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Journal of Microbiological Methods 68 (2007) 639–642
Journal of Microbiological Methods
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Note

Fluorescent measurement of microalgal neutral lipids

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Received 30 August 2006; received in revised form 3 November 2006; accepted 3 November 2006
Available online 26 December 2006

Abstract

Nile Red, a dye that fluoresces at defined wavelengths depending upon the polarity of the surrounding medium, has been proposed to determine the neutral lipid content of microalgal cells. Herein we communicate modifications to this technique that facilitate its use as a high-throughput screening technology, as well as improving its accuracy and versatility.

Keywords: Nile Red (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one); Fluorescent probe; Neutral lipids; Microalgae; High-throughput screen

Natural products derived from marine microorganisms have proven to be immensely valuable as sources of pharmaceuticals, fine chemicals (Molina Grima et al., 2003), and nutriceuticals for both human consumption (Belarbi et al., 2000) and aquacultural use (Tzovenis et al., 2003). In the last two decades, however, there has been much research applied to the feasibility of commercial production of microalgal lipid for use as a biofuel. The need for renewable fuels whose feedstock sequesters CO_2 from stationary sources has also become increasingly important (Huntley and Redalje, in press; Lewis et al., 2000). Scaling up to commercial production of biofuel feedstock will require significant screening of lipid content in wild-type and genetically modified microorganisms. In addition, rapid measurements are required for their integration into on-site quality control measurements. Since the identification of high-yielding strains would be important for commercial production and current research efforts involving large-scale microalgal lipid production, it is timely to revisit a high-throughput screening method for algal lipids.

Traditional analysis of lipid content in biological samples has been performed by solvent extraction and gravimetric determination (Bligh and Dyer, 1959). Further analysis and characterization is performed by GC or HPLC (Carvalho and Malcata, 2005; Wiltshire et al., 2000). A disadvantage to these techniques, in terms of high-throughput screening and on-site measurement, is that the steps required to both extract and derivatize the fatty acids for GC analysis are numerous and time consuming. Moreover, adequate amounts of biomass must be cultured for the extraction and derivatization (approximately 10–15 mg wet weight of cells, (Akoto et al., 2005)). The amount of sample and preparation time, however, can be greatly reduced if the lipid content of algal cells is measured *in situ.* The technique should be applicable to culture in miniature bioreactors and use of fluorescence-based microprobes (Harms et al., 2002; Kostov et al., 2001).

Lipid measurement has been previously proposed using Nile Red, a lipid-soluble fluorescent probe that possesses several characteristics advantageous to *in situ* screening. It is relatively photostable, intensely fluorescent in organic solvents and hydrophobic environments, but has a low quantum yield in water (Fowler et al., 1979). Nile Red's emission maximum is blue-shifted as the polarity of the surrounding environment decreases, (Cooksey et al., 1987; Greenspan and Fowler, 1985; Laughton, 1986; Lee et al., 1998) which allows one to differentiate between neutral and polar lipids assuming appropriate choice of the excitation and emission wavelengths. If one

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doi:10.1016/j.mimet.2006.11.008

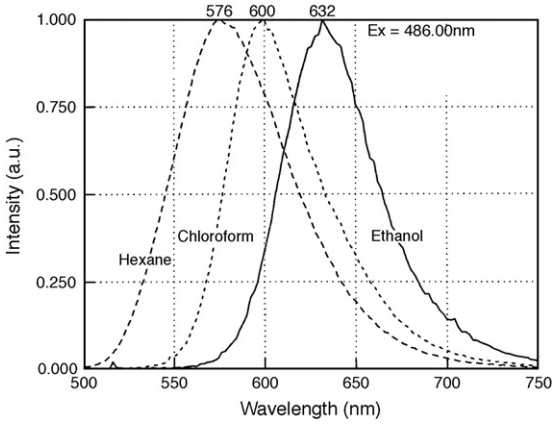

Fig. 1. Technical emission spectra for Nile Red in various solvents. The emission spectra normalized at their maxima to emphasize the wavelength shift. Dielectric constants: hexane, 1.89 at 20 °C; chloroform, 4.8 at 20 °C; ethanol, 24.3 at 25 °C.

couples the measurement with an additional fluorescent stain that binds to cellular protein to determine the actual number of cells per sample, the specific lipid content can also be determined (Laughton, 1986). In our work cell concentrations were determined by using a Z1 Coulter Counter and a hemacytometer.

