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Continuous production of carotenoids from *Dunaliella salina*

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ABSTRACT

During the *in situ* extraction of β-carotene from *Dunaliella salina*, the causal relationship between carotenoid extraction and cell death indicated that cell growth and cell death should be at equilibrium for a continuous *in situ* extraction process. In a flat-panel photobioreactor that was operated as a turbidostat cell numbers of stressed cells were kept constant while attaining a continuous well-defined light-stress. In this way it was possible to study the balance between cell growth and cell death and determine whether both could be increased to reach higher volumetric productivities of carotenoids.

In the two-phase system a volumetric productivity of 8.3 mg β-carotene L_{RV}^{-1} d^{-1} was obtained. *In situ* extraction contributed only partly to this productivity. The major part came from net production of carotenoid-rich biomass, due to a high growth rate of the cells and subsequent dilution of the reactor. To reach equilibrium between cell growth and cell death, sparging rates of dodecane could have been increased. However, already at the applied sparging rate of 286 L_{dod} L_{RV}^{-1} min^{-1} emulsion formation of the dodecane in the aqueous phase appeared.

In a turbidostat without *in situ* extraction a volumetric productivity of 13.5 mg β-carotene L_{RV}^{-1} d^{-1} was reached, solely based on the continuous production of carotenoid-rich biomass.

1. Introduction

Carotenoids from *Dunaliella salina* are produced on a commercial scale in open ponds in e.g. Australia and Israel [1]. Hejazi et al. [2] developed an *in situ* extraction process for production and extraction of carotenoids, the so-called milking process. In this process *Dunaliella* is cultured in closed photobioreactors for the simultaneous production and extraction of carotenoids. After a growth phase the algae are stressed to produce carotenoids. At the same time an organic phase is added to the culture. In this two-phase system the carotenoids are extracted to the organic phase (dodecane). It was shown that with this process a continuous carotenoid production and extraction was obtained for more than six weeks. During this process no biomass growth was observed. The hypothesis was that carotenoids were extracted from the cells and cells kept reproducing carotenoids [3]. However, we recently found that cell-dodecane contact resulted in cell death and carotenoids were only extracted from dead cells [4]. Dead cells fall apart and consequently lose their carotenoids. The lipid globules containing the carotenoids dissolve easily in the lipophilic dodecane. The fact that thus far phase toxicity of dodecane on *Dunaliella* cells was not found might be due to the used methods, namely by measuring e.g. oxygen evolution rates for a total culture and not for single cells with direct contact.

Apparently part of the cells died due to cell solvent contact and cell death was compensated by cell growth. Consequently, in a continuous process constant biomass levels can only be reached if cell death is compensated by cell growth. The objective of this research is to demonstrate that cell growth takes place during the milking process and that the extraction rate increases if the cell death rate is increased. For this we used turbidostat cultivations combined with the *in situ* extraction process. A turbidostat is a continuous culture with controlled turbidity. Lamers et al. [5,6] showed that this approach is very suitable to study the effect of light stress on *D. salina*. In the turbidostat concentration of stressed biomass is constant. In this system the net growth rate is equal to the dilution rate. The sparging rate of dodecane and the light intensity were equal to those used by Hejazi et al. [3]. As a reference we compared the volumetric productivity of this *in situ* extraction experiment with a continuous turbidostat experiment without extraction, solely based on the production of carotenoid-rich biomass.

2. Materials and methods

2.1. Strain and culture medium

D. salina CCAP 19/18 was obtained from CCAP (Culture Collection of Algae and Protozoa, Oban, UK). Stock cultures of the algae were grown in a culture medium as described by Kleinegris et al. [4] with increased sodium

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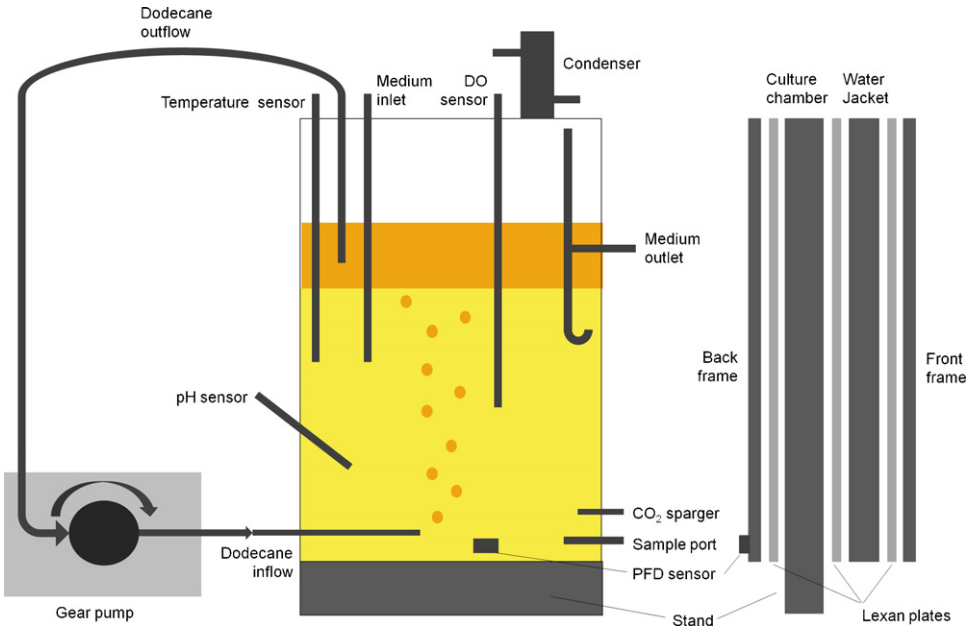

