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Flow cytometry to estimate the cell disruption yield and biomass release of *Chlorella* sp. during bead milling

Emre Günerken[a,b], Els D'Hondt[a,*], Michel Eppink[b], Kathy Elst[a], Rene Wijffels[b]

[a] Flemish Institute for Technological Research (VITO), Boeretang 200, 2400 Mol, Belgium
[b] Wageningen University, Bioprocess Engineering, AlgaePARC, P.O. Box 8129, 6700 EV Wageningen, Netherlands

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ABSTRACT

A number of visual, chemical and fluorescence-based methods are generally employed for monitoring of algae cell growth, culture health and biomass concentration. These methods are often time-consuming, demand destructive and high volume sampling. Rapid, efficient, cost-effective and automated methods which facilitate high-throughput and non-destructive sampling would highly benefit microalgae biotechnology. It is known in literature that with flow cytometry it is possible to monitor microalgae growth and microalgae culture health. Flow cytometry, however, has not been used to estimate biomass release and cell disruption yield. In this study with representative cultures of *Chlorella* sp., flow cytometry data were generated by a long pass filter (> 670 nm) and proved to be a promising technique to rapidly evaluate these parameters during cell disruption by bead milling. Both a laboratory and a commercial culture of *Chlorella* were bead milled. Prior to and during bead milling, biomass release was evaluated gravimetrically and the cell count was determined via hemocytometer manual counting and 3 flow cytometry methods: 1) direct event count of a certain population, 2) quadrant-upper right event count of a long pass filter (> 670 nm) and 3) a calculation based on the long pass filter data. The data of all methods were compared and correlated to gravimetric biomass release data. Manual counting resulted in an underestimation of the cell disruption yield as one of the *Chlorella* cultures manual counts did not agree with biomass release data. Good correlations for both cultures were found for cell disruption yield calculations based on flow cytometry data and gravimetric biomass release data. Flow cytometry is therefore an efficient analytical method to rapidly screen disruption yields during cell disruption in microalgae and can be a substitute for the time-consuming direct methods (e.g. gravimetric, manual counting) for estimating biomass release and cell disruption yield.

1. Introduction

Microalgae have been employed as substrate for industrial biosynthesis of fuel, food, pharmaceuticals, nutraceuticals, cosmetics and a variety of other bioactive molecules [1]. The ease of metabolite recovery and thus cell disruption efficiency are critical for the economic viability of microalgae biorefineries [2]. Evaluation of the cell disruption yield is therefore of great importance.

Most laboratory settings employ direct counting via a standard hemocytometer to determine the cell disruption yield. While direct counting methods are precise and sensitive, they are time-consuming and labor-intensive. Microalgae biomass growth is conventionally monitored by a combination of dry weight-, optical- and fluorescence-based methods for microalgae biomass concentration estimation and culture health monitoring. These analyses are also used for monitoring the yield of downstream processing steps. Estimation of dry weight concentration is the most direct method, but requires milligram/gram dry weight of sample amounts and is time consuming since the result is determined after the sample is fully dried. Additionally, dry weight analysis does not provide specific information. For example, dry weight analysis will result in an overestimation of biomass growth or biomass release during cell disruption due to salt accumulation in the suspension.

Coulter counter, dynamic light scattering or optical density monitors are optical methods that estimate cell particle diameter and correlate the results with biomass volume and dry weight [3]. In general, optical methods are rapid, precise, sensitive and suitable for non-destructive sampling. However, these methods are not suitable to distinguish biologic contaminants, abiotic particles and microalgae cells and are often hampered by morphological and physiological heterogeneity of microalgae cultures and the presence of cellular debris [3,4]. Nephelometry and fluorometric estimation of chlorophyll-*a* have also

* Corresponding author at: Flemish Institute for Technological Research (VITO), Boeretang 200, VITO MAT Building, 2400 Mol, Belgium.
E-mail addresses: els.dhondt@vito.be (E. D'Hondt), michel.eppink@wur.nl (M. Eppink), kathy.elst@vito.be (K. Elst), rene.wijffels@wur.nl (R. Wijffels).

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been employed [4]. Photomicrography followed by automated image analysis is another useful approach, but might not accurately estimate dry mass from the wet volume [5].

Chioccioli et al. [3] reported that OD_{750} values and cell concentrations did not correlate reliably with biomass dry weight in *Chlorella vulgaris* cultures. The authors, however, observed a good correlation between flow cytometer and hemocytometer based measurements. Chlorophyll fluorescence from the microalgae cells was measured in order to monitor microalgae biomass accumulation. It showed a good correlation with the cell counts determined via a hemocytometer, biomass concentration determined by freeze drying and counting data from the flow cytometer. Additionally, Premazzi et al. [6] reported a good correlation between microalgae cell number via manual counting and size estimation by flow cytometry to determine the dry weight concentration. In another study, Wood et al. [7] observed that flow cytometry, compared to OD_{750}, exhibits a greater accuracy in estimation of the specific growth rate when cell numbers were low.

