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Estimating the viability of *Chlorella* exposed to oxidative stresses based around photocatalysis

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ABSTRACT

We examined *Chlorella* cells exposed to several kinds of oxidative stress, namely photocatalysis, UV light, heat, and H_2O_2. After these oxidative treatments, the *Chlorella* cells were stained with neutral red, methylene blue, eosin Y, or fluorescein diacetate (FDA). The results of FDA staining were generally the most consistent with those of colony-forming unit assays. In addition, chlorophyll autofluorescence was responsive to photocatalytic oxidation and may be useful as a simple, rapidly obtained index of the viability of *Chlorella* cells exposed to photocatalysis. We also discussed differences in the stainability of cells in terms of external and internal cellular damage. Photocatalytic oxidation resulted in the greatest degree of cellular damage, including lipid peroxidation and cell deformation.

1. Introduction

Microalgae are photosynthetic microorganisms that convert sunlight, water, and CO_2 to algal biomass (Hurst, 1997). They are expected to be useful renewable energy sources for the production of biodiesel that can potentially displace liquid fuels derived from petroleum (Williams, 2007; Mata et al., 2010; Fairley, 2011). Also, they are regarded as the major primary producers in many ecosystems and thus play an important role in forming environmental biofilms with heterotrophic fungi and bacteria (Hurst, 1997). Environmental biofilms are useful for the bioremediation of air and water, but are sometimes involved in the corrosion or fouling of man-made structures (Gaylarde and Morton, 1999). In either case, estimation of algal viability is essential for controlling biofilm growth. However, complex aseptic operations and long-term culture are generally required to determine algal viability (Hurst, 1997; Linkous et al., 2000; Kim and Lee, 2005; Hund-Rinke and Simon, 2006).

Cell staining is used as a rapid technique for improved visualization of cells and cell components under a microscope. Some dyes can be used to rapidly differentiate between live and dead cells and thus determine the viability of cells (Conn, 1953; Boulos et al., 1999; Davey, 2011). Evans blue (Crippen and Perrier, 1974) and trypan blue (Zuppini et al., 2009) have been used for algae in general. These dyes discriminate the permselective properties of cells and thus selectively color dead cells blue. Recently, a method using fluorescein diacetate (FDA) has been improved for algae by Franklin et al. (2001). FDA is a cell-permeant substrate that can serve as a viability probe to measure intracellular enzymatic activity (Widholm, 1972). Because the object stained differs with the type of dye used, we need to select an appropriate method to evaluate the viability of cells in light of the mechanism by which the cells are damaged. Also, if these assays are to be used with different microorganisms that have basic physiological differences, then they should be revalidated under the new conditions (Brehm-Stecher and Johnson, 2004).

Microalgae sustain their photosynthetic activity under environmental stress. However, high-temperature and UV light, for example, increase the intracellular formation of reactive oxygen species (ROS) and inhibit algal proliferation (Mishra and Singhal, 1992; Lesser, 1996). Harmful artificial biocides, such as heavy metals or tributyltin, have been commonly used as anti-algal agents to prevent environmental biofilm formation, despite their potent toxicity to many other organisms in the ecosystem. In contrast, we

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have been studying the use of TiO_2 photocatalysis as an alternative method with low environmental load (Ohko et al., 2009). The TiO_2 photocatalyst generates ROS under UV light and has a strong antibiotic effect (Matsunaga et al., 1985; Cai et al., 1992; Lu et al., 2003; Sunada et al., 2003). In many studies of algal biofuels and anti-algal effects, and from an industrial perspective, a simple method for rapid analysis of microalgal viability would be useful. However, there has been very little research into finding a compatible, simple, live–dead assay method for use in microalgae exposed to a variety of oxidative stresses.

Here, we used *Chlorella* as a representative environmental alga (Schowanek et al., 1996) and subjected it to oxidative stresses, namely photocatalysis, UV light, heat, or H_2O_2. After the oxidative treatment, the *Chlorella* cells were stained with neutral red (NR) (Crippen and Perrier, 1974), methylene blue (MB) (Huang et al., 1986), eosinY (EY) (Black and Berenbaum, 1964), or FDA. Changes in the autofluorescence intensity (Nancharaiah et al., 2007) of *Chlorella* were also examined by fluorescence microscopy. The data analyzed were compared with those from conventional colony-forming unit (CFU) assays. Morphological changes in the *Chlorella* cells were observed by using scanning electron microscopy (SEM). The amount of lipid peroxidation was measured by the thiobarbituric acid (TBA) method as an index of the oxidative damage to cells (Hodges et al., 1999). On the basis of these results, we discuss the mechanisms by which *Chlorella* are killed under each type of oxidative stress.

We used preliminary range-finding experiments to achieve treatment conditions that would yield roughly a 50:50 ratio of live to dead cells of *Chlorella*. This was important to demonstrate the degree of staining of the damaged cells. We also used the preliminary experiments to achieve staining conditions that were as simple and quick as possible. The resulting data were used in this comparative study. We believe that this kind of orthodox study will help to raise the status of fundamental science and could lead to the development of a next-generation compact and convenient system of algal damage assay that will be readily available to everyone.