Fig. 1. Schematic drawing of the flat-panel turbidostat and gear pump for dodecane sparging. *Left:* front view. *Right:* side view (without gear pump).

chloride concentration. The Hepes-buffered medium (pH 7.5, 4.00×10^2 M Hepes) consisted of 1.50 M NaCl, 9.95×10^{-4} M $Na_2H_2PO_4$, 3.78×10^{-2} M KNO_3, 2.25×10^{-2} M Na_2SO_4, 1.00×10^{-2} M $NaHCO_3$, 4.87×10^{-3} M K_2SO_4, 3.68×10^{-4} M $MgCl_2$, 1.89×10^{-5} M $CaCl_2$, 1.13×10^{-5} M NaFeEDTA, 1.94×10^{-5} M Na_2EDTA, 1.89×10^{-6} M $MnCl_2$, 1.48×10^{-6} M $ZnSO_4$, 6.65×10^{-7} M $CuSO_4$, 1.10×10^{-8} M Na_2MoO_4 and 9.95×10^{-9} M $CoCl_2$.

Suspended cultures were cultivated in 250 mL Erlenmeyer flasks containing 100 mL of medium and were grown on an orbital shaker incubator at 25 °C, under continuous light with an intensity of 70 ± 10 μmol photons m^{-2} s^{-1} and a continuous agitation rate of 100 rpm. The headspace consisted of an air/CO_2 mixture of 95/5%. Every three weeks 10 mL of a culture was transferred to a new flask containing fresh medium.

2.2. Turbidostat system

The turbidostat-operated system with combined *in situ* extraction was performed in a flat-panel photobioreactor as described by Lamers et al. [6] with modifications as shown in Fig. 1.

The bioreactor consisted of two compartments, one for the culturing and *in situ* extraction and the second compartment functioned as water jacket for temperature regulation. The temperature was maintained at 30 °C. The culture chamber had a working volume of 2.5 L and a light path of 0.03 m. The pH of the medium (same as described above but without the addition of Hepes buffer) was controlled at 7.5 by automatically dosing short pulses of carbon dioxide to the cell suspension. The reactor was illuminated from one side with a high-pressure sodium lamp (Philips 400W Master SON-T PIA Green Power). The average light intensity was measured using a PAR 2π quantum sensor (SA-190, Li-cor Biosciences, Lincoln, Nebraska, USA).

In a first step the cells were grown in a batch culture to obtain a high biomass concentration and in the second step the turbidostat was applied together with the two-phase system for the *in situ* extraction.

For cultivation the reactor was inoculated with a stock culture to a concentration of approximately 7×10^8 cells L^{-1}. During the growth phase an average incoming light intensity was used of 200 μmol photons m^{-2} s^{-1}. By gassing the cell suspension via an inlet tube at the bottom of the reactor with nitrogen gas (0.6 L min^{-1}) mixing of the culture and oxygen removal were established. As a cell number of 1.6×10^9 cells L^{-1} was reached, the culture was diluted to reach 0.8×10^9 cells L^{-1} with a total volume of the aqueous phase of 1.9 L (referred to as L_{RV}). Simultaneously the light intensity was increased to 1200 μmol photons m^{-2} s^{-1}.

The inflicted light stress resulted in carotenoid production by the cells. As shown by Lamers et al. [6] the carotenoid producing phase starts almost immediately after applying stress. Within 9 h it reaches the maximum productivity. As noticed earlier (unpublished results), when not applying a turbidostat regime to maintain a constant stress per cell, the stressed cells grow in size so that self-shading results in a lower light perception and consequently the cells can 'grow over' the stress and start multiplying again. At the point where this happened (approximately 24 h after the start of stress) we started the turbidostat.

