Communication

Wave-Mixed and Orbitally Shaken Single-Use Photobioreactors for Diatom Algae Propagation

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Although vertical column, flat-plate, tubular, bubble column and airlift photobioreactors are widely used for the cultivation of diatoms, it has been shown that bubble-induced hydrodynamic stress created in these types of bioreactor can damage cells. Therefore, three single-use surface-aerated bag bioreactors, known for their outstanding results in cultivating shear-sensitive mammalian cells, were for the first time investigated for their suitability in growing the model microalgae Phaeodactylum tricornutum. All of the systems, which were additionally aerated with CO₂ and equipped with illumination systems providing different light qualities, guaranteed a 22- to 43-fold increase in cell density within seven days, without any addition of cell protection agents or changes in cell morphology. Maximum cell density and dry biomass were achieved in the orbitally shaken 2D-bag by using cool-white fluorescent tubes.

Schlagwörter: Diatom, Growth characteristics, LED, Orbitally shaken, Phaeodactylum tricornutum, Single-use bioreactor, Wave-mixed

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1 Problem

Diatoms are one of the largest and most important groups of microalgae [1, 2] and are regarded as a potential source of high-value products such as polyunsaturated fatty acids, natural colorants, biopolymers and therapeutic agents [1 – 5]. Controlled in vitro production processes using diatoms are performed in photobioreactors such as vertical-column, flat-plate, tubular, bubble column and airlift [6, 7]. However, several authors reported that diatoms can be damaged by shear stress induced by mixing and aeration [8, 9]. Interestingly, it has been shown that Phaeodactylum tricornutum is especially sensitive to aeration-induced hydrodynamic stress [9,10], arising from the bursting of small bubbles on the medium surface of bubble column and airlift photobioreactors.

To date, the suitability of surface-aerated single-use bioreactors operating with a ready to use, pre-gamma radiated, light-transmissive flexible plastic bag instead of a sterilized glass vessel has not been described for P. tricornutum. Such surface-aerated single-use bioreactors include wave-mixed bioreactors and orbitally shaken systems [11, 12]. A key component of both bioreactor types is the 2D- or 3D-cultivation bag, which is either moved on a rocker or a shaker unit and is fixed and shaped by a support container (collecting pan or vessel) [12]. The medium containing the cells is efficiently mixed by rocking or shaking the bag, which continuously renews the medium surface and provides bubble-free surface aeration. Power input and oxygen transfer in these single-use bioreactors can be adjusted and controlled via rocking- or shaking rate, rocking angle, shaker diameter, filling level and aeration rate [13, 14]. As described by Werner et al. [15] wave-mixed and orbitally shaken systems are characterized by homogeneous energy dissipation. It is assumed that shear-sensitive cells tolerate this better and it results in reduced cell damage. The phenomenon of homogeneous energy dissipation was already described for airlift bioreactors in the late 1990s [16]. But thereby cell damage from bubble aeration, extensive foaming and resultant flotation can occur. In contrast, in wave-mixed and orbitally shaken single-use bioreactors, shear stress from bubble aeration is not a concern and it is even possible to perform the cultivation without the addition of antifoam agents, something which will simplify downstream processing of diatom-based bioactive substances.

In the subsequently described growth experiments two wave-mixed single-use bag photobioreactor prototypes and one orbitally shaken single-use bag photobioreactor were used. They are equipped with illumination systems providing differing light qualities: cool-white fluorescent tubes (particularly strong at the blue and red ends of the light spectrum), white LEDs, and white and red light LEDs. Photon flux densities of between 56 and 1712 μmol m⁻² s⁻¹ are
known to support *P. tricornutum* growth, therefore, a value of 95 \( \mu \text{mol m}^{-2}\text{s}^{-1} \) was maintained throughout the experiments [5]. The suitability of all three single-use photobioreactors for propagating the model diatom was investigated. Based on the achieved growth results, design recommendations for single-use bioreactors used in diatom-derived production processes are provided at the end of this article.

