYTO9 was determined by incubating a hydrolyzate liquor sample (pH 5.8) with CTC (10 mM) or SYTO9 (3 μ L/mL) at 30°C for 30 min. We observed an intense fluorescence background, indicating a strong auto-CTC reduction and a significant SYTO9 adsorption to the hydrolyzate liquor. A high level of auto-CTC reduction also decreased CTC concentration available for subsequent cell staining. Aged hydrolyzate liquor forms precipitates that might absorb green DNA dye and so generate high background. To reduce the background fluorescence, we separated the hydrolyzate-treated cells from the hydrolyzate using a filter membrane and then stained the cells in situ. *Zymomonas* cells (10^6 cells/mL) were incubated with filtered pretreatment hydrolyzate liquor at 30°C on a rotary shaker (100 rpm) for 4 h. A 0.1-mL aliquot of cells was collected on a black polycarbonate filter membrane with a pore size of 0.2 μ m (Millipore, Bedford, MA, USA), using a vacuum filter assembly and washed with 2 mL deionized water. The membrane loaded with hydrolyzate liquor-treated cells was placed between two O-rings to prevent the dye solution from flowing away. A 25- μ L aliquot of CTC stock solution and an equal volume of 2% glucose were added onto the cell-loading site. After incubation at 30°C on a rotary shaker or in an incubator without any motion for 30–45 min, 3 μ L SYTO9 (1:10 dilution) was added to the CTC-glucose mixture. The membrane was incubated for an additional 15 min. After incubation, the membrane was dried with vacuum suction, washed with 1 mL deionized water, mounted on a microscope slide and examined. Figure 2 shows clear staining of redox-active and inactive cells with little fluorescence background. The interference of hydrolyzate liquor on fluorescent staining was completely eliminated. Membranes incubated without any motion produced similar results. This CTC staining approach provides a sensitive tool for monitoring the toxicity of pretreatment hydrolyzate liquor.

Figure 2. CTC staining of *Zymomonas* cells treated with hydrolyzate liquor. In situ CTC staining on filter membrane showed distinguishable subpopulations—redox active (with red spots) and redox inactive (green only). The effect of pretreatment hydrolyzate liquor on *Zymomonas* cells was indicated by the loss of redox activity. (Bar = 5 μ m)

Figure 3. CTC staining of *Zymomonas* cells treated with lignocellulosic solids. The background interference of lignocellulosic solids on fluorescent staining can be minimized by separating cells and lignocellulosic solids with the filtering steps described in the text. Filter paper alone could remove some of the background (a). When an Acrodisc membrane (5 μ m pore size) was used, the background fluorescence was completely removed (b). (Bar = 5 μ m)

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To examine the effect of the lignocellulosic solids on fluorescent staining, a solid sample was suspended in sterile deionized water at 40% (w/v) concentration. CTC was added to 1 mL of the slurry to a final concentration of 5 mM and incubated at 30°C for 30 min; 3 μ L SYTO9 were then added and incubated for 15 min. The solid matrix produced an intense background fluorescence. Visible light quenchers such as methylene blue and trypan blue failed to reduce background fluorescence. BSA was used as a nonspecific binding agent to block the solid matrix from binding to SYTO9. The solid slurry was incubated with 1% BSA at 30°C for 1 h before the addition of CTC and SYTO9. This treatment also did not reduce background fluorescence. Alternatively, the solid slurry was filtered through both filter paper (Whatman® no. 54) and an Acrodisc® (pore size 5 μ m) before incubating with CTC/SYTO9. The background fluorescence of the filtrate was significantly reduced.

To eliminate the interference of the solid matrix on fluorescent staining, the matrix underwent a separation step, and cells in the filtrate were then captured on the filter membrane and stained *in situ* as previously described. Washed *Zymomonas* cells (approximately 10⁶ cfu/mL) were mixed with the 40% solid suspension at 30°C on a rotary shaker for 2 h. One microliter of the mixture was filtered through either a filter paper or one followed by an Acrodisc. A 0.1-mL aliquot filtrate was then transferred to a black polycarbonate membrane and washed with deionized water. To stain the cells captured on the membrane, 25 μ L each CTC stock solution and 2% glucose were added to the cell site and incubated at 30°C for 45 min. A 3- μ L aliquot SYTO9 (1:10 dilution) was then added and incubated for 15 min. Under an epifluorescence microscope, cells obtained from the filter paper were still surrounded by an intense fluorescence background (Figure 3a), yet cells obtained from the filter paper and Acrodisc were clearly distinguishable from the remaining fluorescence background (Figure 3b). To determine the cell number recovered from filtrate, freshly washed *Zymomonas* cells were mixed with 40% solid at 30°C on a rotary shaker for 2 h. Slurry (1 mL) was

