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Rapid method to screen and sort lipid accumulating microalgae

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HIGHLIGHTS

- Optimized method for detecting lipid rich cells of *C. littorale* with FACS.
- Efficient BODIPY staining dissolved only in ethanol, no assistant necessary.
- Maintenance of cellular viability after staining and cell sorting.
- The process can be applied to sort cells with high lipid content.

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ABSTRACT

The present work established an efficient staining method for fluorescence activated cell sorting (FACS) with *Chlorococcum littorale* maintaining cellular viability. The method was designed to detect high-lipid cells and to guarantee cellular viability. $BODIPY_{505/515}$ (BP) was more suitable to FACS when compared to Nile red. The optimum concentrations were 0.4 μg ml^{-1} of BP, 0.1% DMSO or 0.35% ethanol. Both ethanol and DMSO were equally efficient and assured cellular viability after the staining and sorting. Here a method is presented to rapidly screen and sort lipid rich cells of *C. littorale* with FACS, which can be used to produce new inoculum with increased cellular lipid content.

1. Introduction

Microalgae are among the most promising and suitable sources to achieve sustainable production of commodities such as proteins, carbohydrates and specially oil as feedstock for food, feed, chemicals and biofuels (Chisti, 2007, 2008; Wijffels and Barbosa, 2010). However, the lipid productivity of the known strains needs to increase to make the production of algal lipids economically feasible (Norsker et al., 2011). Higher lipid productivity can be achieved with fast growing strains with high oil yield, which can reduce the costs of microalgae bulk production (Wijffels and Barbosa, 2010; Norsker et al., 2011). One option to develop an industrial culture of algae for production of lipids is via rapid sorting of lipid rich cells from a mixed population (Pragya et al., 2013).

High throughput cell sorting can be done by coupling the fluorescence detection of intracellular lipid bodies (LB) with flow cytometry (FC) to carry out Fluorescence Assisted Cell Sorting (FACS) (Brennan et al., 2012; Cooper et al., 2010; Elliott et al., 2012; Yen Doan and Obbard, 2011). This technique allows the selection of a certain profile and the use of it to sort targeted cells, which can be grown and be used to establish a new cell lineage (Yen Doan and Obbard, 2011). However, there are still challenges to apply it to sort lipid rich microalgae: (i) the staining must be designed to distinguish efficiently the high-lipid content cells; (ii) the dye must be homogeneously distributed in the population and (iii) the process must assure cellular viability to allow growth of sorted cells.

Nile red (NR – 9-diethylamino-5-benzo[α]phenoxazinone) is still the most common lipophilic fluorophore used for microalgae (Chen et al., 2009, 2012; Cooper et al., 2010; Doan and Obbard, 2012; Montero et al., 2010; Yen Doan and Obbard, 2011). Yet, this dye has a series of drawbacks, especially when aiming at FACS. It may require a combination of cell destructive methods to increase dye

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homogeneity and the accuracy is highly strain dependent (Chen et al., 2009). Also, it can interfere with the chlorophyll molecule on the lipid specific fluorescence, since it is also excited by a green laser (530/25 nm) and emits fluorescence in the yellow spectra (590/10) (Laurens and Wolfrum, 2010). However, the ultimate inadequacy is its limited photo stability resulting in signal which is not constant in time. The application of $BODIPY_{505/515}$ (BP) probe in microalgae has become much more popular over the last few years (Brennan et al., 2012; Cooper et al., 2010; De la Hoz Siegler et al., 2012; Elliott et al., 2012; Govender et al., 2012; Velmurugan et al., 2013; Xu et al., 2013). $BODIPY_{505/515}$ (4,4-Difluoro-1,3,5,7-Tetramethyl-4-Bora-3a-Diaza-s-Indacene) has a high oil/water coefficient, which allows it to infuse membranes and makes the labeling of cell components faster than NR. It is excited by a blue 488 nm laser, with maximum emission in the green spectrum at 515 nm, which makes it spectrally distinguishable from algal chlorophyll spectra (Brennan et al., 2012; Cooper et al., 2010).

In the current research *Chlorococcum littorale* was the species of choice. Previous results of screening tests showed, under nitrogen stress: (i) high photosynthetic efficiency, (ii) biomass production ($0.3\ g\ l^{-1}d^{-1}$) and (iii) lipid accumulation (10–35 g fatty acids per g biomass $l^{-1}\ d^{-1}$). This strain was first described by Chihara (1998), *C. littorale* showed fatty acids accumulation of 34% DW high and a resilient CO_2 uptake (up to 50% CO_2) (Ota et al., 2009, 2011). These features remark the potential – not thoroughly investigated - of this strain for lipid production. The aim of this work was to optimize a fluorescence method to efficiently assist cell sorting of *C. littorale,* assuring cellular viability.

2. Methods

2.1. Microorganism and culture conditions

C. littorale (NBRC 102761) was acquired from the microbial culture collection of NIES, Japan (Chihara, 1998). For the optimization phase of the experiments it was cultivated batch-wise in the following medium (composition in $g\ l^{-1}$): NaCl 24.55; $MgSO_4{\cdot}7H_2O$ 6.60; $MgCl_2{\cdot}6H_2O$ 5.60; $CaCl_2{\cdot}2H_2O$ 1.50; $NaNO_3$ 1.70; HEPES 11.92; $NaHCO_3$ 0.84; EDTA-Fe (III) 4.28; K_2HPO_4 0.13; KH_2PO_4 0.04; trace elements in $mg\ l^{-1}$: $Na_2EDTA{\cdot}2H_2O$ 0.19; $ZnSO_4{\cdot}7H_2O$ 0.022; $CoCl{\cdot}6H_2O$ 0.01; $MnCl_2{\cdot}2H_2O$ 0.148; $Na_2MoO_4{\cdot}2H_2O$ 0.06; $CuSO_4{\cdot}5H_2O$ 0.01). The cultivation was carried out in an incubator (Multitron Pro orbital shaker incubator INFORS HT, Switzerland) under controlled conditions and carried out in 0.2 l borosilicate Erlenmeyer flasks. The operational conditions were: mixing at 120 rpm, light intensity $150\ \mu mol\ m^{-2}\ s^{-1}$, temperature at 25 ± 0.2 °C and CO_2 supply of 2% v/v over air flow in the headspace.

