Milking of microalgae revisited

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This research was conducted under the auspices of the Graduate School VLAG
Milking of microalgae revisited

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Submitted in partial fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 29 October 2010
at 4.00 p.m. in the Aula
Dorinde M.M. Kleinegris
Milking of microalgae revisited

With propositions, and summaries in Dutch and English

ISBN: 978-90-8585-791-4
## Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Mechanism of extraction of β-carotene from microalga <em>Dunaliella salina</em> in two-phase bioreactors</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>The selectivity of milking of <em>Dunaliella salina</em></td>
<td>27</td>
</tr>
<tr>
<td>4</td>
<td>Carotenoid fluorescence in <em>Dunaliella salina</em></td>
<td>45</td>
</tr>
<tr>
<td>5</td>
<td>Mechanism of <em>in situ</em> carotenoid extraction from <em>Dunaliella salina</em></td>
<td>55</td>
</tr>
<tr>
<td>6</td>
<td>Continuous production of carotenoids from <em>Dunaliella salina</em></td>
<td>77</td>
</tr>
<tr>
<td>7</td>
<td>Two-phase systems: potential for <em>in situ</em> extraction of microalgal products</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Summary</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>Samenvatting</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>Dankwoord</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>Curriculum Vitae</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>Training activities</td>
<td>143</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
Algae are of huge importance to the world. More than 40% of the earth’s carbon is fixed by algae and they provide the world with oxygen. Their ecological significance lays in their abundance, extreme biodiversity and capability to live in a variety of aqueous environments, ranging from very extreme ones such as soda lakes, and desert soils, to more moderate environments such as freshwater lakes and saline oceans (Norton et al. 1996). Furthermore mankind profits from algae via direct use. Algae are very attractive for the purpose of producing various compounds as they do not have to compete with cultivated farmland and can make use of waste streams as nutrient source. Although they are not superior to higher plants concerning photosynthetic efficiency, microalgae have high growth rates and give higher oil yields than higher plants such as palm or soybean (Rodolfi et al. 2009).

As there is a huge biodiversity of algae, there are many possible products that can be produced with or extracted from algae. Macro-algae or seaweeds are used for human consumption and for the production of alginates, agar and carrageenan (Gunstheimer and Jahreis 1998; Norton et al. 1996). Micro-algae are more and more of interest for production of various products such as e.g. carotenoids with Dunaliella salina or Haematococcus, health food with Chlorella and Spirulina, and several species for aquaculture usually rich in ω-3 poly-unsaturated fatty acids (Borowitzka 1999; Cardozo et al. 2007; Radmer and Parker 1994). Some examples of production systems are given in Figure 1.1.

Furthermore there is an increased worldwide attention for the production of microalgae because of their potential as biodiesel feedstock producers, as many species are well known for their high lipid content (Dismukes et al. 2008; Schenk et al. 2008).

Carotenoids

As carotenoids play an important role in photosynthesis they are found in almost all higher plants, algae and photosynthetic bacteria. Located in the chloroplast, they contribute to light harvesting, dissipation of excess light energy, scavenging
of triplet chlorophyll and singlet oxygen, and maintaining photosystem structure (Bartley and Scolnik 1995; Demmig-Adams and Adams 2002). With β-carotene as accessory pigment in the chloroplast the range of light that can be absorbed is broadened, as can be seen in Figure 1.2.

There are more than 600 carotenoids classified in nature and all consist of a C40 skeleton. This backbone consists of carbon atoms with alternating single and double bonds. The ends of the backbone can be cyclic and can contain oxygen-based functional groups. The groups without oxygen are called carotenes, such as α-carotene and β-carotene (Figure 1.3), whereas the group with oxygenized derivatives is known as the xanthophylls, with lutein and astaxanthin as well-known examples (Figure 1.3).

The chemical properties and light-harvesting characteristics of the carotenoids are dependent on the molecular structure of their backbone. Carotenoids appear in
the range from yellow to red and typically absorb light from 400 to 500 nm (Armstrong and Hearst 1996; Britton 1995). Due to their capability to quench singlet oxygen and other radicals, carotenoids are well-known anti-oxidants. Moreover, some carotenoids, such as α-carotene and β-carotene, consist of a β-ionone ring, the so called retinyl group. Most omnivore and herbivore animals possess the required enzymes to convert these carotenoids into vitamin A, hence their name pro-vitamin A.

Due to their pro-vitamin A activity and their anti-oxidant capacity carotenoids play an important role in human health. They are considered to prevent cancer and other diseases that are believed to be induced by free radicals, such as atherosclerosis, cataracts, age-related macular degeneration and multiple sclerosis (Edge et al. 1997; Demmig-Adams and Adams 2002).

All these properties make carotenoids a very interesting ingredient for food and feed products, as well as pharmaceutical, cosmetic, or other health care products (Borowitzka and Borowitzka 1988; Lorenz and Cysewski 2000).
Figure 1.3: Molecular structure of β-carotene and astaxanthin.

**Dunaliella salina**

*Dunaliella salina* is used worldwide as the main source of natural β-carotene due to its extreme carotenoid-producing capacity. *D. salina* is a halotolerant, unicellular green alga (Chlorophyta, Dunaliellales). It has two flagella and a large, single chloroplast that comprises most of the cell. If the cells are stressed, viz, by exposure to extreme environmental conditions such as high light intensity, high salinity, low temperature or nutrient deprivation, the cells can grow in size and can accumulate large amounts of carotenoids, up to 10% of their dry weight (Ben-Amotz et al. 1982; Borowitzka and Borowitzka 1988), as shown in Figure 1.4. The chloroplast shrinks, chloroplast membranes decrease in size, and carotenoid containing lipid globules are formed and stored in the interthylakoid spaces of the chloroplast (Ben-Amotz et al. 1982).

Commercial production of β-carotene is done by growing and stressing the algae in open ponds (Ben-Amotz 1995; Borowitzka 1999). During this process, the algae are first grown to obtain a high biomass concentration. Next, the cells are stressed to induce β-carotene accumulation. When high levels of β-carotene are reached, the biomass is harvested and further processed. Either the biomass is dried and directly applied as carotenoid-rich biomass, or the carotenoids are ex-
tracted from the biomass and purified, yielding pure β-carotene as product (Figure 1.4).

**Figure 1.4:** Left: processing scheme of carotenoid production with *Dunaliella salina* in open ponds. Right: *Dunaliella salina* cells at different stages of growth and stress (scale bars indicate 50 μm).

**In situ extraction in organic-aqueous two-phase systems**

Hejazi *et al.* (2002) developed a new method for the simultaneous production and extraction of carotenoids from *Dunaliella salina* in a closed system. After a biomass production step, the algae are stressed to produce and accumulate a high quantity of carotenoids in oily globules. At the same time organic phase (dodecane) is added to the culture. The carotenoids are extracted into the upper, organic, phase while the biomass remains in the aqueous phase. This process could be run for over six weeks, with constant biomass density and increasing levels of extracted carotenoids (Hejazi *et al.* 2004a). Moreover, the first production step in which the biomass is grown does not need to be continuously repeated, yielding an increased overall volumetric productivity of carotenoids.