Keeping in line with the aim of this assay, which is to select for candidate organisms for large-scale production, we wanted a method which is sensitive to the nonpolar lipid content of cells. Fig. 1 shows the blue shift of the emission maxima of Nile Red as the solvent polarity decreases. The peak emission intensity of Nile Red in hexane occurs near 576 nm when excited at 486 nm (Fig. 1). We used these excitation and emission conditions on the assumption that these conditions would permit one to preferentially observe Nile Red in the most hydrophobic environment, which presumably reflects the neutral lipid fraction.

Using this approach we measured the relative neutral lipid content of two different algal strains: *Nannochloropsis* sp. and *Tetraselmis* sp. The algae were cultured in f/2 media (Guillard, 1975) and incubated at 23 °C–25 °C under incandescent lighting to provide 150–200 μE m^{-2} s^{-1} of light. They were grown in 500 mL Erlenmeyer flasks containing up to 250 mL of liquid culture medium. The samples for measurement were taken from cultures that had been incubated for around 3 to 6 days. If the

Table 1
Microalgae oil % FA composition of sample

Dodecanoic (Lauric)	C12:0	1.09
Tetradecanoic (Myristic)	C14:0	2.57
Unknown	–	11.68
Hexadecanoic (Palmitic)	C16:0	9.81
Hexadecanoic (Palmitoleic)	C16:1n-7	9.22
Unknown	–	16.39
Hexadecadienoic	C16:2n-4	0.09
Hexadecatrienoic	C16:3n-4	0.12
Octadecanoic (Stearic)	C18:0	0.28
Octadecenoic (Oleic)	C18:1n-9	1.62
Octadecadienoic (Linoleic)	C18:2n-6	0.56
Octadecatrienoic (Linolenic)	C18:3n-3	Nd[a]
Octadecatrienoic	C18:3n-4	0.15
Ecosenoic (Gadoleic)	C20:1n-9	0.46
Ecosatetraenoic	C20:4n-3	0.97
Ecosatetraenoic (Arachidonic)	C20:4n-6	0.86
Eicosapentanoic (EPA)	C20:5n-3	8.36
Docosanoic (Behenic)	C22:0	8.42
Decosapentanoic	C22:5n-3	1.35
% identified peaks ()		46.9
% unidentified peaks		53.0

Of total fatty acid.
[a] Nd ≤ 0.10%.