Non-stressed cultures were grown under nitrogen-replete conditions (i.e., with nitrogen available in de growth medium), while stressed cultures where kept under nitrogen-depleted conditions (i.e., no nitrogen available in the media). To carry out the stress experiments the progressive starvation approach was used, i.e. cells were allowed to consume all nitrogen available (150 mgN-NO_3/l). Nitrogen starvation was used and a factor to stimulate lipid accumulation inside the cells. For the sorting and viability experiments, microalgae were cultivated in a flat panel reactor as described by (Breuer et al., 2013) for both stressed (without nitrogen in the medium) and non-stressed cultures (with nitrogen in the medium). Both stressed and non-stressed experiments were performed aseptic and batch-wise. The liquid volume in the reactors was 380 ml with a light path of 14 mm. Operation was run at continuous illumination (incident light $410\ \mu mol\ m^{-2}\ s^{-1}$) using LED lamps with a warm white light spectrum (Bridgelux, BXRA W1200). The pH was set at 7.0 and controlled on demand by CO_2 addition to the airflow.

Nitrogen starvation was progressively imposed in the experiments. All available nitrogen was allowed to run out in the systems. Nitrogen replete experiments were carried out using samples from the exponential growth phase. The nitrogen content (N-NO_3 $mg\ l^{-1}$) was checked daily by the Sulphanilamide N-1-naphthyl method (APHA 4500-NO3-F) using the automatic analyzer SEAL AQ2.

2.2. Experimental set-up and analytical methods

The experimental set-up followed the flow chart presented in Fig. 1. For every trial the measurements were performed on samples taken from the reactor under the same experimental conditions. All analysis were carried out with 3–5 replicates. Cytometer measurements were terminated when 10.000 individual particles were measured.

The purpose of this approach was to select the best suitable protocol to effectively stain *C. littorale*. The process also had to guarantee two key points: to detect high lipid content cells and to sustain the viability of the cells.

2.2.1. Fluorescence analysis

A Microplate reader FLx800 (BioTek Instruments) was used to assess fluorescence emission after excitation on predetermined wavelengths. For Nile red (NR) (CAS#: 7385-67-3, Sigma Aldrich) a baseline protocol was established. Microalgae cell suspension was set at 0.8 of optical density (750 nm) and NR and glycerol (Sigma Aldrich, CAS#: 56-81-5) were directly added to the cell suspension. NR was kept in a stock solution, in acetone, at a concentration of $100\ \mu g\ ml^{-1}$. Fluorescence emission measurements were taken from 0 to 15 min, with 20 s intervals. The set of filters used were: excitation of 530/25 nm and emission of 590/10 nm. The sensitivity was set at 70 mV. The optimization approach tested the following parameters: NR concentrations: 0.4; 0.6; 0.8; 1.0 and 1.2 $\mu g\ ml^{-1}$, glycerol: 5; 10; 25; 50; 100; 125; 150 $\mu g\ ml^{-1}$.

A baseline protocol for $BODIPY_{505/515}$ (BP) (Life Technologies®) in the micro plate reader could not be established due to excessive noise (data not shown). For that reason the BP analysis was done directly in the flow cytometer (FACSCalibur, BD Biosciences, San Jose, California). BP stock solutions were prepared in all tested carriers: Dimethyl sulfoxide (DMSO, Sigma Aldrich CAS#: 67-68-5), ethanol (EtOH, Sigma Aldrich CAS#: 64-17-5) and glycerol ($C_3O_8H_3$, Sigma Aldrich CAS#: 56-81-5). The effectiveness of glycerol as a carrier was tested by addition of glycerol to the algae suspension in the following concentrations: 0; 0.10; 0.2; 0.3; 0.4; 0.5; 0.75 $\mu g\ mL^{-1}$.

Carriers were kept at the lowest possible concentration, in order to not affect the cellular viability. Microalgae samples were diluted until a concentration in which approximately 200 cells min^{-1} could be measured by the cytometer (between 1:50 and 1:200 dilution). Sterilized PBS (phosphate saline buffer – 1.0×) was used as sheath fluid. The readings were carried out with an argon ion laser with excitation at 488 nm and emission at 530/30 (BP, FL1 channel). All readings were logarithmic and the sensitivity of the apparatus was set at 300 mV. The cells were sorted under these settings. To actually sort cells it is necessary to set a sorting gate, a selected fraction of the acquisition plot that establishes the thresholds which will be used to activate the sorting line, and perform the physical separation of targeted cells. An example of a sorting gate is given in Fig. 4.

The flow cytometer cannot provide imaging of the assessed cell population. Therefore, the FlowCam (Fluid Imaging Technologies, Yarmouth, Maine) was used to provide both fluorescence measurements and imaging of the population. This approach had the objective to provide more descriptive information of the population: auto fluorescence and BP-dependent fluorescence per cell, cell diameter, presence of reproductive cells, contamination control