The mechanism of this *in situ* extraction process was never unravelled. The process was described only at macroscopic level and it remained unclear whether in the extraction process carotenoids were released from the cells into the water phase and extracted from the water phase or whether direct cell-solvent contact was required.
It is important to understand the mechanism of extraction to develop an effective \textit{in situ} extraction process. This is not only of importance for carotenoid extraction from \textit{Dunaliella salina} but also for extraction of oils from different algae. In the case of biodiesel production from algal oil it was shown that this will only be economically feasible if several components from the algae are extracted and fractionated while maintaining their functionality (Wijffels \textit{et al.} 2010). In such a biorefinery, mild extraction techniques, such as the \textit{in situ} extraction process will be very important.

In this thesis we report on the research of this \textit{in situ} two-phase extraction process further with special attention to the actual mechanism behind the extraction process. The consequences of understanding the mechanism for \textit{in situ} carotenoid extraction are analyzed. Finally we discuss the possibilities of \textit{in situ} extraction for different algal products.
Outline of thesis

In chapter 2 a first study on the mechanism of the extraction of β-carotene from Dunaliella salina cells is described. Macro-scale and micro-scale experiments were used to gain insight in the extraction process and two possible hypotheses describing the mechanism were formulated.

In chapter 3 the selectivity of in situ extraction is discussed. By varying stress conditions (e.g. low temperature or high light intensity) the profiles of carotenoids produced by three different Dunaliella strains were changed. We compared the profiles of produced and extracted carotenoids and chlorophylls in the cells and the organic phase for each situation.

Chapter 4 describes a method for visualization of the carotenoid globules inside the cells. Endogenous fluorescence from isolated carotenoid containing globules was measured and their intracellular presence was determined by confocal laser scanning microscopy.

In chapter 5 the mechanism of the extraction process was studied again in order to formulate a final hypothesis. The visualization method described in chapter 4 was used, together with macro-scale extraction experiments in small reactor flasks. Next to dodecane as organic solvent, the combination of dodecane and very small amounts of the more toxic solvent dichloromethane was studied.

Chapter 6 shows the productivity of in situ extraction in an aqueous organic two-phase system operated as turbidostat. The turbidostat was used to keep stressed cell numbers constant in order to study the equilibrium between cell growth and extraction related cell death. As a reference we compared the volumetric productivity of this in situ extraction experiment with a continuous turbidostat experiment without extraction, so without the addition of an organic phase.

In chapter 7 we describe the possibilities of in situ extraction with two-phase systems and discuss its application for future algal cultivation combined with product extraction.
Chapter 2

Mechanism of extraction of β-carotene from microalga *Dunaliella salina* in two-phase bioreactors

Abstract

We show that it is possible to extract β-carotene selectively from *Dunaliella salina* in two-phase bioreactors. The cells continue to produce β-carotene and the extracted part is substituted by newly produced molecules. This process is called 'milking'. We performed several experiments to understand the exact mechanism of the extraction process. The results show that direct contact between the cells and the biocompatible organic solvent was not a requirement for the extraction but it accelerated the extraction. Electron microscopy photographs showed an undulated shape of the cell membrane and a space between the cell and the chloroplast membrane in the cells growing in the presence of dodecane (a biocompatible solvent). Extra-chloroplast β-carotene globules located in the space between the cell and the chloroplast membranes were observed in these cells as well. It was shown that dodecane was taken up by the cells. The concentration of dodecane in the cells was about 13 pg cell⁻¹. It can be concluded that dodecane uptake by the cells is responsible for the morphological changes in the cells and leads to more activity in the cell membrane. The results suggest two possible modes of extraction. One of the mechanisms is transport of the globules from the chloroplast to the space between the cell and the chloroplast membranes and subsequently from there to the outside by exocytosis. Another possible mode for the extraction could be release of β-carotene from the globules as a result of alterations in the membrane in response to the uptake of dodecane. β-Carotene molecules diffuse from the chloroplast to the space between the cell and the chloroplast membranes and from there to the medium either by diffusion or by exocytosis after accumulation in the vesicles.

**Introduction**

*Dunaliella salina* is a unicellular microalga with the ability to produce and accumulate β-carotene (Ben-Amotz 1995). β-Carotene is an orange pigment with several applications in food, pharmaceutics, and cosmetics. β-Carotene is one of the photosynthetic pigments and is produced in the thylakoid membranes in the chloroplast. It has two different functions in the cells: 1) a role in energy transfer in the photosystem of the cells (Tanada 1951), and 2) a role in the protection of the cells against oxidation (Lers *et al.* 1990). The production of β-carotene is enhanced by stress factors (e.g. high light intensity, high salinity, and nutrient deficiency) and overproduced β-carotene is accumulated in oil globules in the cells (Rabbani *et al.* 1998).

Whole-cell biocatalysis in two-phase bioreactors is used for the production of high-value metabolites with a greater affinity to another phase, immiscible with the aqueous cell phase. Application of this system in microalgal biotechnology has been reported previously. Frenz *et al.* (1989a) collected a substantial fraction of hydrocarbons (hydrophobic hydrocarbons) from the microalga *Botryococcus braunii*, by exposing the biomass (cells) for a short time to hexane.

We have shown that it is possible to extract β-carotene selectively from *D. salina* in two-phase bioreactors (Hejazi *et al.* 2002). In this process the cells are first grown and then stressed to produce larger amounts of β-carotene. In the stress stage the biocompatible organic phase is added and β-carotene is extracted selectively. The solvent is continuously re-circulated through the aqueous phase containing the cells. The cells continuously produce β-carotene and newly produced molecules substitute the extracted part. Due to the substitution process it is possible to produce larger amounts of β-carotene than in the commercial production process. This process is called ‘milking’ (Hejazi *et al.* 2004a). The exact mechanism of this extraction process is not known. Understanding this mechanism would be important in optimization of the process and its extension for production of other high-value compounds from microalgae.

Our previous results showed that the extraction rate was affected at least by two parameters: mixing rate of the aqueous and organic phases in the bioreactor (Hejazi *et al.* 2003a) and β-carotene content of the cells (Hejazi *et al.* 2003b). These results suggested that interaction between the solvent and the cell
membrane as well as β-carotene fraction and location inside the cells could play an important role in the extraction. The membrane of *D. salina* is continuously active in an endo- and exocytosis process (Ginzburg *et al.* 1999). During this process the medium in which the algae grow is taken up in small vesicles. After a few seconds the vesicles are released again. We think this physiological property of the cells might play a role in the extraction process.

In this article we will look in more detail into the mechanism of the extraction process. We studied whether direct contact between the cells and the organic solvent is important for extraction. The effect of the presence of the organic phase on the distribution and location of β-carotene globules was determined. In addition, we looked to the effect of the solvent on the cells and, more specifically, the cell membrane. For this study we performed experiments in bioreactors and analyzed the cells with microscopic techniques.

**Materials and methods**

**Cell preculture**

*Dunaliella salina* (19/18, CCAP) was cultivated in three Erlenmeyer flasks of 50 mL each. The alga was grown in a climate cabinet at a temperature of 20°C and a light intensity of 50-80 μmol photons m$^{-2}$ s$^{-1}$. Every week new Erlenmeyers were inoculated with 1 mL of the old cultures. The medium contained 1 M NaCl, 5 mM KNO$_3$, 0.5 mM Na$_2$PO$_4.2H_2$O, trace elements (5 mL L$^{-1}$ of stock solution containing 12.3 mM Na$_2$EDTA.2H$_2$O, 4.66 mM FeCl$_3.6H_2$O, 42.0 mM CuSO$_4.7H_2$O, 60.6 mM ZnSO$_4.7H_2$O, 17.0 mM CoCl$_2.6H_2$O, 366 mM MnCl$_2.4H_2$O, and 1.04 mM Na$_2$.MoO$_4$), and 25 mmol Tris buffer. The pH of the medium was adjusted to 7.5 by 3 M HCl. Before inoculation the medium was autoclaved for 40 min at 121°C. To avoid precipitation, the phosphate was autoclaved separately.