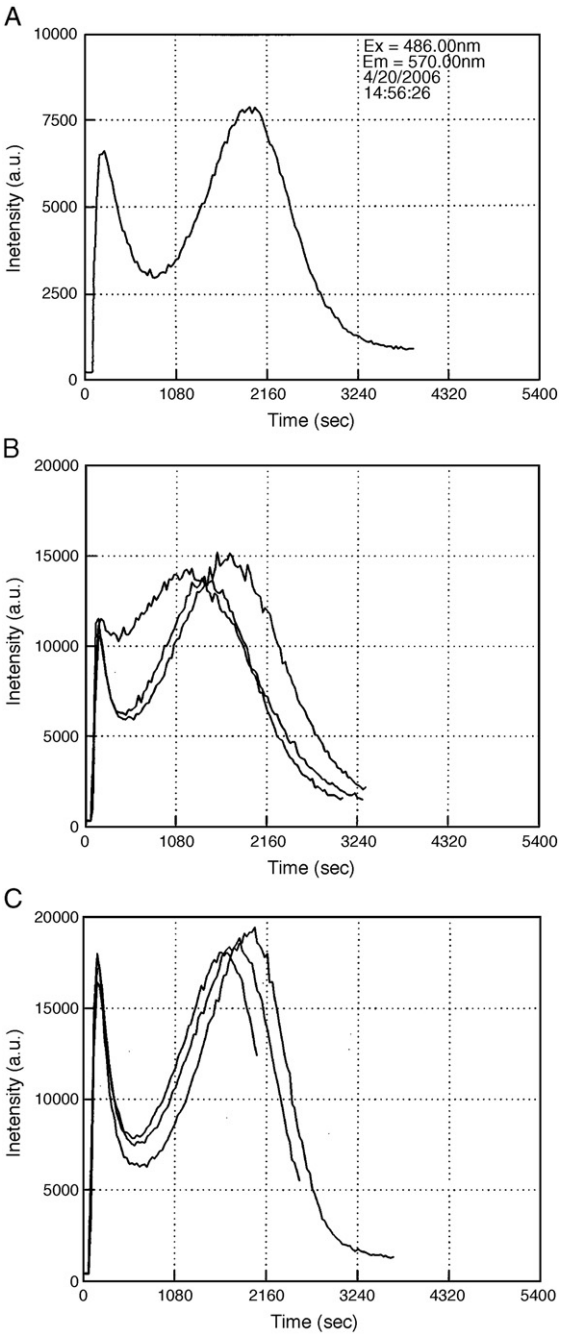

Fig. 2. Typical time-varying intensity emission profile of Nile Red excited at 480 nm and recorded at 570 nm after addition to a solution of live *Nannochloropsis* sp. cells (A). Multiple responses of live *Nannochloropsis* sp. cells at an OD_{720} of 0.1 (B) and live *Tetraselmis* sp. cells at an OD_{720} of 0.1 (C).

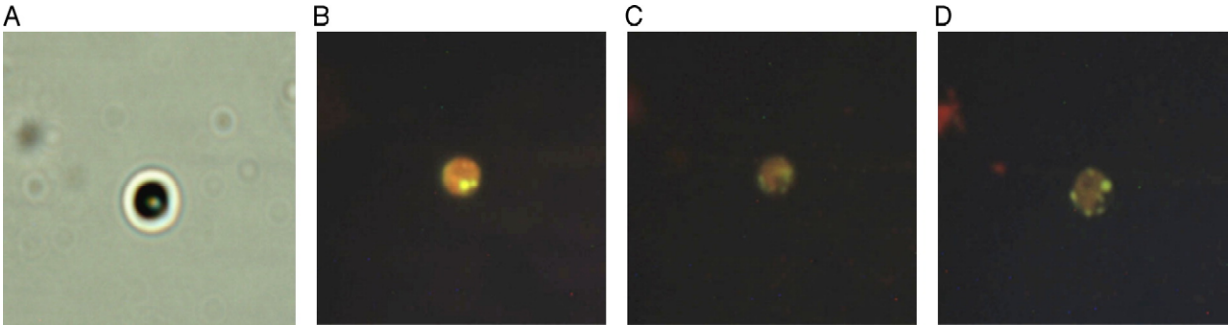

Fig. 3. Wide field epifluorescence images of *Nannochloropsis* sp. cells infused with Nile Red. (A) shows the cell under bright field microscope at 100×. (B–D) show cells after addition of Nile Red. (A) Bright field (100×), (B) after 2.5 min. (C), after 10 min, (D), after 19 min.

optical density of the culture was above of 0.1–0.3 at 720 nm, it was diluted accordingly with media. Otherwise, the cultures were used as they were. Per sampling, 3 mL of this algal suspension was stained with 10 μL of 7.8×10^{-4} M Nile Red dissolved in acetone (final concentration 0.26 μM) and then excited at 486 nm before measuring the emission at 570 nm (using excitation and emission slits set at 8 nm FWHM) using an ISS PCI spectrofluorimeter (ISS Inc., Champaign, IL). We note that 570 nm was used since this wavelength is typical of common interference filters used in filter fluorimeters and we anticipate transferring this method to that instrument platform. A typical profile of lipids extracted from dried cells of *Nannochloropsis* sp. using hexane is given in Table 1.