filtered through a filter paper. The standard viable count procedure was carried out for the filtrate after serial dilutions in PBS, pH 7.0. A recovery of 46% was obtained from an inoculum of 6.6×10^7 cfu/mL and 41% for 6.6×10^5 cfu/mL. Quantitative analysis of CTC-labeled cells offers a potentially useful tool for the rapid detection of *Zymomonas* viability during the fermentation process. The redox assay detected the redox activity of only those cells recovered in the filtrate. We assumed that they represented the whole cell population in the solid matrix (i.e., free cells and cells bound to solids).

By coupling the sensitivity of both CTC and green fluorescent DNA dyes, we successfully developed a simple and rapid fluorescence microscopy technique that eliminates the background fluorescence of the biomass matrix and determines the subpopulations of *Z. mobilis* treated with hydrolyzate liquor and lignocellulosic solids within 1 h. We plan to further evaluate its suitability for predicting both the viability and ethanol productivity of *Z. mobilis* during the fermentation process.

REFERENCES

1. Altman, S.A., L. Randers and G. Rao. 1993. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. *Biotechnol. Prog.* 9:671-674.
2. Combs, N. and C. Hatzis. 1996. Development of an epi-fluorescence assay for monitoring yeast viability and pretreatment hydrolyzate toxicity in the presence of lignocellulosic solids. *Appl. Biochem. Biotechnol.* 57/58:649-657.
3. Cook, K.L. and J.L. Garland. 1997. The relationship between electron transport activity as measured by CTC reduction and CO₂ production in mixed microbial communities. *Microbial Ecology* 34:237-247.
4. Jones, R.P. 1987. Measures of yeast death and deactivation and their meaning: part I. *Process Biochem.* 22:117-127.
5. Lebaron, P., N. Parthuisot and P. Catala. 1998. Comparison of blue nucleic acid dyes for flow cytometric enumeration of bacteria in aquatic systems. *Appl. Environ. Microbiol.* 64:1725-1730.
6. Lloyd, D. and A.J. Hayes. 1995. Vigour, vitality and viability of microorganisms. *FEMS Microbiol. Lett.* 133:1-7.
7. Rodriguez, G.G., D. Phipps, K. Ishiguro and H.F. Ridgway. 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Appl. Environ. Microbiol.* 58:1801-1808.
8. Schaule, G., H.C. Flemming and H.F. Ridgway. 1993. Use of 5-cyano-2,3-ditolyl tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water. *Appl. Environ. Microbiol.* 59:3850-3857.
9. Severin, E., J. Stellmach and H.M. Nachtigal. 1985. Fluorimetric assay of redox activity in cells. *Anal. Chim. Acta.* 170:341-346.
10. Sherr, B.F., P. del Giorgio and E.B. Sherr. 1999. Estimating abundance and single-cell characteristics of respiring bacteria via the redox dye CTC. *Aquat. Micro. Ecol.* 18:117-131.
11. Sieracki, M.E., T.L. Cucci and J. Nicinski. 1999. Flow cytometric analysis of 5-cyano-2,3-ditolyl tetrazolium chloride activity of marine bacterioplankton in dilution cultures. *Appl. Environ. Microbiol.* 65:2409-2417.
12. Ullrich, S., B. Karrasch and H.-G. Hoppe. 1999. Is the CTC dye technique an adequate approach for estimating active bacterial cells? *Aquat. Micro. Ecol.* 17:207-209.
13. Vaija, J., C. Ghommidh and J.M. Navarro. 1993. *Zymomonas mobilis* cell viability: measurement method comparison. *Antonie Leeuwenhoek* 64:57-66.
14. Yu, F.P. and G.A. McFeters. 1994. Rapid *in situ* assessment of physiological activities in bacterial biofilms using fluorescent probes. *J. Microbiol. Methods* 20:1-10.

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