**Contact between cells and organic phase during extraction**

We performed experiments to investigate whether direct contact between the cells and the organic phase is a requirement for the extraction. Four small bottles (100 mL) were filled with 40 mL of cell culture (suspended or immobilized). For immobilization, the cell suspension was added to a 2% agarose (ultra-low gelling
temperature) solution at a temperature of 38°C and hardened after cooling as a layer in the bottles. The final concentration of agarose in the layer was 0.5%. On top of the layer either 25 mL artificial seawater and 20 mL biocompatible solvent (dodecane) (Hejazi et al. 2002) or only 20 mL dodecane was added to bring the cells in indirect or direct contact with organic solvent. Bottles containing suspended cells were mixed continuously to bring the cells in contact with the organic phase, or left to sink to the bottom to prevent them from direct contact with the dodecane. The bottles were illuminated from the side and the upper part of the bottles (organic phase) was covered with aluminum foil to prevent the extracted β-carotene from degradation (Hejazi et al. 2003a).

Every 3 days samples of 1 mL were taken from the organic phase to measure the β-carotene and chlorophyll concentration spectrophotometrically. The samples were returned after measurement. At the end of the experiment microscopic samples were prepared and observed by light microscopy.

**Electron microscopy (EM)**

The aim of this experiment was to determine the molecular and phase effects of the solvent on the ultrastructure of the cells. Three 1 L bottles containing 300 mL of culture media were inoculated with 20 mL cell suspension of a preculture. This medium had about the same composition as used for the precultures. However, to obtain faster cell growth phosphate and nitrate concentrations were doubled (10 mM KNO₃ and 1 mM NaH₂PO₄·2H₂O). In addition, 3 mL of sterilized carbon source (0.5 M NaHCO₃) was added to avoid carbon limitation. The solid carbon source (NaHCO₃) was sterilized in an oven at 120°C overnight and then dissolved in sterilized water.

The cells in each bioreactor were grown under different circumstances. The cells in the first reactor were grown in only aqueous phase (medium). In two other reactors dodecane was added. To separate molecular and phase effects of the organic solvent on the cells the second and third bioreactor were run under two different conditions. In the second reactor the aqueous phase was saturated with a biocompatible organic solvent. To be sure that the aqueous phase was saturated with dodecane, we added dodecane drop wise until the moment that the droplets of dodecane appeared on top of the medium. The total volume of dodecane added in this way was 1 mL. The third reactor contained 80% aqueous phase and 20% organic phase (75 mL). Dodecane was used as a biocompatible
organic phase. The temperature was controlled using a water bath and kept at 25°C. The bottles were illuminated with fluorescent lamps (Sylvania CF-EL 55W/840) with an average light intensity of 700 μmol photons m⁻² s⁻¹ (measured with a light meter: Licor model Li-250). Mixing of the culture media and the organic phase was done with two different mixers, fixed on one axis. A magnetic stirrer (diameter 6.0 cm, bar width 1.0 cm) was placed 0.6 cm from the bottom. The second stirrer was placed in the interface between the water phase and the dodecane. This was a six-bladed turbine stirrer (diameter 3 cm, paddle width and height 0.5 cm). Temperature and pH were controlled at 25±0.5°C and 7.8±0.2, respectively.

The cells, which were obtained from the three bioreactors, were concentrated by centrifugation and fixed for 30 min in 0.2% glutaraldehyde. Further fixation with aldehydes and osmium tetroxide, dehydration, and embedding in LR White (London Resins Co.) was essentially as described by van Lent et al. (1990). Ultrathin sections of ~60 nm were cut and mounted on 150 mesh copper grids covered with a formvar film. Sections were stained for 5 min with 2% (w/v) uranyl acetate and lead citrate according to Reynolds (1963). Sections were observed and photographed with a Philips CM12 transmission electron microscope.

To observe the effect of direct contact of pure dodecane on the cells a part of the harvested cells from the two-phase bioreactor was centrifuged and the pellets were suspended in pure dodecane for 5 min. Afterwards, electron microscopy samples were prepared using the abovementioned procedure.

**Confocal laser scanning microscopy (CLSM)**

Although carotenoids have been considered nonfluorescent for a long time, weak fluorescence from all-trans β-carotene in fluid solution at ambient temperature has been shown (Jorgensen et al. 1992). Chlorophyll fluorescence is very well known. Therefore, in addition to electron microscopy the CLSM technique was applied to investigate the location and distribution of the cell pigments (chlorophyll and β-carotene) in green and orange (stressed) cells of *D. salina*. The cells were stabilized with a lugol solution. The argon ion laser was excited at wavelengths of 450 and 480 nm, since the absorption spectrum of β-carotene has peaks at these wavelengths. At these wavelengths chlorophyll is autofluorescent.
as well. Green and red fluorescence, from β-carotene and chlorophyll, respectively, was detected with a photomultiplier tube. From these data 3D pictures were formed with the computer and software packages LSM Image Browser (Zeiss) and 3D Viewer (Zeiss).

**Determination of dodecane concentration in the medium**

In order to determine whether dodecane is taken up by the cells, 50 mL cell suspension samples from a two-phase bioreactor and a bioreactor without dodecane (as a control) were taken. To determine the solubility of dodecane in the fresh medium, dodecane was also added to a bottle containing fresh medium. It was left for 24 h to saturate. Then samples were taken from the lower part of bottle (saturated fresh medium) and analyzed for dodecane concentration. The concentration of dodecane in the medium was determined with a gas chromatograph (GC), based on the method described by Kropp et al. (2000). Extraction of dodecane from the samples was performed by addition of 15 mL of dichloromethane to the samples three times. The samples were then centrifuged (3 min, 1500 rpm) and decanted. Ten μL tetradecane was added as an internal standard to each sample. The extraction samples were pooled and concentrated in a rotary evaporator (40°C, no vacuum) to volumes of ~2 mL. The analysis was performed using an HP-5 column and a flame ionization detector. The temperatures of the injector and detector were 200°C and 250°C, respectively. The oven was held at 90°C for 2 min before the temperature was increased at 4°C/min to a temperature of 200°C. Helium was used as the carrier gas.

**β-Carotene and chlorophyll analysis**

β-Carotene and chlorophyll concentrations were determined spectrophotometrically. A sample of 1 mL was taken from the organic phase and the absorbance was measured at the wavelength maxima for β-carotene and chlorophyll and compared with standard curves (Hejazi et al. 2002).

**Fatty acid analysis**

The fatty acid composition of the cells growing under different circumstances was determined using the method described by Rodriguez-Ruiz et al. (1998). A 150 mL of cell suspension was harvested from each of the reactors and centrifuged
(10 min, 10,000 rpm). The pellet was resuspended in 5 mL of the supernatant, moved to reagent tubes, and centrifuged again (8 min, 2500 rpm). A 3 mL solution of methanol and acetyl chloride in a ratio of 20:1 (v/v) and 1.5 mL hexane were added to the pellet and mixed. The total solution was heated in a heating block at 100°C for 30 min. Every 10 min the solution was carefully shaken. After 30 min the reagent tubes were cooled to ambient temperature. Two phases were separated from each other. The top layer was hexane containing the fatty methyl esters. This layer was moved to GC tubes and analyzed overnight with a GC using a CP-SIL-88 column and a flame ionization detector. The injector and detector temperature were 250°C and 270°C, respectively, and the oven was held at 160°C for 4 min before its temperature was increased at 4°C/min to 220°C. Hydrogen (125 Pa) was used as carrier gas.