The light intensity at the backside of the culture was detected with a PAR photon flux density sensor that was connected to an ADAM-5000 data acquisition card. The light transmitted through the culture (and consequently the turbidity) was evaluated every minute and compared to the set point, i.e. the amount of light transmitted 24 h after the start of light stress (see above). This set point was controlled via a Lab View virtual instrument running on a PC (Lab View 7.1, National Instruments), by automatically switching on or off the feed medium pump. The culture volume was maintained constant by continuous removal of culture broth via a horizontal outlet tube connected to a long vertical tube. This vertical tube had one end (bended upwards) in the culture broth and the top end was situated high above the surface level. This way a constant level of the aqueous phase could be realised even with an organic phase on top.

At the same time as the turbidostat was started, 0.5 L organic phase (dodecane, referred to as L_{dod}) was added to the reactor to apply a two-phase system. The sparging with nitrogen gas of the reactor was stopped and the dodecane was sparged through the reactor to provide mixing and extraction. The dodecane was pumped from the top layer to the bottom inlet by means of a gear pump (Verdergear, VG-045.8), viton tubing and stainless steel connectors. The sparging rate of the organic phase (0.286 L_{dod} L_{RV}^{-1} min^{-1}) was calculated based on the volumetric sparging rate applied by Hejazi et al. [2] of 0.200 L_{dod} min^{-1} for a system of 0.7 L of aqueous phase.

As a reference we performed the turbidostat also without the combination with *in situ* extraction. The flat-panel photobioreactor was operated as described by Lamers et al. [6] with minor modifications and set-points for temperature, aeration, pH as described above. The reactor was inoculated at a lower cell concentration of 7×10^7 cells L^{-1}. This time both during the cultivation period and the turbidostat period nitrogen gas was sparged (0.6 L min^{-1}) through the reactor. This was done via needles at the bottom of the reactor to create an improved flow pattern compared to the first run. The total volume of the aqueous phase was now 2.5 L (L_{RV}).

2.3. Analytical procedures

Cell growth and stress were followed during cultivation by optical density measurements at 530 nm, 680 nm and at 750 nm on a spectrophotometer (Spectronic® 20 Genesys, Spectronic Instruments, UK). Cell number and cell volume (referred to as L_{CV}) were measured with a Beckman Coulter Multisizer 3 (Beckman Coulter Inc., Fullerton, USA, 100 μm orifice). Diameter of the cells was calculated from their volume.

Dry weight of the cell suspension was determined at the end of the cultivation, as previously described by Kleinegris et al. [7] but with 1.5 M ammonium formate as a washing buffer.

Carotenoid levels of biomass and dodecane were determined spectrophotometrically as described by Kleinegris et al. [4]. One milliliter aliquots of cell suspension were centrifuged at $3220 \times g$ for 10 min (Allegra X-22R, Beckman Coulter). After centrifugation the supernatant was discarded and 3 mL dodecane was added to the pellets. After vigorous shaking to re-suspend the pellet 9 mL of methanol was added, again vigorously shaken and then centrifuged for 3 min at $3220 \times g$. The dodecane phase containing lipophilic carotenoids (upper layer) was analysed with a spectrophotometer (Ultrospec 2000, Pharma Biotech) at 453 nm and 665 nm and dodecane as reference.

Finally the β-carotene concentration in the biomass was calculated via the equation below (as previously described by Kleinegris et al. [4])

$$C_{\beta\text{-car}} = \left(\frac{ABS_{453} - ABS_{665}}{3.91}\right) \times 3.657 \times 3 \times X \quad (\text{mg L}^{-1})$$

with($ABS_{453} - ABS_{665}$/3.91): absorbance of β-carotene corrected for chlorophyll contamination.3.657: calibration factor derived from HPLC analysis of β-carotene concentration.3: amount of milliliter dodecane added for extraction.*X*: the dilution factor to measure absorbance on spectrophotometer.

The amount of extracted β-carotene in the dodecane was measured according to the same calibration curve without applying the factor 3.

2.4. Microscopy

Bright field microscopy pictures from cells were made during the whole period of cultivation with a CK 40 bright field microscope (Olympus), equipped with an Olympus AX 70 camera.

3. Results and discussion

We combined *in situ* extraction with biomass cultivation in a turbidostat-controlled flat panel photobioreactor. After a growth phase to obtain high biomass levels, the cells were stressed by applying a high incident light intensity. As was shown by Lamers et al. [6] the cells use the first 4 h to adjust to the inflicted stress by increasing cell size and simultaneously increasing production of carotenoids. We started the turbidostat after the maximum cell size and carotenoid content were reached in order to be able to keep cell numbers of the stressed cells constant (24 h after the switch to high light). Furthermore, dodecane was applied as an organic phase on top of the aqueous phase and pumped through the reactor to yield a high contact area between the organic and the aqueous phase.

