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Single-cell-based monitoring of fatty acid accumulation in *Crypthecodinium cohnii* with three-dimensional holographic and *in situ* microscopy

Anna-Maria Marbà-Ardébol[a], Jörn Emmerich[b], Peter Neubauer[a], Stefan Junne[a,*]

[a] *Chair of Bioprocess Engineering Department of Biotechnology Technische Universität Berlin Ackerstrasse 76 ACK 24, D-13355 Berlin, Germany*
[b] *SOPAT GmbH Ackerstrasse 76 D-13355 Berlin, Germany*

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ABSTRACT

To date, *on line* monitoring in bioprocesses is restricted to conventional parameters. Presently, advances in microscopy allow the monitoring of single-cell size distributions in a bypass or *in situ*. These data provide information regarding population heterogeneity, substrate conversion, or product synthesis as these parameters are related to the size of the cells. In this study, changes in the single-cell size distribution of the heterotrophic microalgae *Crypthecodinium cohnii* were tracked with holographic microscopy and a photo-optical microscopy probe, which is applicable *in situ*. This algae produces the polyunsaturated fatty acid docosahexaenoic acid (DHA). On the basis of the cell size and broadness of the size distribution, the applied methods enabled to distinguish between cells in the growth and production phase. Under conditions of low growth and high fatty acid accumulation, the cell size kept concomitantly changing. The correlation between cell size measurements and the intracellular DHA content was confirmed by regression analysis. The phase heterogeneity, which was measured by holographic microscopy, changed simultaneously with the DHA synthesis. The amount of information obtained by both digital holographic and *in situ* microscopy is similar to that obtained by flow cytometry but with reduced effort for a real-time analysis.

1. Introduction

The present study evaluates the suitability of two techniques, namely three-dimensional digital holographic microscopy (DHM) [1] and photo-optical *in situ* microscopy (ISM) [2], for monitoring lipid accumulation in heterotrophic algae without using traditional staining methods or chromatographic analysis. ISM can be used *in situ* for real-time monitoring. In contrast to many previous reports of *on line* microscopy in bioprocesses, the microscopes used in this study are commercially available and thus relevant for broader application beyond research.

The cell size of the heterotrophic microalgae *Crypthecodinium cohnii* increases concomitantly with the intracellular content of the polyunsaturated fatty acid docosahexaenoic acid (DHA) [3]. The traditional method of measuring fatty acid content in a cell by gas chromatography is time consuming, and it only represents an average value of the entire sample. In contrast, single-cell-based analyses such as flow cytometry (FCM) provide more information but also require a large amount of sample preparation time if staining is necessary. Automated FCM can reduce this time of operation, but the method is still required to be conducted *at line* to enable automated sampling, dilution and staining [4]. Moreover, the time of operation could also affect the physiology or viability of sensitive cell types. This can be circumvented with the expression of fluorescent compounds inside the cell; however, genetic modification will be required. Another technique that can be applied to rapidly detect changes in the cell size is microscopy coupled with auto-

Abbreviations: DHA, docosahexaenoic acid; DHM, digital holographic microscopy; ISM, *in situ* microscopy; FCM, flow cytometry; *C. cohnii*, *Crypthecodinium cohnii*; DO, dissolved oxygen; BOX, Bis-(1,3-dibutylbarbituric acid) trimethine-oxonol; NR, Nile Red; ID, inner diameter; R_s, substrate uptake rate in a time interval; $\triangle c_s$, difference in substrate concentration in a time interval; c_{s0}, feed substrate concentration; c_{sm}, average substrate concentration in a time interval; V_l, average working volume in a time interval; $\triangle t$, time interval; F, feed rate; R_p, production rate; $\triangle c_p$, difference of the product concentration in a time interval; C/N ratio, ratio in between carbon and nitrogen concentration; FPIA, flow particle image analysis; GC-FID, gas phase chromatograph equipped with a flame ionization detector; R^2, correlation coefficient of determination; FSC, forward scatter; SSC, side scatter; DHA_{pred}, DHA predicted with a non linear correlation; D_c, cell diameter measured with the photo-optical microscopy probe; DCW, dry cell weight; σ, variability; e, admitted error; 1−α, amplitude of the confidence interval; n, sample size.

* Corresponding author.
E-mail address: stefan.junne@tu-berlin.de (S. Junne).

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Table 1
Substitution of NaCl in shake-flask experiments.