Results and discussion

Contact between cells and organic phase during extraction
This experiment was performed to investigate whether direct contact between the cells and organic phase is a requirement for extraction of carotene or the extraction takes place via the aqueous phase to the organic solvent. We conducted a series of experiments in small bottles with suspended and immobilized cells in direct and indirect contact with the organic phase. Immobilized cells were used to prevent contact between cells and the organic phase. To show that the extraction was not a result of the contact between dead cells and organic phase the amount of chlorophyll present in the solvent phase was measured as well.
As shown in Figure 2.1, in all bottles extraction took place. However, the extraction from cells in direct contact with the solvent was higher than in the case of indirect contact. This means that direct contact between the cells and the organic solvent accelerates the extraction process, and thus is an important parameter in an efficient extraction process. The results are in agreement with our previous results, which showed that the extraction rate was enhanced by an increase of the mixing rate (Hejazi et al. 2003a). No detectable amounts of chlorophyll were found in any of the samples, showing no damage of cells during the extraction.
Cell characteristics

To investigate ultrastructural features of the cells growing under different circumstances, cells were observed with electron microscopy. The effect of both carotenogenesis and the organic phase on the accumulation and location of $\beta$-carotene containing globules in the cells were investigated. We also studied the effect of the organic phase on the cell membrane. The cells growing in the three bioreactors in the presence of organic phase, in the saturated aqueous phase, and in the absence of organic solvent were studied.

Effect of carotenogenesis on the ultrastructure of the cells

Obvious differences were visible between green and orange cells. Green cells in all cases showed a large chloroplast and a very small amount of $\beta$-carotene containing lipid globules inside the chloroplast (Figure 2.2). When carotenogenesis started, in all cells (in the presence or absence of dodecane) more of these globules appeared. This effect has also been described in the literature (Ben-Amotz 1995; Lers et al. 1990; Rabbani et al. 1998). The globules containing $\beta$-carotene were located around thylakoid membranes and around the outer membrane of the chloroplast.

Figure 2.1: $\beta$-Carotene extraction from *D. salina* in direct and indirect contact with the organic phase (dodecane).
We determined the fluorescence of the cells by CLSM. With CLSM it is possible to follow the changes in distribution of the cell pigments in different physiological stages (non-stressed and stressed cells). The cells were excited at 450 and 480 nm and green and red fluorescence could be detected. Green fluorescence was related to β-carotene and red fluorescence was from chlorophyll. The non-stressed cells showed very strong red fluorescence which covered almost the whole cell (*D. salina* has a very big chloroplast) and almost no green fluorescence. In the stressed cells, however, the red fluorescence was not distributed homogeneously in the chloroplast anymore. It was seen as spots in different places in the chloroplast. In the stressed cells strong green fluorescence

Figure 2.2: Electron microscopy picture of non-stressed (A) and stressed (B) cells, grown in absence of dodecane. Left: whole cell, Right: enlarged part (white rectangle in whole cell) 1: pyrenoid with surrounding starch molecules, 2: vacuoles 3: nucleus, 4: chloroplast, 5: globules between thylakoid membranes of chloroplast.
around the cell membrane was detected (Figure 2.3). Since β-carotene is fluorescent in lipophilic environments (Jorgensen et al. 1992), we assumed the fluorescence came from β-carotene accumulating in oil globules. This together with electron microscopy pictures showed that in stressed cells most of the β-carotene-containing globules were located in the outer part of the chloroplast. This part of β-carotene seems to be available for extraction. The changes in red fluorescence of chlorophyll after stressing the cells confirmed the damage in the photosystem, which has been reported before (Ben-Amotz 1995; Lers et al. 1990; Rabbani et al. 1998).

Figure 2.3: CLSM of non-stressed (left) and stressed (right) cells of *D. salina*. Green and red fluorescence indicate β-carotene and chlorophyll, respectively.

Electron microscopy pictures showed that both gray and white globules were present. This means that the globules are probably filled with different compounds. There are three possible explanations for the color difference: uptake of dodecane by the cells, a difference in the composition of fatty acids, and a difference in β-carotene concentration. Since white globules were also seen in the cells growing in the absence of the organic phase, we do not think the effect was caused by dodecane uptake.

It is known that the production of fatty acids by *D. salina* is related to cultivation conditions such as salt concentration and light intensity (Azachi et al. 2002; Mendoza et al. 1999). The fatty acid composition of the cells grown under different circumstances was determined to investigate the possible effect of the
Mechanism of extraction

presence of dodecane on the fatty acid composition. The results showed that all the samples contain about the same composition of fatty acids (results not shown). We therefore think that the difference in the color is because of a difference in β-carotene concentration in the globules.

**Effect of the organic solvent on the ultrastructure of the cells**

Two main differences were noticed between the stressed cells growing in the presence or absence of dodecane. The first difference was observed in the cell membrane. The membrane of the cells growing in the presence of the organic phase and in the saturated phase showed an undulated shape. A space between the cell and the chloroplast membranes was observed in these cells as well (Figure 2.4). This space is not visible in the cells growing in the absence of the organic phase. This indicates that the cell membrane is more active when the cells are treated with the organic solvent.

![Figure 2.4: Parts of a cell growing in the presence of dodecane (a biocompatible organic solvent) which shows active cell membrane (1) and wider space between the cell membrane and chloroplast membrane (2). 3: Shows chloroplast membrane.](image)

The second observation was the location of the β-carotene globules. According to the literature β-carotene accumulation occurs within oil globules inside the chloroplast (Ben-Amotz et al. 1982). Our EM pictures showed that all the β-carotene globules were located inside the chloroplast next to the thylakoid
membrane when cells were grown in the absence of the organic phase (Figure 2.2). In the cells grown in the presence of the organic phase and in the medium saturated with solvent, however, some globules were observed in the space between the cell and the chloroplast membranes, which became wider. These globules were in contact with the cell membrane and looked like they were being extracted. When the cells were brought in contact with pure dodecane, the movement of the globules towards the outside of the chloroplast and in the direction of the outer cell membrane was observed more clearly (Figure 2.5).

Figure 2.5: The space between the cell and chloroplast membrane in the cells of *D. salina* in which β-carotene globules (D) are observed. Part of cells: growing in saturated phase (A), in the presence of dodecane phase (B), and in the presence of dodecane phase brought to direct contact with dodecane (C) are shown.
Our previous results showed that β-carotene extraction in the milking process is selective (Hejazi et al. 2002). Lers et al. (1990) showed that β-carotene is the main pigment of the globules and they have almost no chlorophyll. Apparently the extracted β-carotene comes from the globules.

**Mechanism of extraction**

The results indicate that the extraction occurs in cases of direct and indirect contact between the cells and organic phase. Figures 2.4 and 2.5 show some morphological changes in the cells grown in the presence of dodecane. These together with the literature (Sikkema et al. 1995) suggest that the extraction process is most probably accompanied by uptake of the solvent molecules by the cells. To prove this uptake, a set of experiments was carried out. As can be seen in Figure 2.6, the amount of dodecane in the fresh medium saturated with dodecane was not detectable, whereas in medium containing the cells and dodecane detectable amounts of dodecane present. The concentration of dodecane in the cells was about 13 pg cell⁻¹. This indicates that molecules of dodecane are absorbed by the cells. Literature studies suggest that the cell membrane would be a first place that dodecane can accumulate (McIntosh et al. 1980). It seems that dodecane subsequently diffuses into the space between the cell and chloroplast membranes (as shown in Figure 2.4).