β-Carotene was continuously extracted from the aqueous phase to the organic phase with an extraction rate of approximately 2.75 mg β-carotene L_{dod}^{-1} d^{-1} (equivalent to 0.7 mg β-carotene L_{RV}^{-1} d^{-1}) as shown in Fig. 2. This rate is comparable to values obtained by Hejazi et al. [2] who found extraction rates varying between 2.5 and 4.1 mg L_{dod}^{-1} d^{-1} for comparable sparging rates of organic phase.

As the reactor was operated as a turbidostat the turbidity was kept constant by pumping medium into the reactor while keeping the total aqueous volume constant via an overflow tube. As can be seen in Fig. 3 this resulted in the first days of the turbidostat period in a small increase in cell number and a decrease in cell size until steady state was attained.

In previous research it was shown that cell death, caused by sparging the organic phase through the reactor, and consequent break up of the cells is the underlying mechanism of carotenoid extraction [4]. Therefore, the constant cell number achieved here together with the significant extraction rate of carotenoids must have been a result of cell division going together with cell death and cell lysis. Moreover, to keep cell numbers constant it was necessary to continuously dilute the aqueous phase with fresh medium, at a dilution rate of 0.55 d^{-1}, resulting in a net specific growth rate of 0.55 d^{-1}. If we assume that all extracted carotenoid were completely withdrawn from cells with a carotenoid content of 9 pg $cell^{-1}$, we can calculate a cell death rate of 0.05 d^{-1} due to dodecane sparging. This yields a total growth rate of 0.60 d^{-1} for run 1 and shows that cell growth was considerably higher than cell death.

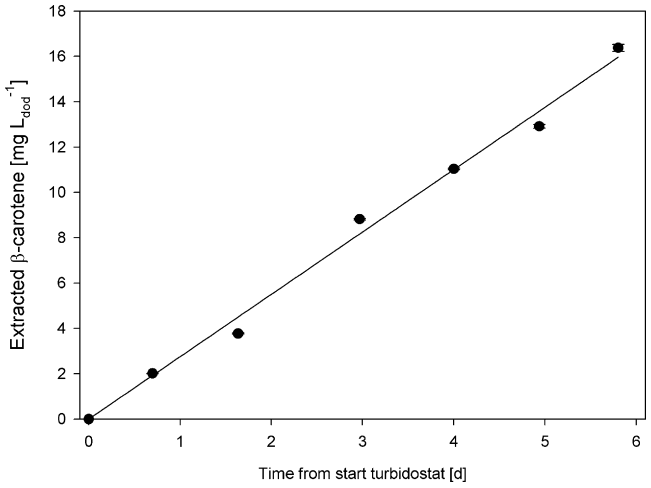

Fig. 2. Extracted β-carotene [mg L_{dod}^{-1}] in time. The bullets show the measured values, with error bars depicting the standard deviation, $n = 2$. The slope of the line gives the extraction rate of 2.75 mg L_{dod}^{-1} d^{-1}, with a R^2 of 0.99.

In Table 1 all steady state data for the experiments performed are summarized. With a cell concentration of 1.6×10^9 cells L_{RV}^{-1} and a cellular β-carotene content of 9 pg β-carotene $cell^{-1}$ combined with the aforementioned dilution rate of 0.55 d^{-1} a daily volumetric production rate of 7.6 mg β-carotene L_{RV}^{-1} d^{-1} is obtained. As a comparison, β-carotene extraction by the dodecane yielded 0.7 mg β-carotene L_{RV}^{-1} d^{-1}, which was 10 times less than the 7.6 mg of β-carotene removed from the reactor via the biomass overflow. Consequently, we can conclude that a much higher pro-