A typical time-dependent emission trace of *Nannochloropsis* sp. is given in Fig. 2A. The most immediate and noticeable result is that the maxima are independent of time. This, in fact, becomes the most significant signature of this assay. Cooksey et al. (1987) measured the Nile Red fluorescence at a single time point almost immediately (i.e. 30 s) after the Nile Red had been added to a suspension of cells. In our work, however, we found the maximum in emission intensity (at 570 nm) varied with time for both algal strains (Fig. 2B, C). That said, we suggest a more viable assay should track the emission intensity over a period of between 30 and 40 min after addition of Nile Red, and the maximum emission intensity recorded as opposed to recording the intensity at a predetermined time point. Although more investigation is needed to fully explain this phenomenon, we suspect that the lack of precise reproducibility regarding the time at which the maximum in the emission intensity occurs is a consequence of varying dye diffusion rates through intracellular regions. This observation was supported by wide field epifluorescence microscopy images of a single *Nannochloropsis* sp. cell at various times after addition of the Nile Red (Fig. 3).

We also found that replication experiments performed on live *Nannochloropsis* sp. and *Tetraselmis* sp. cells suggest that the upper limit of error associated with any given measurement, at least when executed according to our protocols, is ±12%. This was determined from a number of independent measurements, in which anywhere from two to six replicate measurements were taken, and the RSD ranged from 0.5% to 12%. Specifically, algal cells were cultivated in batch culture, harvested and diluted to the appropriate OD, and an aliquot of this solution measured for its lipid content according to the protocol described above. Although we have not determined the reason for variability up to 12%, we have noted that the Nile Red dye is not taken up by the dead cells. It is therefore possible that observed variation in the maximum emission intensity is due to varying proportions of dead cells across samples.

Daily calibration of the fluorimeter is essential. Cooksey et al. (1987) calibrated his fluorimeter using an aqueous suspension of latex beads dispersed in a known concentration of Nile Red. We used a stock solution of Nile Red in hexane for day-to-day calibration of our fluorimeter, which is a more specific and tailored calibration for the use of Nile Red in detecting lipids in various cellular systems. In general, the appropriate solvent can be chosen so that its emission maximum of Nile Red matches its emission maximum in the target medium.

To test our modified Nile Red assay as a screening tool, we used it to screen a single strain of *Nannochloropsis* sp. cells for relative lipid content when grown under nitrogen-deficient and nitrogen-sufficient conditions. Nitrogen-limited growth can stimulate the cells to produce more lipids per cell since protein biosynthesis is limited (Suen et al., 1987). Using our Nile Red protocol we found that the cells grown under nitrogen-deficient conditions exhibited 6.7 times more fluorescence than cells grown under nitrogen-sufficient conditions. To get absolute measurements, direct calibration curves correlating emission intensity to lipid concentration can be easily generated, as has been reported previously (Cooksey et al., 1987; Lee et al., 1998; Alonzo and Mayzaud, 1999).

In this work we have reported modifications to the Nile Red assay that improve its ability to screen for neutral lipids, either across multiple microalgal strains grown under identical conditions, or across varying growth conditions applied to a single strain. Specifically, the application of Nile Red to measure neutral lipids should calibrate the instrument using dyes dissolved in organic solvents, and appropriately account for the nonlinear intensity emission with respect to time. For absolute measurements of lipid per unit cell, one simply needs to develop the requisite calibration curve that correlates fluorescence to lipid content, whether determined gravimetrically or by use of lipid standards.

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