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Optimization of a Nile Red method for rapid lipid determination in autotrophic, marine microalgae is species dependent

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ABSTRACT

Several studies have been conducted to develop rapid methods for quantification of lipid content in microalgae, as an alternative for time consuming gravimetric methods. Different studies showed that lipid staining with Nile Red in whole cell suspensions and subsequently quantification by the use of a spectrofluorometric device is a promising method, but a profound optimization and validation is rare. It has already been proven that the correlation curve for quantification is species dependent, but it has not yet been investigated whether this is also the case for the optimization of the Nile Red assay protocol. Therefore, two autotrophic, marine microalgae, *Nannochloropsis oculata* and *T-Isochrysis lutea*, strongly differing in e.g. cell wall structure, were selected in this study to investigate whether optimization of the Nile Red assay is species dependent. Besides this, it was checked for one of these species, *Nannochloropsis*, whether the lipid content, determined by the Nile Red assay, could indeed be correlated with the neutral and/or total lipid content determined by gravimetric methods.
It was found that optimization of the Nile Red assay was strongly species dependent. Consequently, optimization has to be done for each species before using the assay. For *Nannochloropsis*, a good correlation was found between total and neutral lipid content obtained by the Nile Red assay and by gravimetric methods.

1. Introduction

During the last decades, lipids from microalgae have been experiencing an increasing interest as a promising source for food, feed and fuel. Microalgal cells contain 10 to 40% lipids, depending on the species and the nutritional and environmental factors during cultivation (Ryckebosch et al., 2012a). It is therefore important to conduct experiments to maximize the lipid content of microalgae. Traditionally, the total lipid content is being determined by solvent extraction and gravimetric determination. Extraction of total lipids from microalgae should be performed with chloroform/methanol (1:1), as optimized by Ryckebosch et al. (2012b). To obtain the neutral lipid content, which is particularly interesting for biodiesel applications, the lipid extract can be separated by thin-layer chromatography (TLC), solid-phase extraction (SPE), high performance liquid chromatography (HPLC) or gas chromatography (GC) (Bligh and Dyer, 1959; Christie, 2003; Ryckebosch et al., 2012b). Although very reliable and reproducible, these analyses are also very time consuming, are labour intensive and require large amounts of algal sample. This makes it difficult to screen large numbers of algal samples or to follow up a culture of algae to determine optimal harvesting time or culturing conditions. A more rapid screening method is thus necessary.

Several rapid screening methods have been proposed in the past, being fluorometric methods using Nile Red or BODIPY 505/515 as fluorescent dyes (e.g. Cooksey et al., 1987; Elsey et al., 2007; Greenspan et al., 1985; Govender et al., 2012), colorimetric methods (Cheng et al., 2011; Wawrik and Harriman, 2007), and methods using Fourier Transform Infrared (FTIR) spectroscopy (Liu et al., 2013; Miglio et al., 2013) or Nuclear Magnetic Resonance (NMR) (Gao et al., 2008).

Nile Red (NR, 9-diethylamino-5H-benzo[α]phenoxazine-5-one) is a chromophore that emits light in the visible spectrum. It is well soluble in a hydrophobic environment and therefore has been used for many purposes, from visualization of lipid droplets via microscopy to quantification of lipid content in whole cells (e.g. Cooksey et al., 1987; Elsey et al., 2007). The NR assay can also be combined with flow cytometry to sort cells containing different amounts of lipids (Greenspan et al., 1985). However, the photostability in the latter application is limited and there is no possibility to measure an excitation or emission spectrum

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(Cabanelas et al., 2015). A more recently developed dye, BODIPY 505/515, has been used as an alternative for NR because of its higher sensitivity, reproducibility and photostability for flow cytometry applications (e.g. Cabanelas et al., 2015; Benito et al., 2015). However, De La Hoz Siegler et al. (2012) reported that fluorescence intensity caused by BODIPY dyes is not proportional to the lipid content.

Because microalgal species with a tough cell wall (e.g. *Chlorella* species) can be impermeable to NR (Pick and Rachutin-Zalogin, 2012), several studies investigated modifications of the NR assay by introducing glycerol (Doan and Obbard, 2011), DMSO (Doan and Obbard, 2011; Chen et al., 2009), ethanol (De La Hoz Siegler et al., 2012) or microwave treatment (Chen et al., 2011) to improve the penetration of the dye through the cell wall.

Although NR has been used in many studies for quantification of lipid content in whole (microalgal) cells, a thorough optimization and validation is often lacking (e.g. Elsey et al., 2007; Liu et al., 2008; Wahidin et al., 2014). Studies that have performed a thorough optimization and validation are only known for heterotrophic microalgae (De La Hoz Siegler et al., 2012; Huang et al., 2009; Isleten-Hosoglu et al., 2012) and focus on the use of the NR assay to optimize heterotrophic culturing conditions and to determine the optimal harvesting time in order to maximize the lipid content of the microalgae.

For a profound validation, a calibration curve has to be made, correlating the lipid content of independent samples determined by fluorometry and by gravimetry. However, several studies used algal samples from the same batch, harvested at different time intervals, which are not independent samples (e.g. Cabanelas et al., 2015; Feng et al., 2013; Wahidin et al., 2014; Wu et al., 2014). It has already been stated by De La Hoz Siegler et al. (2012) that the correlation curve is species dependent, as the staining capacity of NR differs strongly upon different species. However, some studies expanded the correlation curve to other species (Chen et al., 2009) or made a correlation curve with different species together (Sitepu et al., 2012). Several studies even did not make a correlation curve (Eltgroth et al., 2005; Gardner et al., 2012; Doan and Obbard, 2011).