![Figure 2.6: Dodecane content of the fresh medium without cells saturated with dodecane (medium), The cells growing in the absence of dodecane (AP), and the cells growing in the presence of dodecane (OP).](image-url)
As a result, two possible mechanisms for the extraction of β-carotene in the milking process are suggested: 1) the globules of β-carotene are moved from the chloroplast to the space between the cells and chloroplast membranes (Figure 2.5) and afterwards released from there to the medium by exocytosis; and 2) molecules of β-carotene are released from the globules as a result of alterations in the membranes of the globules in response to the uptake of dodecane. The molecules diffuse from the chloroplast to the space between the chloroplast and cell membranes. From there the molecules either directly diffuse to the medium or first are accumulated inside the vesicles (Figure 2.7) and then released from the cells by exocytosis.
**Conclusion**

Although direct contact between the cells and organic phase is not a requirement for extraction, extraction is enhanced by direct contact. Dodecane uptake by the cells causes changes in the ultrastructure of the cells. The cell membrane is more active in the presence of the biocompatible organic phase. We think that one of the mechanisms of the extraction of β-carotene is movement of the globules from the chloroplast membranes and subsequently from there to outside by exocytosis. Another possible mode for extraction could be the release of β-carotene from the globules as a result of alteration in the membrane of the globules. β-Caratene molecules subsequently diffuse from the chloroplast to the space between the cell and the chloroplast membranes and from there to the medium either by diffusion or by exocytosis after accumulation in vesicles.

**Acknowledgements**

We thank Dr. Jan van Lent, Laboratory of Virology, Wageningen University, for help with electron microscopy, Mr. Jan Willem Borst for help with CLSM techniques, and Ir. Rouke Bosma for fatty acid analysis.
Chapter 3

The selectivity of milking of *Dunaliella salina*

Abstract

The process of the simultaneous production and extraction of carotenoids, milking, of *Dunaliella salina* was studied. We would like to know the selectivity of this process. Could all the carotenoids produced be extracted? And would it be possible to vary the profile of the produced carotenoids and, consequently, influence the type of carotenoids extracted? By using three different *Dunaliella salina* strains and three different stress conditions we varied the profiles of the carotenoids produced. Between *Dunaliella bardawil* and *D. salina* 19/18 no remarkable differences were seen in the extraction profiles, although *D. salina* 19/18 seemed to be better extractable. *D. salina* 19/25 was not ‘milkable’ at all. The milking process could only be called selective for secondary carotenoids in case gentle mixing was used. In aerated flat-panel photobioreactors extraction was much better, but selectiveness decreased and also chlorophyll and primary carotenoids were extracted. This was possibly related to cell damage due to shear stress.

Introduction

Carotenoids, a group of natural, fat-soluble, yellow to red pigments, are mainly found in plants, amongst which algae, and photosynthetic bacteria, where they play an important role in photosynthesis. Carotenoids protect the cell from damage by light and oxygen. Due to its capacity of accumulating large amounts of carotenoids, the alga *Dunaliella salina* is used worldwide as main source of natural β-carotene to meet with the increasing demand of β-carotene by food, pharmaceutical and cosmetic industries.

Hejazi *et al.* (2002) developed a new method for the simultaneous production and extraction of carotenoids from *Dunaliella salina*. In a two-phase bioreactor, cells are cultivated under stress conditions so that they start to produce and accumulate a high quantity of carotenoids in oily globules. The carotenoids are extracted from the cells to the organic phase in a continuous process, the so-called milking of microalgae. Since mainly carotenoids are extracted and not other cell components, Hejazi *et al.* (2004a) called the process selective. We would like to know the selectivity of this milking process. Can all the carotenoids that are produced be extracted? And is it possible to vary the profile of the produced carotenoids and, consequently, influence the kind of carotenoids extracted?

To obtain a better insight into the selectivity of the milking process, we used three strains of *Dunaliella salina*, i.e. *D. bardawil*, *D. salina* CCAP 19/18, and *D. salina* CCAP 19/25. *D. salina* CCAP 19/18 was chosen because the original milking process was based on this strain (Hejazi *et al.* 2002). *D. bardawil*, deposited at Culture Collection of Algae and Protozoa (CCAP) as *D. salina* CCAP 19/30, was selected because of its manifold applications by other researchers (e.g. Ben-Amotz *et al.* 1982). *D. salina* CCAP 19/25 was chosen because of its production of relatively high amounts of zeaxanthin (Yokthongwattana *et al.* 2005).

Next to the use of different strains, we also varied the type of stress applied, namely, high light intensity or low temperature (with and without the combination with high light intensity). We expected these different stress factors to result in the expression of different carotenoid profiles in the cells; for example Ben-Amotz (1996) found that, at low temperature, more 9-cis β-carotene is produced than all-trans β-carotene in *D. bardawil*. The physical-chemical properties of 9-cis β-carotene (high solubility in hydrophobic solvents and lack of crystal formation)
Selectivity of extraction

may result in different extraction kinetics than for all-trans β-carotene.

Materials and Methods

Strain and culture medium

*D. bardawil* was kindly provided by Dr. Ami Ben-Amotz (Nature Beta Technologies). *D. salina* CCAP 19/18 and *D. salina* CCAP 19/25 were obtained from CCAP (Oban, UK).

Stock cultures of the algae were grown in a culture medium containing 1.00 M NaCl, 9.95·10^{-4} M Na_{2}H_{2}PO_{4}.2H_{2}O, 3.78·10^{-2} M KNO_{3}, 2.25·10^{-2} M Na_{2}SO_{4}, 1.00·10^{-2} M NaHCO_{3}, 4.87·10^{-3} M K_{2}SO_{4}, 3.68·10^{-4} M MgCl_{2}.6H_{2}O, 1.89·10^{-5} M CaCl_{2}.2H_{2}O and 1.13·10^{-5} M FeEDTA. In addition, the medium contained micronutrients: 1.94·10^{-5} M Na_{2}EDTA.2H_{2}O, 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_{2}MoO_{4}, and 9.95·10^{-9} M CoCl_{2}. The solution was buffered with 4.00·10^{-2} M HEPES acid and adjusted to pH 7.5 with 3 M NaOH.

The medium was sterilized at 121°C for 30 min. The phosphate, magnesium, calcium, and carbonate salts were separately sterilized and added after autoclaving to avoid precipitation.

All strains were maintained as suspended culture in 250 mL Erlenmeyer flasks containing 100 mL of medium. Cultures were kept in a growth cabinet at 25°C under continuous light with an intensity of 120-150 μmol photons m^{-2} s^{-1} and were continuously shaken. The headspace consisted of an air/CO_{2} ratio of 95/5%. Every week, 10 mL of a culture was transferred to a new flask containing fresh medium.