## 2 Experimental

### 2.1 Algae Culture

An axenic culture of the diatom *P. tricornutum* CCMP2928 (isolated in the North Pacific Yellow Sea, near Dalian, China) was obtained from the National Center for Marine Algae and Microbiota. Maintenance culturing and inoculum production for the subsequent bioreactor experiments was carried out in 1-L polycarbonate shake flasks (Corning) with vented caps under light exposure (16:8 h light:dark cycle, 95 \( \mu \text{mol m}^{-2}\text{s}^{-1} \), white fluorescent tubes). The orbitally shaken flasks were kept in a Multitron Cell incubator (Infor HT) in batch mode at 23 °C, with a shaking frequency of 110 rpm and shaking diameter of 25 mm. Guillard f/2 medium (Sigma Aldrich) [17] with a salinity of 30 % (Aqua Medic Reef Sea Salt) and a pH value of 8.0 ensured an optimal physiological cell environment, whereby an initial cell density of about 5 \( \cdot \) 10^5 cells mL \(^{-1} \) (from exponential growth phase) was adjusted in all shake flasks. Subcultivation of the cultures was performed weekly. Under these experimental conditions the cells grow with a maximal specific growth rate \( (\mu_{\text{max}}) \) of 0.6 d \(^{-1} \) until they achieve a cell density of 1.3 \( \cdot \) 10^7 cells mL \(^{-1} \). Visual inspection under the microscope confirmed a fusiform morphotype, as commonly reported for in vitro cultures of *P. tricornutum* [18, 19].

### 2.2 Process Parameters of the Single-Use Bioreactors and Process Analysis

Batch cultivations of *P. tricornutum* were performed in three single-use bioreactor types, whose main parameters and specific features are delineated in Tab. 1. The single-use photobioreactors differed in their power input generators, bag sizes, designs, instrumentation, and illumination (in particular the light color, light source and its position). The average light intensity impinging on the medium surface in the bag was measured using a PAN-LX-1308 Luxmeter. In all the bioreactor runs, the f/2 medium was used (see Section 2.1). Cells were harvested after the stationary phase had been reached. As in the shake flasks, an initial cell density of 5 \( \cdot \) 10^6 cells mL \(^{-1} \) and the day/night illumination rhythm (16 h with and 8 h without illumination) were adjusted.

For both wave-mixed single-use bioreactors, the BIOSTAT CultiBag RM 20 and AppliFlex (Figs. 1a, b), the setup of the process parameters was performed on the basis of our experience with plant and animal cells in these bioreactors and extensive bioengineering data determined in earlier studies [20]. The process parameters for the 2D-bag, orbitally shaken in the Multitron Cell (Fig. 1c), were selected to ensure a similar fluid flow and a \( k_La \) value exceeding 8 h \(^{-1} \). Neither antifoam nor cell protecting agents were added because foaming is negligible and shear stress is low in all three of the single-use photobioreactors.

Biomass increase was monitored daily on aliquots (3 mL) of the suspension culture, using a Fuchs-Rosenthal counting chamber and a Leica DMLB microscope (200x magnification). Reported cell density values corresponded to total cell numbers and were calculated by taking the mean of three samples.

Dry biomass concentrations were calculated using Eq. (1).

\[
C_b = 3 \cdot 10^{-8} n \ [\text{g cells L}^{-1}]
\]

(1)
A calibration curve established the dependency between the dry biomass concentration \( C_b\) \( \text{g L}^{-1} \) and the cell density \( n\) \( \text{cells mL}^{-1} \). For this purpose, cells were grown in 1-L polycarbonate shake flasks (Corning) and incubated in the Multitron Cell for 11 days in batch mode. Cell dry weight was determined daily after harvesting 10 mL of the culture broth and subsequent centrifugation at 4000 rpm for 15 min (Heraeus Multifuge X1R). Afterwards, the fresh cell biomass was dried at 65 °C for 48 h (Heraeus Vactherm).

Maximum specific growth rate and cell division rate were also calculated to characterize microalgal growth. Finally, pH was analyzed offline (Mettler Toledo Five Easy) and morphology was microscopically observed every day.

### Results and Discussion

As obvious from Fig. 2 all three single-use photobioreactors supported growth of \( P. tricornutum \) cells. pH changed to 6.2 and cell morphology was similar for all of the cultivations (Fig. 3), and thus independent on the bioreactor type. Maximum specific growth rates \( 0.6697 \text{d}^{-1} \leq \mu_{\text{max}} \leq 0.9816 \text{d}^{-1} \) and cell division rates \( 0.71 – 1.04 \text{d}^{-1} \) were calculated. These results are in the same range as those observed in orbitally shaken flasks (300 mL culture volume) made from plastic (Corning and ThermoFisher Scientific) and glass (data not shown). Moreover, the results are even up to 17 % higher than or correspond quite well to the maximum specific growth rates and cell division rates reported by other...
research groups for the same culture medium and reusable photobioreactors, such as stirred [8], helical tubular [5] and airlift [21, 22]. Moreover, it can be assumed that leachables and extractables that inhibit diatom growth are not secreted from the bag contact layer of the single-use photobioreactors. This interpretation is also supported by the fact that no abnormal growth behavior and no morphological changes appeared during the 11 days of cultivation in that moderate illumination intensities.