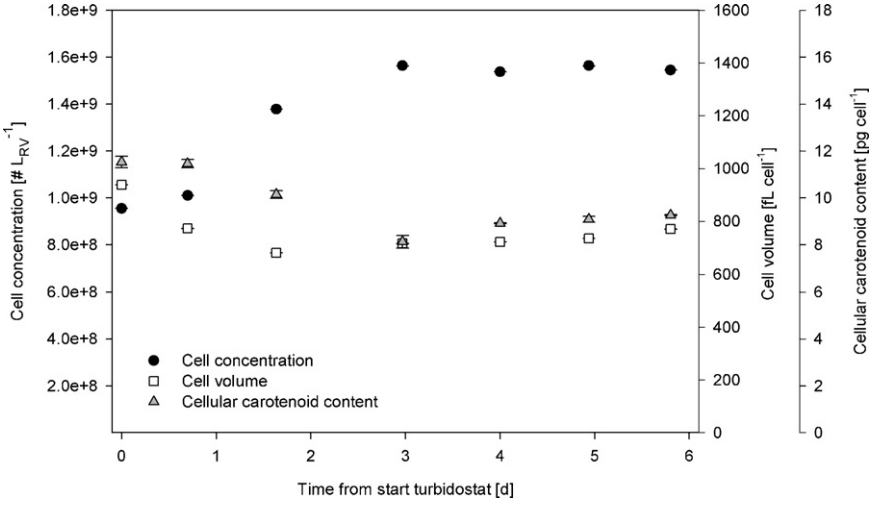

Fig. 3. Cell concentration in the reactor [# L_{RV}^{-1}], cell volume [fL $cell^{-1}$], and cellular carotenoid content [pg $cell^{-1}$] in time for run 1: turbidostat mode combined with *in situ* extraction. Errors bars depict standard deviations obtained from duplicate samples.

Table 1
Comparison of steady state values of the *in situ* extraction experiment of Hejazi et al. [2], the *in situ* extraction turbidostat experiment (run 1) of this article, and the turbidostat without *in situ* extraction (run 2).

	Hejazi et al. [2]	With *in situ* extraction	Without *in situ* extraction
Light intensity ($\mu mol\ m^{-2}\ s^{-1}$)	1200	1200	1200
Light path (m)	0.03	0.03	0.03
Transmission (%)	–	16	6
Sparging rate ($L_{dod}\ L_{RV}^{-1}\ min^{-1}$)	0.286	0.286	0.286
Reactor volume (aqueous phase) (L_{RV})	0.7	1.9	2.5
Cell concentration (cells L_{RV}^{-1})	1.2×10^9	1.6×10^9	1.2×10^9
Total cell volume ($L_{CV}\ L_{RV}^{-1}$)	–	1.1×10^{-3}	1.5×10^{-3}
Average cell volume ($fL\ cell^{-1}$)	–	7.4×10^2	1.2×10^3
Intracellular carotenoid concentration (g β-carotene L_{CV}^{-1})	–	12.0	12.2
Cellular carotenoid content[a] (pg β-carotene $cell^{-1}$)	4–51	8.9	15.0
Carotenoid content per dry weight (mg β-carotene g_{DW}^{-1})	–	28.1	19.0
Net specific growth rate (d^{-1})	0	0.55	0.75
Produced β-carotene in biomass overflow (mg β-carotene $L_{RV}^{-1}\ d^{-1}$)	0	7.6	13.5
Extracted β-carotene in dodecane (mg β-carotene $L_{RV}^{-1}\ d^{-1}$)	2.5	0.7	0

[a] The cellular carotenoid content increased in time from 4 to 51 pg $cell^{-1}$.

ductivity can be reached in this system in case the carotenoid-rich biomass from the overflow can also be extracted. In case we take all produced β-carotene into account the total volumetric productivity of the system was 8.3 mg β-carotene $L_{RV}^{-1}\ d^{-1}$.

Possibly a higher cell death rate and carotenoid extraction rate could have been achieved by increasing the dodecane sparging rate. However, the sparging rate applied (0.286 $L_{dod}\ L_{RV}^{-1}\ min^{-1}$) resulted in the formation of a dodecane emulsion in the aqueous phase after 6 days of operation, as shown in Fig. 4. Dead cell material such as amphipolar membrane components will have acted as surfactants and have lead to strong emulsification of the two-phase system. Consequently, the small dodecane droplets were stabilized and hardly coalesced anymore [8]. These droplets interfered with the turbidity measurements of the system and resulted in an apparent increase of the turbidity, causing an increased dilution rate. Consequently cells were washed out (data not shown). Moreover, due to increasing emulsion formation it was not possible to separate the aqueous and organic phase anymore (Fig. 4d).

With these experiments we showed that it was possible to shift the equilibrium between cell growth and cell death obtained by Hejazi et al. [2] towards a higher cell growth rate (Table 1). The process, however, was not stable on the long run due to emulsion formation. To determine the maximum growth rate of stressed cells and, as such, maximum carotenoid production rates, we performed this experiment again without *in situ* extraction. Emulsion formation was eliminated and it was possible to obtain a constant cell growth rate and stable values for cell concentration, cell size and cellular carotenoid content, as summarized in Table 1.

In this run without *in situ* extraction the medium had to be continuously refreshed with a dilution rate of 0.75 d^{-1} to keep cell numbers constant, giving a net specific growth rate of 0.75 d^{-1}. This is 1.3 times higher than the total growth rate in the first run with *in situ* extraction.