2. Materials and methods

2.1. Algal strain and culture condition

The strain of *Chlorella sorokiniana* NIES-2169 was obtained from the National Institute for Environmental Studies, Tsukuba, Japan. *C. sorokiniana* was inoculated into MM4N medium (Imase et al., 2008) (pH 6) and incubated with shaking at 28 °C under 24-h continuous illumination with white fluorescent light (20 μmol m^{-2} s^{-1}). To remove culture components from the cell suspension, the cell suspension was centrifuged (3000 g) for 5 min and the supernatant liquid was replaced with distilled water (DW). The *Chlorella* cells were resuspended in DW with a vortex mixer. These operations were done a total of 3 times.

2.2. Photocatalytic treatment and UV illumination

TiO_2-coated glass plates (5 cm × 5 cm × 2 mm, 50SQB, Photocatalytic Materials Inc., Aichi, Japan) were used for photocatalytic treatment. A 0.1-mL portion of the cell suspension (7.0×10^7 cells mL^{-1}) was pipetted and dripped onto each test piece. A contact film made of polypropylene (4 cm × 4 cm × 60 μm, Kokuyo, Japan) was used to cover the dripped cell suspension and was held down lightly so that the suspension became distributed over the entire film. The test piece was placed in an airtight illumination chamber to prevent drying, in accordance with the method of Japanese Industrial Standards JIS R1702 (Japanese Industrial Standards Committee, 2006). It was then illuminated with a 10-W black-light bulb (FL10 BL-B, Toshiba, Japan) at a light intensity of 1.5 mW cm^{-2}. The light emitted was in the UV-A region with a peak wavelength of 351 nm; the light intensity was measured with a UV radiometer (UVR-36, Topcon). After the sample had been illuminated for a set period of time, the contact film was peeled off with tweezers. The cell suspension was collected into a phosphate buffer solution (per liter of distilled water: 3.04 g $NaH_2PO_4 \cdot 2H_2O$ and 10.92 g $Na_2HPO_4 \cdot 12H_2O$; pH 7.0, 5 mL). The test piece was brushed gently with a polypropylene toothbrush into the phosphate buffer solution. Treatment with UV alone was performed on Pyrex glass plates (5 cm × 5 cm × 1 mm) in the same manner as above but without the TiO_2.

2.3. Heat treatment

We investigated the influence of heat treatment (50 °C for 25 min and 70 °C for 10 min) by using a 1-mL cell suspension (1.4×10^6 cells mL^{-1}) placed in a test tube. The test tubes were soaked in a water bath at 50 or 70 °C.

2.4. H_2O_2 treatment

One microliter of H_2O_2 (5 vol%) was added to 1 mL of a cell suspension (1.4×10^6 cells mL^{-1}), and the mixture was stirred in the dark. After 2 h, catalase was added to a final concentration of 100 U mL^{-1} in order to stop the reaction of H_2O_2 (~1 μmol mL^{-1}) with cells.

2.5. Dye-staining and autofluorescence

Control and oxidatively treated cells were stained with NR, MB, or EY. The concentration of each stain in DW was 10 mg mL^{-1}. The samples were incubated for 20 min in the dark at room temperature. FDA was dissolved in dimethyl sulfoxide as a stock solution of 5 mg μg mL^{-1} and used at a final concentration of 5 μg mL^{-1} in DW. Samples treated with FDA were incubated for 5 min in the dark for staining. Before being imaged, the stained cells were collected on a polycarbonate membrane filter (Isopore, Millipore, Billerica, MA).

Cells stained with NR, MB, or EY were observed under an optical microscope (BX51, Olympus, Japan) equipped with a CCD camera (AxioCam HRc, Carl Zeiss MicroImaging, Tokyo, Japan). Cells stained with FDA were examined in epifluorescence mode. A bandpass filter (510–550 nm, U-MNIBA3, Olympus) was used to observe the cells stained with FDA. This filter allows only the green emission of FDA to pass through; the red autofluorescence of chlorophyll is blocked, so that only the green emission of FDA becomes clearly visible under blue excitation (470–495 nm). The chlorophyll autofluorescence of unstained cells was observed with a fluorescence mirror unit (U-MWIB3, Olympus; excitation filter, 460–495 nm; emission filter, 510 nm long-pass; dichromatic filter, 505 nm) on the microscope. Cell survival was calculated relative to that of control cells. At least 1000 cells were manually counted from random fields in each experiment. We used a cell solution in which over 99% of the cells were viable. Survival rates were calculated by comparing the numbers of living cells observed before and after the treatments.

NR and FDA stain living cells, whereas MB and EY stain dead cells. The principle of each staining method is shown briefly in Table 1. Cells that stained red or pink with EY and those that stained blue or pale blue with MB were considered to be dead. In the autofluorescence testing, cells emitting red fluorescence were considered to be live; in the FDA fluorescence testing, cells emitting green fluorescence were considered to be live. Because the fluorescence intensity of damaged cells was dramatically reduced