Cultivation systems

The first set of extraction experiments was carried out in 1 L glass bottles containing 300 mL of culture medium (as described above, but without the addition of HEPES buffer). The bottles (diameter 9 cm) were equipped with two magnetic stirrers fixed on one axis, stirring at a rate of 110 rpm. The lower stirrer (diameter 6.0 cm, width 1.0 cm) was placed 0.5 cm from the bottom and the upper one (six-bladed turbine stirrer, diameter 3 cm, paddle width and height 0.5 cm) was placed at the surface of the cell suspension (liquid height 6 cm). The pH
was continuously measured and adjusted by giving short pulses of carbon dioxide to the reactors. The bottles were illuminated from the bottom side with fluorescent lamps (Sylvania CF-EL 55W/840) with an average light intensity of 109±10 μmol photons m⁻² s⁻¹ for the growth period. During light stress, light intensity was increased to 800±67 μmol photons m⁻² s⁻¹ from the bottom and an extra light source (slide projector with halogen lamp) was placed at the side of the reactor to reach higher light intensities (1899±708 μmol photons m⁻² s⁻¹ additional illumination from one side). The bottles were placed in a large water bath to regulate temperature at 25.0±0.3°C for the growth period and light stress and 16.5±1.5°C for cold-stress.

The second set of extraction experiments was carried out in flat-panel FMT150 photobioreactors (PSI, Brno, Czech republic). These panel bioreactors have a width of 10 cm, depth of 2.5 cm, and height of 19 cm, containing 300 mL of culture medium (as described above, but without the addition of HEPES buffer). Cell suspension was stirred by aeration (0.3 L min⁻¹). The pH was online-measured and continuously adjusted by giving short pulses of carbon dioxide to the reactors. The bottles were illuminated from one side with light-emitting diodes (red: 627 nm and blue: 455 nm) with an average light intensity of 100±1 μmol photons m⁻² s⁻¹ for the growth period. During light-stress, light intensity was increased to 1700±22 μmol photons m⁻² s⁻¹. Temperature was regulated via a Peltier element, directly cooling the bottom side of the bioreactor. Temperature was maintained at 25.0±0.1°C for the growth period and for the cold-stress: *D. bardawil* at 14.0±0.1°C and *D. salina* 19/18 at 17.0±0.1°C.

Reactors were inoculated with a 1-week-old stock culture to a concentration of approximately 7·10⁵ cells mL⁻¹. When a cell number of more than 1·10⁶ cells mL⁻¹ was reached in the reactor, stress conditions were applied. Per strain, seven cultivations were performed. One control without stress or dodecane, two with light stress, two with cold stress and two with light and cold-stress (for each stress one with and one without dodecane). In the first set of experiments, dodecane (75 mL) was added to milk the cells from the start of stress. Dodecane was gently mixed with the aqueous phase by means of the upper stirrer, as described before. The dodecane layer was shielded from illumination from the side by an aluminum foil cover around the reactor.

In the second set of experiments, the dodecane could not be added at the same time as stress was induced. The aeration caused the dodecane layer to emulsify.
and spread through the whole reactor. Consequently, the dodecane with extracted carotenoids would be in the illuminated zone and extracted carotenoids could be broken down, which would influence our results. To overcome this problem, dodecane was added at the end of the experiment, after lights were shut off. Emulsification still appeared, but carotenoids were no longer exposed to light. Time was limited to 2 h, to prevent the cells from overgrowing stress, since cells were no longer light-stressed.

For the first set of experiments, we made use of all three algae strains, *D. bardawil*, *D. salina* 19/18, and *D. salina* 19/25. For the second set of experiments, we only made use of *D. bardawil* and *D. salina* 19/18. *D. salina* 19/25 was left out because the first set of experiments already showed that milking this strain did not give good results.

**Analytical procedures**

Cell growth and stress were regularly followed during cultivation by optical density measurements at 530 nm and at 680 nm on a spectrophotometer (Spectronic® 20 Genesys, Spectronic Instruments, UK), measured against a reference of demineralised water. Cell number and volume were measured with a Beckman Coulter Multisizer 3 (100 μm orifice; Beckman Coulter, Fullerton, CA, USA). The diameter of the cells was calculated from their volume.

The dry weight of the cell suspension was determined at the end of the cultivation, as described by Zhu and Lee (1997) with adaptations as described. Five mL of cell suspension was washed three times with 25 mL ammonium formate (1 M) over a pre-dried and weighed filter (pre-combusted (450°C, 2 h) glass fibre filter, Whatman GF/F, retention size 0.7 μm). Filters were dried in an oven for 24 h (98°C), allowed to cool down in a dessicator for at least 2 h and reweighed. Measurements were performed in triplicate.

**Pigment analysis**

Samples were taken at the end of the stress period. Five mL of cell suspension was centrifuged for 10 min at 2500 rpm and at 4°C. The dodecane was divided over freeze-drying flasks (10 mL per flask) and stored at -80°C. All dodecane samples were freeze-dried after the last run of each set-up. Freeze-drying was performed in a Christ Epsilon 2-60 freeze-dryer (Salmenkipp, Breukelen, The
Netherlands) to obtain a pellet of the extracted substances. Frozen dodecane samples were transferred from -80°C to the pre-cooled freeze-dryer (-20°C). Next, vacuum was started (1.03 mbar) and the samples were freeze-dried for 20 h at -20°C. After this a second drying step was performed at -10°C for at least another 20 h and at 0.001 mbar. After all dodecane was evaporated, the flasks containing pellets were again transferred to a freezer (-80°C) awaiting the next steps of pigment analysis.

The extraction of lipids was performed according to the method of Fraser et al. (2000), with adaptations described. Whenever possible, all manipulations were carried out on ice and shielded from strong light. For extraction of lipids, 4 mL of methanol/chloroform (2.5:2, including 0.1% butylated hydroxytoluene (BHT)) was added to the tubes, which contained pellets of approximately 1-5 mg dry weight (cell suspension) or remaining pellets of 10 mL freeze-dried dodecane. The suspension was mixed and incubated for 10 min in a sonicator bath. Tris-HCl/NaCl solution (2.5 mL, 50 mM Tris-HCl, 1M NaCl, pH 7.5) was added to the suspension, mixed, and incubated for 10 min in a sonicator bath. To obtain a clear phase separation, the tubes were centrifuged for 10 min at 2500 rpm. The lower phase (chloroform) was transferred to a new clean tube. To the remaining phase, 1 mL of chloroform (including 0.1% BHT) was added for further extraction. The suspension was mixed, incubated for 10 min in a sonicator bath, and centrifuged for 10 min at 2500 rpm. The lower phase was pooled with the previous chloroform phase. This extraction step was repeated two more times. The pooled chloroform extracts were dried by flushing with nitrogen gas. The dried residues were dissolved in ethylacetate (including 0.1% BHT) to prepare them for high-performance liquid chromatography (HPLC) analysis. To remove the remaining impurities, samples were incubated in the sonicator bath for 10 min and centrifuged for 10 min at 2500 rpm. For the analysis, dark glass HPLC bottles were used. HPLC with photodiode array analysis was performed as described by Bino et al. (2005). The first set-up experiments were performed and analysed separately from the second set of experiments with the flat-panel reactors. During the first HPLC run, the neoxanthin peak was separated from the violaxanthin peak. During the second run, the neoxanthin and violaxanthin peaks were inseparable. Here the total surface of the combined peak of neoxanthin/violaxanthin was determined and used as if one peak.
Results and discussion

The so-called milking of micro-algae has proved to be a good method for the production of carotenoids by *D. salina*. This process was called selective since mainly carotenoids are extracted (Hejazi *et al.* 2004a). By using three algae strains and three different types of stress, we expected expression of varying carotenoid profiles by the algae and, consequently, varying profiles of carotenoids extracted through milking. In this way we would like to obtain insight in the selectivity and controllability of the milking process of *D. salina*.