In Fig. 5 the cell concentration in the reactor for this run is shown, together with the cellular carotenoid content and cell size. With a cell concentration of 1.2×10^9 cells L_{RV}^{-1} and a cellular β-carotene

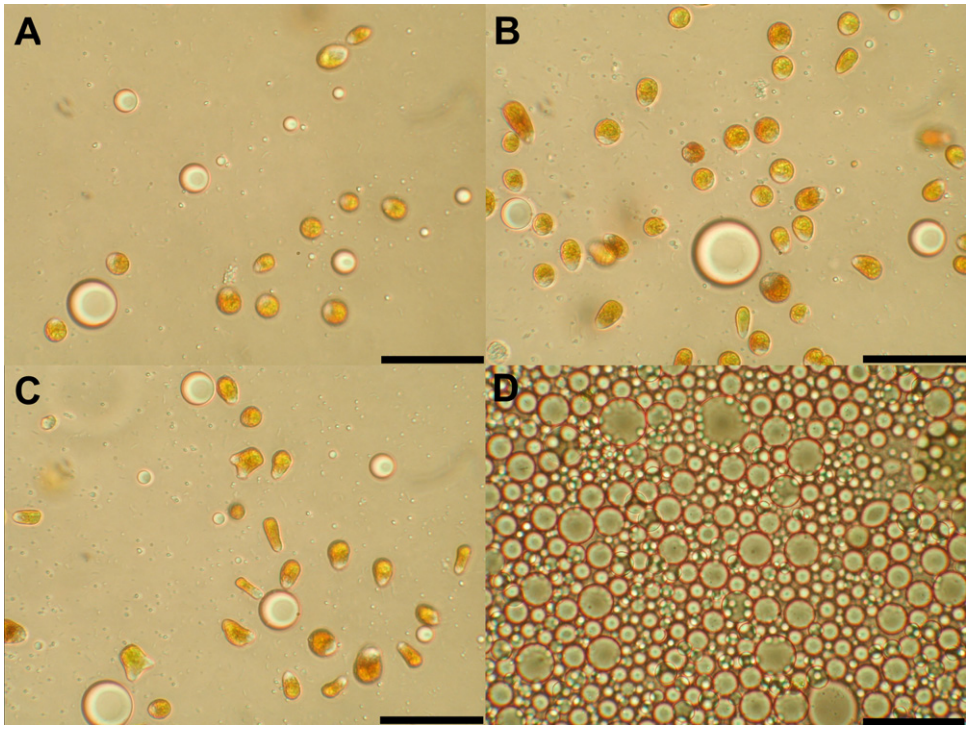

Fig. 4. Bright field microscopy pictures of reactor samples of run 1. (A)–(C) shows stressed cells and dodecane droplets in aqueous phase. (D) shows total emulsion of dodecane in aqueous phase, almost no living cells left. Size bars indicate 50 μm.

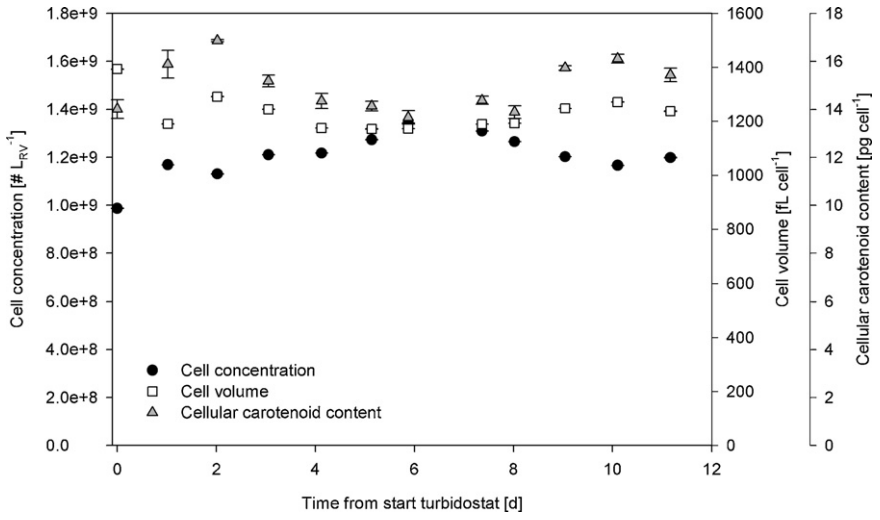

Fig. 5. Cell concentration in the reactor [# L_{RV}^{-1}], cell volume [fL cell^{-1}], and cellular carotenoid content [pg cell^{-1}] in time for run 2: turbidostat mode without *in situ* extraction. Errors bars depict standard deviations obtained from duplicate samples.

content of 15 pg β-carotene cell^{-1} combined with the aforementioned dilution rate of 0.75 d^{-1} a daily volumetric carotenoid production of 13.5 mg β-carotene L_{RV}^{-1} d^{-1} was reached, which was more than half as much as the daily volumetric carotenoid production of 8.3 mg β-carotene L_{RV}^{-1} d^{-1} of run 1 performed with *in situ* extraction.