Although it is known that the correlation curve is dependent on the microalgal species, the dependence of the assay optimization on the species has not been studied thoroughly. Parameters that should be optimized to obtain a high fluorescence intensity and reproducibility are the cell and NR concentration, incubation time and eventually the concentration of glycerol, ethanol or DMSO, as carrier molecules for the dye.

The aim of this study was therefore to investigate whether optimization of the NR assay is species dependent. Therefore, a comparison was made between representatives of *Nannochloropsis* and *T-Isochrysis*, two autotrophic, marine microalgal genera strongly differing in e.g. cell wall structure. For one of these species, *Nannochloropsis*, a correct validation was performed to check whether the lipid content, determined by the NR assay, can indeed be correlated with the neutral and/or total lipid content determined by a gravimetric method.

2. Materials and methods

2.1. Cultivation of microalgae

Two marine microalgal species were selected for this study: *T-Isochrysis lutea*, formerly *Isochrysis sp.* (CCAP 927/14) and *Nannochloropsis oculata* (SAG 38.85). The microalgae were cultured in Wright's Cryptophyte (WC) medium (Guillard and Lorenzen, 1972) to which artificial sea salt (Homarsel, Zoutman Industries, Roeselare, Belgium) was added in a concentration of 30 gL^{-1}. The pH of the medium was adjusted to 8–8.1 and it was autoclaved. An inoculum of the microalgae was added in a ratio of 1:10 under a laminar flow hood. Cultivation was carried out in 2 L bottles incubated at controlled temperature (20 °C) or in 1 L flasks (further described in Section 2.3.1). The bottles and flasks were irradiated with daylight fluorescent tubes in a cycle of 16 light hours and 8 dark hours and were aerated with filtered air.

2.2. Optimization of NR assay for N. oculata and T-I. lutea

Incubation time and NR and cell concentration were independently varied for both microalgal species. Furthermore, it was tested whether the addition of glycerol could improve penetration of the dye and consequently increase the fluorescence intensity and the sensitivity of the assay as suggested by Doan and Obbard (2011). All fluorometric measurements were carried out on a Perkin Elmer LS 55 fluorescence spectrometer with a temperature controlling system set at 25 °C. Visualization of the NR staining in *T-Isochrysis* is shown in Fig. 1.

2.2.1. Optimization of incubation time

To optimize the incubation time, 10 μl of a NR solution in acetone (0.5 mg/mL) was added to 3 mL of an algal suspension with optical density at 750 nm ($OD_{750\ nm}$) of 0.2. According to the protocol of Doan and Obbard (2011), glycerol (0.25 mL) was added to this suspension to improve the transfer of NR through the cell wall. The fluorescence intensity (excitation wavelength 486 nm, emission wavelength 570 nm) was recorded during the 3 h following the addition of NR.

2.2.2. Optimization of cell concentration and NR concentration

For each species, two cell concentrations (corresponding to an $OD_{750\ nm}$ of 0.4 and 0.2 for *N. oculata* and 0.2 and 0.1 for *T-I. lutea*) were chosen, for which the NR concentration added to the algal suspension was varied. A dilution series of the NR stock solution was made, resulting in solutions with concentrations 1, 0.5, 0.25, 0.05, 0.025, 0.0125 and 0.0025 mg/mL in acetone. Following Doan and Obbard (2011), 0.25 mL glycerol was added to 3 mL of the algal suspension. 10 μl of the appropriate NR solution was added to this mixture, in order to keep the volume of acetone constant. This resulted in NR concentrations of 3.33, 2.50, 1.67, 0.83, 0.17 and 0.03 μg NR/mL algal suspension. The emission spectrum between 400 and 800 nm was recorded (excitation wavelength 486 nm) after a minimum incubation time of 90 min (selected in Section 2.2.1). Fluorescence intensity was determined by calculating both the relative peak height (at wavelength 570 nm) and the relative peak area (between wavelengths 520 and 670 nm). The recorded intensities were corrected for both the blank sample without NR and a NR solution in water.

2.2.3. Optimization of glycerol concentration

An optimal cell and NR concentration were selected from 2.2.2. To optimize the glycerol concentration, different volumes (0.1 to 0.5 mL) were added to an algal suspension with $OD_{750\ nm}$ of 0.2. Subsequently 10 μl of a NR solution (0.5 mg/mL) was added to this mixture. The emission spectrum between 400 and 800 nm was recorded (excitation wavelength 486 nm) after a minimum incubation time of 90 min. Fluorescence intensity was calculated as described in Section 2.2.2.

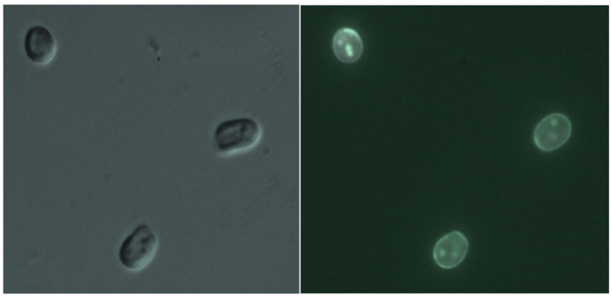

Fig. 1. Visualization of NR staining in *T-Isochrysis* with light microscopy (left) and fluorescence microscopy (right).