If similar fluid flow, oxygen supply, average illumination intensity and light quality was guaranteed, as in the case of the BIOSTAT CultiBag RM (Fig. 2a) and the AppliFlex (Fig. 2b), which were both operated with white light LEDs, this resulted in identical growth curves. In both cases the exponential phase began after three days and linear growth ended after six days. The maximum dry biomass concentration was 0.4 g L⁻¹, and was reached on day 7. Consequently, the maximum specific growth rates in the BIOSTAT CultiBag RM and AppliFlex were comparable (average $\mu_{\text{max}} = 0.7729$ d⁻¹).

The highest dry biomass concentration (0.645 g L⁻¹), cell density ($2.11 \cdot 10^7$ cells mL⁻¹) and maximum specific growth rate ($\mu_{\text{max}} = 0.9816$ d⁻¹) were found in the Multitron Cell, operating with cool-white fluorescent tube illuminated CultiBags. Here the peak dry biomass was between 38 and 40% higher (Fig. 2c), and the maximum specific growth rate was 20% higher than in the BIOSTAT CultiBag RM and the AppliFlex, which were both operated with white light LEDs. This observation may be explained by the main difference between the cultivations, which was the higher red and blue light spectrum ranges provided by the Multitron Cell. It is known from genome analyses [23] that diatoms possess red light absorbing phytochromes and blue light absorbing cryptochromes in addition to aureochromes. Schellenberger Costa et al. [24] described similar growth and biomass production with blue and red light for *P. tricornutum* cultures, however, other groups showed that blue light promoted growth for diatoms including *Skeletonema costatum* [25], *Haslea ostrearia* [26, 27] and *Chaetoceros* sp. and *Skeletonema costatum* with blue-green light [28].

Unexpectedly, a longer lag phase (3 days) and the lowest biomass growth occurred in the BIOSTAT CultiBag RM with red and white light LEDs. The peak cell density ($1.09 \cdot 10^7$ cells mL⁻¹) as well as dry biomass concentration (0.326 g L⁻¹) were measured one day later than in the other experiments (Fig. 2a). The maximum specific growth rate was 14% lower ($\mu_{\text{max}} = 0.6697$ d⁻¹) and the maximum dry biomass concentration was 19% lower than in the BIOSTAT CultiBag RM, which was only illuminated with white light LEDs.

### 4 Concluding Remarks

The results clearly indicate that *P. tricornutum* cells can be successfully grown in wave-mixed and orbitally shaken single-use photobioreactors operating with 2D- and 3D-cultivation bags at benchtop scale. Moreover, they can attain maximum cell densities, dry biomass concentrations and specific growth rates that are comparable to values typically achieved in reusable stirred, helical tubular and airlift photobioreactors. Investigations focusing on the influence of power input and illumination (color, intensity, duration) as well as on the quantity and quality of diatom-derived secondary metabolites are planned for the future.

However, a current weakness of the tested single-use systems – excluding the AppliFlex prototype in which reusable sensors to control and measure pH and DO were installed –
is the lack of single-use sensors that are suitable for phototrophic applications. Bleaching effects caused by light from the currently installed optical pH- and DO sensors mean that the development of electrochemical single-use versions for phototrophic cultivations is required.

Since higher red and blue light spectrum ranges not only contribute to improved biomass growth, but also have the potential to support protein and metabolite expression of diatoms, additional illumination of both of the wave-mixed photobioreactor prototypes with blue light LEDs would appear to be advantageous. It is a matter of fact, that LEDs, which guarantee homogeneous illumination with low heat generation, will increasingly replace light tubes. For this reason, the use of LEDs should also be taken into account for novel Multitron Cell shaker systems.

If these new features become available in the future, single-use photobioreactors will be suitable for diatom-based manufacture of high-value products. Based on the required scale, the user will have a choice between production processes in either an orbitally shaken (scalable up to 10 L culture volume) or a wave-mixed (scalable up to 300 L culture volume) single-use photobioreactor illuminated with LEDs.

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Symbols used

- $C_b$ [g L$^{-1}$] biomass concentration
- $n$ [cells mL$^{-1}$] cell density
- $T$ [°C] temperature
- $\mu_{\text{max}}$ [h$^{-1}$] maximal specific growth rate
- $k_{l,a}$ [h$^{-1}$] volumetric oxygen transfer coefficient

Abbreviations

- DCU digital control unit
- DO dissolved oxygen
- LEDs light emitting diodes
- $P.\ tricornutum$ Phaeodactylum tricornutum
- vvm volume of air per volume of medium per minute

References