The differences in daily volumetric productivity for both runs can be explained by differences in cell concentration, carotenoid content and growth rates. For the first run (with *in situ* extraction) the cell concentration was higher than for the second run (without *in situ* extraction), namely 1.6×10^9 cells L_{RV}^{-1} and 1.2×10^9 cells L_{RV}^{-1}, respectively. The intracellular carotenoid concentration was for both runs comparable (12 g L_{CV}^{-1}), but as the cells in the first run were much smaller than in the second run, the cellular carotenoid content was different (8.9 pg cell^{-1} for run 1 and 15.0 pg cell^{-1} for run 2). Moreover, the growth rate for the first run was 0.60 d^{-1}, whereas for the second run it was 0.75 d^{-1}.

The differences between the cell concentration, cellular carotenoid content and growth rates are partly related to a difference in transmitted light. In the first run the light transmitted was 16% of the incoming light, whereas in the second run the transmitted light was 6%. The set points for the light transmitted through the reactor were based in both runs on the cell concentration of 1.0×10^9 cells L_{RV}^{-1} at the start of the turbidostat. However, cell size and cellular carotenoid concentrations differed already at the start of both turbidostats. This might have resulted in differences in stress experienced by the cells and consequently could have induced further changes in cell size and cellular carotenoid content towards the steady state period.

Furthermore, the oxygen partial pressure in the first run (with *in situ* extraction) was very high when compared to the second run (without *in situ* extraction). The oxygen partial pressure in the first run even reached 1.2 bar, equivalent to 5.5 times air-saturated values of oxygen. In the second run oxygen removal was more efficient leading to an oxygen partial pressure of 0.3 bar (equivalent to 1.4 times air-saturation). The absence of aeration and the presence of dodecane in the first run resulted in difficulties in the removal of oxygen from the culture. Although oxygen dissolves very well in dodecane, as the dodecane formed an emulsion it was inseparable from the aqueous phase and the oxygen could not be removed from the dodecane phase. As too high oxygen concentrations lead to photorespiration and photooxidative damage this will have had a diminishing effect on the growth rate [9,10].

Another explanation for the increased volumetric β-carotene productivity in the second run compared to the first run with *in situ* extraction, might be that extracted carotenoids in the organic phase were broken down when the dodecane was sparged through the illuminated zone of the reactor. Carotenoids degrade within days when exposed to light [11,12]. Light-induced carotenoid degradation might have resulted in an underestimation of the carotenoid extraction rate and consequently of the cell death rate.

In both experiments we found steady cell growth for stressed cells, much higher than expected for cells with high carotenoid content. Carotenogenesis in stressed *Dunaliella* cells is related to sub-optimal growth conditions and the specific growth rate of the algal cells is inversely correlated to the carotenoid content of the cells [13,14]. However, it was found before that cells with increased cellular carotenoid concentrations compared to non-stressed cells can still show significant specific growth rates compared to non-stressed cells and, consequently, yield high volumetric productivities [6,15–17]. Leonardi and Cáceres et al. [18] found that stressed cells that were grown at low salt concentrations or under nutrient deprivation started to reproduce sexually instead of vegetative reproduction by longitudinal fission which is the main variant of reproduction for non-stressed cultures. Based on comparisons of our microscopy pictures with literature, we determined forms of both vegetative and sexual reproduction, as can be seen in Fig. 6 [19–21]. Most cells were clearly orange, containing a high intracellular carotenoid concentration, although some green cells remained present as well.

Hejazi et al. [2] found a volumetric productivity of 2.5 mg β-carotene L_{RV}^{-1} d^{-1} for a similar system (Table 1). In the research of Hejazi et al. [2] the cell density remained approximately constant yielding a net cell growth of zero, without the application of an actively controlled turbidostat. Therefore, the total volumetric yield consisted only of the extracted β-carotene in the organic phase and not of β-carotene from the biomass overflow. However, the β-carotene concentration of the biomass increased in time from 3 to 60 mg β-carotene L_{RV}^{-1} in 46 days. If we take this accumulation of carotenoids into account we can deduce an additional productivity of 1 mg β-carotene L_{RV}^{-1} d^{-1} for the work of Hejazi. As a comparison, the maximum volumetric productivity of the turbidostat system used in our study was 8.3 mg β-carotene L_{RV}^{-1} d^{-1} with *in situ* extraction and 13.5 mg β-carotene L_{RV}^{-1} d^{-1} without the combination with *in situ* extraction.