Flow cytometry can yield valuable information on microalgae replicative cycles, and can determine cell concentration and cell size to evaluate biomass production. However, such estimations are based on the assumption that the entire population has the same cell size which may cause an over- or under-estimation. Environmental microalgae cultures exhibit significantly different physiological and biochemical characteristics compared to unialgal cultures employed in industrial bioprocesses. Several authors have reported the use of specialized flow cytometers that accommodate the great variation in the cellular concentrations, chlorophyll α content, and DNA content between algal species [8]. Flow cytometry could thus yield reliable estimates of biomass for either type of microalgae populations [9–11] since biomass concentration can be estimated by correlating known data on carbon content to forward scatter pulse area (FSC-A), or species-specific chlorophyll fluorescence or DNA content of the cultured algae. It can also yield data on several indicators of microalgae growth and physiology, such as the cell size, DNA, protein and lipid content and offers the possibility to monitor microalgae metabolites by fluorescent staining. Dominguez Teles et al. [12] reported a method based on flow cytometry to screen and sort lipid accumulating microalgae. Petrescu et al. [13] reported the use of flow cytometry to evaluate physiological alterations induced in *Chlorella fusca* by heavy metal exposure. Most of the earlier works have used the FSC-A and side scatter pulse area (SSC-A) data for these purposes [14–18]. Chioccioli et al. [3] reported for the first time the use of pulse width data in estimating the biomass dry weight of *Chlamydomonas reinhardtii* and *Chlorella vulgaris* cultures. Moreover, Green et al. [17] and Toepel et al. [18] studied flow cytometry to estimate the biomass concentration via data from cell diameters using forward scatter and corrected chlorophyll fluorescence signal data.

All the above mentioned techniques are used to differentiate strains or monitor algae cultivation. The current study, however, is focused on monitoring the cell disruption efficiency since cell wall and cytoplasmic membrane disruption are essential to recover intracellular biomolecules. A number of mechanical and non-mechanical methods are employed for cell disruption of which bead milling is the chosen technique in this study [19]. Conventionally, cell disruption is evaluated by monitoring cell counts, particle size and release of metabolites (e.g. protein, lipids) [20]. Cell counts are used to estimate the intact microalgae population after the disruption process, but suffer from some drawbacks. In general, manual counting assays tend to cause an under- or overestimation of the cell disruption yield because the disrupted cells that have secreted constituents but remain intact can be counted as healthy cells. Dyes that color living and death cells differently, can tackle this problem, but this is usually labor intensive and requires experience. Particle size estimation yields similar data, but is time-consuming and prone to misinterpretation due to clumping of the biomass [21,22]. Nephelometric monitoring is also used, but may not be suitable for all types of algae [23]. In comparison, methods for monitoring metabolite release (e.g., protein estimation by Bradford method, UV absorptiometry for metabolites) are simple and yield quicker results. However, the underlying analytical procedures frequently yield inconsistent results and the inter-species and inter-strain variations in microalgae productivity further compromise data accuracy [24]. Metabolite denaturation at high levels of cell rupture is another concern which may cause an underestimation of cell disruption [25]. Similarly, at low levels of cell rupture ($>$ 30% of cells remaining), metabolite release indicators may estimate higher cell rupture rates than by cell counting. Spiden et al. [26] have proposed a model of continuum of cell rupture, in which cells become leaky to metabolites prior to their fragmentation. This applies in particular to *Chlorella* in which the cell rupture may be underestimated through cell counting.

Flow cytometry represents a promising approach in this context since the benefits include direct processing of samples, fast generation of results (2 min vs approximately 20 min for manual counting), feasibility for counting larger number of cells (5000–10,000 vs about 400 cells in manual counting), automation and low sample requirements [27]. In this study, the possibility of using a flow cytometer with standard configurations to monitor the cell disruption of two *Chlorella* cultures was evaluated. Flow cytometer data was compared with other methods, e.g. number reduction through direct cell count and gravimetric measurements to validate the relevance of this fast method.

2. Materials and methods

2.1. Microalgae biomass

The study was performed with culture specimens of two species of *Chlorella*. *Chlorella* sp. cultured under photoautotrophic conditions was obtained from Wageningen University Research Center Bioprocess Engineering department (Wageningen, The Netherlands). The microalgae cultured according to Postma et al. [28]. The second specimen consisted of commercially grown *Chlorella sorokiniana* biomass obtained from Phycom (Ochten, The Netherlands). In order to minimize variations induced by growth and nutrient conditions, samples of the microalgae cultures were collected during the late logarithmic phase of growth. In the following, the culture specimens are referred to as WUR and PHY, respectively.

2.2. Dry weight determination of the untreated algae cultures

15 mL of culture samples were transferred to pre-weighed 50 mL tubes and were frozen at − 20 °C overnight and subsequently at − 80 °C for 6–8 h. This was followed by freeze-drying for 72 h under vacuum conditions, and subsequent storage in a desiccator until they attained room temperature. Finally, the dry samples were weighed at room temperature, and stored in sealed sample tubes at − 20 °C until further analysis. The ash-free dry weight of the culture samples was not determined since the reported ash content of the used strains was $<$ 5% [29]. The result was used to prepare the 6% (w/w) microalgae suspension for bead milling.

2.3. Cell disruption by bead milling

The protocol of Schwenzfeier et al. [30] was used for cell disruption by bead milling. Briefly, the 6% (w/w) microalgae suspension was recirculated through the grinding chamber of the DYNO®-MILL Model MULTI LAB RL (Willy A. Bachofen AG Maschinenfabrik, Muttenz, Switzerland) for 50 min at 1.5 L/min with mill rotation of 2000 rpm. The 0.6 L grinding chamber contained Yttria-stabilized zirconia beads (0.4–0.6 mm) at maximum level of bead filling. Subsequently, the jacket of the bead mill and the microalgae suspension vessel were cooled to 5 °C with the intention to maintain the temperature of the microalgae suspension at the mill outlet below 30 °C. All experiments were performed in duplicate with intermediate cleaning of the bead

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