In Figure 3.1, cell number, optical density, and cell diameter of *D. bardawil* are shown during the growth period and the subsequent stress periods with high light intensity (Figure 3.1a), low temperature (Figure 3.1b), and the combination of high light intensity and low temperature (Figure 3.1c). *D. salina* 19/18 and *D. salina* 19/25 gave similar results (not shown here).

As can be seen, the cells were in a lag-phase for the first 12 h. Cell diameter increased, as the cells experienced their transfer from the Erlenmeyer bottle to the reactor as stressful. Hereafter, they started dividing. After cell numbers of approximately $1 \cdot 10^6$ cells mL$^{-1}$ were reached, stress was started. High light intensity resulted in an increase in cell diameter. The first-hour stress was severe enough to be growth limiting and cell numbers kept constant. After these first hours, the cells started to overgrow the stress and cell numbers increased again. However, cell diameter remained high and the ratio of the optical density at 530 nm over the optical density at 680 nm (OD$_{530}$/OD$_{680}$) increased, indicating stress-related carotenoid production.

The curves for low temperature stress and the combination of high light and low temperature stress show a similar profile, though cells were less capable of overgrowing the stress implied.

Production of carotenoids

*D. bardawil / D. salina* 19/18

The profiles of the carotenoids produced by *D. bardawil* and *D. salina* 19/18 were quite similar for each stress variant (Figure 3.2 and 3.3). The presence of dodecane did not influence carotenoid production. Therefore, these results are not shown.

Chlorophyll levels and the carotenoids neoxanthin, violaxanthin, and lutein were
Figure 3.1: Growth curves of *D. bardawil* during cultivation and stress with A) high light intensity, B) low temperature and C) the combination of high light intensity and low temperature. Dashed lines indicate start stress. Error bars represent the standard deviation, $n = 2$. 
produced in lesser amounts or remained constant in comparison to cells grown without stress. At high light intensities the de-epoxidation reaction in the violaxanthin reaction will take the upper hand and violaxanthin will be converted to antheraxanthin and, subsequently, zeaxanthin, which will be accumulated in the chloroplast thylakoids (Jin et al. 2002; Yamamoto 1979). This is in agreement with our results when high light intensities were used for stress. The xanthophylls neoxanthin and violaxanthin decreased with high light intensity and a sharp increase in zeaxanthin was seen.

However, in case of low temperatures used for stressing, concentrations of all xanthophylls remained approximately constant. This does not coincide with the suggestion that cold stress can be seen as light stress, assuming that the stress inflicted is not about the irradiance intensity itself, but about the amount of photons that are received per cell division time (Ben-Amotz and Avron 1983; Krol et al. 1997).

All three stress conditions resulted mainly in the production of extra β-carotene in its various isomers and, in some cases, in accumulation of zeaxanthin and cis-lycopene. In case of light stress, the β-carotene/chlorophyll ratio increased from 0.16 to 2.56 for D. bardawil and from 0.07 to 2.35 for D. salina 19/18. For low temperature, this effect was less pronounced, but still, the ratio β-carotene/chlorophyll increased from 0.16 to 0.37 for D. bardawil and from 0.07 to 0.15 for D. salina 19/18. The combination of light stress and low temperature resulted in an increase of the β-carotene/chlorophyll ratio to 3.19 for D. bardawil and to 3.33 for D. salina 19/18.

In case high light intensity was used to stress the cells, the ratio of 9-cis/all-trans β-carotene increased from 0.55 to 1.27. For stress by low temperature, this effect was less obvious, but still apparent (0.55 to 0.67).

The results obtained in this study are quite comparable to the literature. For example, several researchers found that the ratio of 9-cis to all-trans β-carotene increased when cells were stressed (Ben-Amotz 1996; Ben-Amotz et al. 1988; García-González et al. 2005). Jimenez and Pick (1994) found that this increase in 9-cis to all-trans β-carotene ratio was mainly due to an accumulation of carotenoid containing globules and a decrease in thylakoid-bound carotenoids. The globules contained over 50% 9-cis β-carotene, whereas the thylakoids contained mainly all-trans β-carotene. However, in some cases, literature is contradictory concerning the production of certain carotenoids in reaction to
certain stress conditions. For example García-González et al. (2005) and Krol et al. (1997) found that lutein levels increased with both light stress and cold stress. However, Ben-Amotz et al. (1988) and Leon et al. (2003) found that levels decreased when cells were stressed with light stress or nitrogen starvation, respectively. We found that lutein levels remained quite constant or decreased slightly with light stress involved and seem to increase slightly when cells were

Figure 3.2: Carotenoids produced by Dunaliella bardawil under stress conditions: no stress, HL: high light intensity, LT: low temperature, HL/LT: combination of high light intensity and low temperature. Error bars represent the 95% confidence interval.

Figure 3.3: Carotenoids produced by Dunaliella salina 19/18 under stress conditions: no stress, HL: high light intensity, LT: low temperature, HL/LT: combination of high light intensity and low temperature. Error bars represent the 95% confidence interval.
A more detailed overview on why cells start to produce certain carotenoids under stress conditions and what regulatory mechanisms concerning carotenogenesis are understood so far is given by Lamers et al. (2008).

**D. salina 19/25**

*D. salina* 19/25 clearly showed a very different carotenoid profile compared to *D. bardawil* and *D. salina* 19/18. *D. salina* 19/25 produced only very low amounts of carotenoids (Figure 3.4). Yokthongwattana et al. (2005) measured approximately 1.1 pg carotenoids per cell, whereas in this study, we found 1.4 pg carotenoids per cell.

The amount of chlorophyll, and primary carotenoids neoxanthin, violaxanthin, and lutein decreased per gram biomass when cells were stressed, independent of the type of stress invoked. Light stress resulted in an increase of the carotenoids β-carotene, zeaxanthin, and *cis*-lycopene. In case temperature stress was involved, amounts of β-carotene decreased as well and only zeaxanthin and *cis*-lycopene were accumulated.

Previous research showed that both chlorophyll and carotenoid content decreased per cell after light stress (Yokthongwattana et al. 2005). This carotenoid decrease accounted for all determined carotenoids (neoxanthin, violaxanthin, anteraxan-
thin, lutein, and β-carotene), except for zeaxanthin where an increase was found from 0.25 pg to 2.0 pg per cell. In this research, an increase in zeaxanthin for light stress was found from 0.02 pg to 0.08 pg per cell. This is little less than found by Jin and Melis (2003), who found that light stressed cells of *D. salina* 19/25 produced 0.22 pg zeaxanthin per cell, also an almost eightfold increase compared to nonstressed cells (0.03 pg zeaxanthin per cell).

### Extraction

The profile of the extracted carotenoids was as expected. Mostly, β-carotene (various isomers) was extracted and next to that zeaxanthine, cis-lycopene, and lutein (Figure 3.5). These are secondary carotenoids that are not only coupled to the photo-system in the thylakoid membranes to help with photosynthesis but moreover they are extra produced in large quantities under stress conditions and function as protective layer against stress. Secondary carotenoids are characterized by their localization in extraplastidic globules (Skulberg 2004). These globules consist mainly of neutral lipids of which 30% carotenoids and less than 3% chlorophyll. The carotenoids in these globules exist mainly as β-carotene.