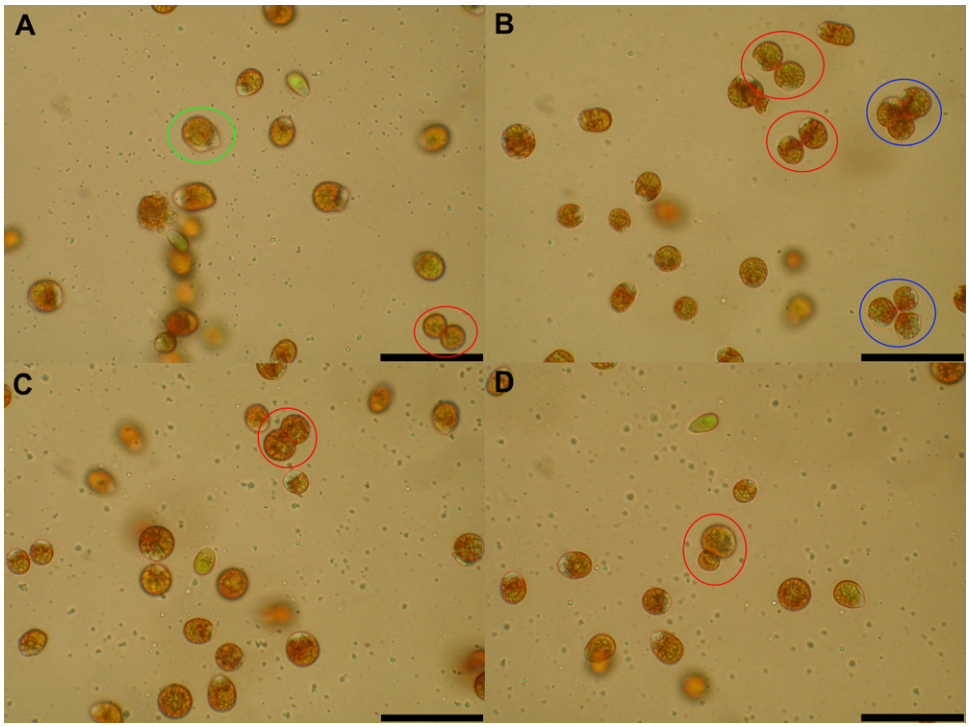

Fig. 6. Bright field microscopy pictures of stressed cells during turbidostat mode of run 2, on days 1 (A), 2 (B), 3 (C) and 5 (D). Most cells appear orange, though some green cells can be seen. In the red circles dividing cells via longitudinal fission are shown, in the green circle a cell with two 'heads' each with 2 flagella (not visible) can be seen, and in the blue circles gamete fusion and zygote forming is depicted. Size bars indicate 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

As we did not optimize the system yet, there is reason to believe that these values can be increased. A lead for optimization is the amount of stress inflicted (incoming light intensity and transmitted light). As was shown by Lamers et al. [6] increasing the light intensity to 1400 $\mu mol\ m^{-2}\ s^{-1}$ in a similar system and with a light transmission of 13% a much higher maximum volumetric β-carotene productivity was reached (37 mg β-carotene $L_{RV}^{-1}\ d^{-1}$). However, after a first increase the dilution rate decreased to zero in 4 days. This decrease in growth is probably related to a higher light intensity applied and the fact that the algae were not allowed to acclimate and increase in concentration after switching to high light. To reach the highest productivity which can be maintained over time an optimum should be found between the incoming light intensity and cell concentration.

4. Conclusions

The turbidostat was a useful system to study the balance between cell growth and cell death. Simultaneously a two-phase system was applied for *in situ* extraction of carotenoids from the biomass. Next to cell death related carotenoid extraction, a high dilution rate was necessary to ensure constant cell numbers. The daily volumetric productivity was 8.3 mg β-carotene $L_{RV}^{-1}\ d^{-1}$, where the major part of this productivity was not achieved by *in situ* extraction, but by the net production of carotenoid-rich biomass. In a turbidostat without *in situ* extraction a daily volumetric productivity of 13.5 mg β-carotene $L_{RV}^{-1}\ d^{-1}$ was reached. In both systems the carotenoids from the produced biomass still have to be extracted. For system 1 increase of the sparging rate was not an option as it would lead to increased emulsion formation, which was already a problem at the here applied rate. Further disadvantages of the two-phase system were the decreased growth rate due to high oxygen concentrations and the light-related carotenoid degradation. Emulsion formation and oxygen accumulation will become even worse after scale up because the ratio between area and volume will be decreased. Hence we can conclude that a two-phase system for carotenoid extraction from *Dunaliella* is not suited for *in situ* extraction, viz the combination of fermentation and extraction in one step, but it might be very suited as down-stream processing step.

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