NaCl Substitute	Mol eq. [mol L^{-1}]	Concentration [g L^{-1}]
None (control)	0.34	20.00
$NaNO_3$	0.17	14.45
NaH_2PO_4	0.17	20.40
$K_2S_2O_8$	0.17	45.00

mated software-based cell detection. Photo-optical measurements *in situ* or in a bypass have been described in several reports [5,6]. Some approaches included the use of a stop-flow device to capture the sample before a picture is taken, thus minimizing the flow of the cell suspension. However, the involvement of micromechanical parts directly confronted with media components and cell suspension may cause problems. Other approaches allow to capture the cell suspension directly, *e.g.* by applying immersion lenses; thus, further optical adjustment during the measurement is not necessary [7]. Microscopy tools are applied for determinations beyond biomass concentration [8–12]. The morphological features of a cell can allow to draw a conclusion about its physiological state [13]. The application of *in situ* microscopy in a phototrophic culture of the microalgae *Chlamydomonas reinhardtii* was described earlier [14]. The same algorithms that are applied for *off line* measurements are usually applicable for *in situ* measurements, although some adaptations may be required. However, the accurate detection of morphological features of undiluted samples at cell densities typically achieved in bioreactor cultivations remains a challenging task. The advantages of a high measurement frequency and the possibility of obtaining a tool for process control when applied *in situ* have to be compared with the disadvantages of a higher background signal and a concomitant loss of accuracy.

Therefore, this study evaluates the monitoring of lipid accumulation in heterotrophic algae in real time by DHM and ISM without the use of traditional staining methods. DHM uses the absorption of red light from an LED at the edges of particles in comparison to a reference beam that passes through the sample towards a photo-detector to determine the cellular size, volume and phase homogeneity among other parameters. This method is applied for diluted samples *ex situ*. Recent developments enable the user to connect the holographic microscope to a flow cell in such a way that a bypass measurement becomes feasible. ISM as applied in this study consists of a single-rod sensor probe that allows the capture of images within a known focus area in a measurement gap using a high-resolution CCD sensor. Cells continuously pass through this gap because of the movement of the liquid phase and are illuminated with a white flash light.

In our study, various phases of the DHA production process are monitored during lab-scale reactor cultivations with the single-use bioreactor CELL-tainer©. In addition, the effect of different media on growth and lipid accumulation is described. If applied successfully, novel process analytical tools are available for parallel and automated process development and for process control in the case of *in situ* applicability. DHM and ISM could provide suitable information on intracellular DHA accumulation in lipid droplets based on single-cell size distribution in real time.

Table 3
Parameters for the detection of algal cells in the OsOne software version 4.3 (Ovizio) and SOPAT detection software (SOPAT); recipe file: insitu1lim1k.pss.

Parameter	SOPAT	OsOne
Background	–	2.15
Median cell size (d_{50}) [μm]	18	32
Background detection algorithm	–	Phase variance
Cell detection algorithm	v1R Algo	Local maximum
Apply refocus	yes	yes
Detect invalid areas	yes	yes
Cell minimum size [μm]	8.4	9
Cell maximum size [μm]	23.5	–
Remove image defects	adjusted	9
Split neighbour cells	adjusted	6
Invalid area-sensitivity	–	4

2. Materials and methods

2.1. Media preparation and pre-cultivation

C. cohnii culture CCMP 316 was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota, ME. Precultures were prepared using a previously published method [15].

2.2. Bioreactor cultivations

Cells were cultivated in the single-use rocking-motion bioreactor CELL-tainer® CT 20 (Cell tainer Biotech, the Netherlands) for 7.5 days. The media composition and cultivation procedure have been described previously [17]. To operate the CELL-tainer at 1 L working volume, expansion channels (Cell tainer Biotech) were used throughout the cultivation to fully cover the electrodes with a sufficient amount of liquid during rocking. In total, 100 mL of pre-culture was used for inoculation. The bioreactor process was started in a batch mode followed by a non-limited fed-batch mode in which the glucose concentration (main carbon source) was maintained between 10 and 25 g L^{-1}. The temperature was maintained at 25 °C. The pH was automatically maintained at 6.0 with 1 M HCl and 1 M NaOH. The dissolved oxygen (DO) levels were maintained by regulating the rotational speed to avoid values below 20% of dissolved oxygen saturation. In the production phase, sodium acetate was added, and the feed operation was changed to a pH-auxostat mode controlled by acetic acid as described elsewhere [15]. The addition of sodium acetate induced the expression of the enzymes required for the assimilation of acetic acid, which are not active in cells grown with glucose. Previous studies have proven that acetic acid is a suitable additive to enhance DHA synthesis in *C. cohnii* [17]. The liquid volume increased by approximately 1.8 L during the entire fed-batch phase.

Table 2
Overview of the main characteristics of the applied microscopes.

Parameter	SOPAT MM 1	oLine-OT40GA
Field Depth	2.32 μm	1.5 μm
Camera	2750 × 2200 CCD with 19 fps	2456 × 2058 CCD with 15 fps
Interface	GigE Vision	–
Magnification	10 × with an adaptive TV lens with a magnification factor of 1.6	63 ×
Numeric aperture	0.1	0.7
Illumination	Transmission, Xenon flash lamp, 2.6 J, pulse duration 8 μs	Transmission, Monochromatic LED at 630 nm
Measuring Gap	200 μm	not applicable
Probe length	270 mm	not applicable
Probe diameter	24.5 mm	not applicable
Software Version	SOPAT v1R.002.0053	OsOne-4.3

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