![Figure 3.5: Extracted carotenoids and chlorophylls as percentage of produced, either by *D. bardawil*, *D. salina* 19/18 or *D. salina* 19/25: (db): *D. bardawil*, (19/18): *D. salina* 19/18, (19/25): *D. salina* 19/25. Stress by L: high light intensity, T: low temperature, LT: combination of high light intensity and low temperature. Error bars represent the standard deviation, n=3.](image)

38
in the form of 9-cis and all-trans β-carotene. Further, small amounts of 15-cis, some unidentified other isomers of β-carotene and α-carotene were described (Ben-Amotz et al. 1982; Jimenez and Pick 1994). The fact that we find approximately equal amounts of 9-cis and all-trans β-carotene as extracted carotenoids and some other β-carotene isomers (among which also probably α-carotene) feeds the idea that it are the carotenoids accumulated in globules that are extracted during milking. Also zeaxanthin, lutein, and cis-lycopene were found among the extracted carotenoids. Thus far, it has not been reported that these three carotenoids consisted in carotenoid-containing globules in _D. bardawil_. However, various researchers (Yokthongwattana _et al._ 2005; Bassi _et al._ 1993; Bassi and Caffari 2000; Jin _et al._ 2001; Tardy and Havaux 1996) found that the zeaxanthin and lutein that _D. salina_ formed in excess during stress is not bound to chlorophyll-proteins in the thylakoid membrane as in low light grown cells. Yet, this excess zeaxanthin and lutein is located in the lipid bilayer or at least somewhere in the chloroplast thylakoids where it can be easily separated from the chlorophyll. Extraction of lutein is previously found by others as well in their two-phase cultivation system of _D. bardawil_ (Leon _et al._ 2003).

In some cases, not all secondary carotenoids were extracted; this could be due to the very low amounts produced and, consequently, even lower – undetectable – amounts of carotenoids extracted. No carotenoids were extracted during the milking of _D. salina_ 19/25 (Figure 3.5). It is not clear whether this is caused by the very low amounts of carotenoids produced or whether cells of _D. salina_ 19/25 are not extractable via the milking process. Only low percentages carotenoids were extracted when milking was performed in the stirred reactors (Figure 3.5). Though amounts of carotenoids produced for _D. bardawil_ and _D. salina_ 19/18 were approximately the same, the amounts of extracted carotenoids were little higher for _D. salina_ 19/18. We produced maximally 10 mg carotenoids per reactor in case of _D. salina_ 19/18 and the stress combination of high light intensity and low temperature, which corresponds with approximately 33 mg L<sub>culture</sub><sup>-1</sup>. In the dodecane phase, we extracted approximately 0.1 mg L<sub>dodecane</sub><sup>-1</sup>, which corresponds to approximately 1% of the total carotenoids, the maximum value found in this research. In the research performed by Hejazi _et al._ (2003a) in the same set-up, similar results were found for the same time span.
Chapter 3

The low extraction percentages can be explained by 1) the fact that we only stressed and milked the cells for 48 h, and 2) the fact that we used simple systems that were stirred gently by magnetic stirrer bars, resulting in a small surface contact between the dodecane and the cell suspension. The extraction percentages can be improved by increasing the extraction time and, more importantly, by increasing the contact area between the dodecane and the biomass.

This is shown in the research performed by Hejazi et al. (2002), where values of 12 mg carotenoids \( \text{L}_{\text{culture}}^{-1} \) and approximately 2 mg carotenoids \( \text{L}_{\text{dodecane}}^{-1} \) were obtained. In these experiments, Hejazi and coworkers made use of flat-panel photobioreactors in which the mixing was performed by recirculation of the dodecane itself through the aqueous phase. This resulted in an increased contact between cells and organic phase. Elsewhere, approximately 8% of the total carotenoids were extracted after 4 days of stressing and milking (Leon et al. 2003).

Therefore, we also used a second set-up in which we performed almost the same experiments as in the first set-up. Only, in this set-up, we used small flat-panel photobioreactors, stirred by aeration. We chose not to re-circulate the dodecane to prevent carotenoid degradation by light. Two algal strains were used (\( D. \ bardawil \) and \( D. \ salina \) 19/18), again with three stress variants; high light intensity, low temperature, and the combination of both. The time used for stressing was equal to the time used in the first set-up, but the extraction time was shortened to 2 h. Extraction was done with aeration but without illumination. With these reactors, much higher extraction values than in the first set-up were reached (Table 3.1). These values are comparable to the aforementioned values of Hejazi et al. (2004a) and Leon et al. (2003).

As can be seen in Figure 3.6, in this set-up, chlorophylls were also extracted, mainly chlorophyll \( a \), but also neoxanthin/violaxanthin (in this HPLC run, the peaks of neoxanthin and violaxanthin overlap, so we measured the combined peak). Hejazi et al. (2003a) also found low amounts of chlorophyll to be extracted (up to 3% of the total amount of chlorophyll). It is not known whether this extracted chlorophyll is the result of cell death due to the more vigorous stirring (Hejazi found that cell death was up to 10% in his set-up) or whether chlorophyll can be milked from the cells as well. Ben-Amotz et al. (1982) found low amounts of chlorophyll in the separated globules. Chlorophyll is heterogenically bound to
other compounds in the chloroplast with mainly strong hydrophilic bonds. However, Deroche and Briantais (1974) and Öquist and Samuelsson (1980) report that there exist some forms of chlorophyll a that are loosely bound in the cell. The fact that chlorophyll is very lipophilic and has a log P value similar to the log P value of β-carotene (chlorophyll 17.2, β-carotene 17.6) makes this

Table 3.1: Extraction values for both reactor set-ups.

<table>
<thead>
<tr>
<th>Set-up</th>
<th>Cells</th>
<th>Stress</th>
<th>Carotenoids produced [percent of biomass]</th>
<th>Carotenoids extracted [percent of produced]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stirred reactor</td>
<td><em>D. bardawil</em></td>
<td>HL</td>
<td>3.0</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT</td>
<td>1.2</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HL/LT</td>
<td>2.7</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td><em>D. salina 19/18</em></td>
<td>HL</td>
<td>4.2</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HL/LT</td>
<td>4.1</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td><em>D. salina 19/25</em></td>
<td>HL</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HL/LT</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>Aerated reactor</td>
<td><em>D. bardawil</em></td>
<td>HL</td>
<td>6.1</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT</td>
<td>1.7</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HL/LT</td>
<td>4.4</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td><em>D. salina 19/18</em></td>
<td>HL</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT</td>
<td>1.5</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HL/LT</td>
<td>0.4</td>
<td>1.9</td>
</tr>
</tbody>
</table>
chlorophyll $a$ a plausible candidate for extraction. However, the fact that chlorophyll $b$, neoxanthin and violaxanthin were extracted in some cases as well suggests cell death as reason for extraction.

In this research, our main objective was to compare the produced carotenoid profile with the extracted carotenoids and see whether all produced carotenoids could be milked. In the first set-up with low extraction percentages, all extra produced secondary carotenoids were extracted. In the second set-up, much higher extraction percentages were obtained, but next to that, the extraction was not purely selective for secondary carotenoids. Also, chlorophyll was extracted due to cell death. This cell death was most probably caused by shear as a result of aeration.

In our opinion, a most optimal milking process in the future circumvents cell death by shear (caused by, e.g., aeration or agitated mixing), but at the same time creates a large contact area between the organic phase and the biomass. Also, the dodecane (containing extracted carotenoids) should be prevented from being illuminated, to prevent light-induced carotenoid degradation. If all these
important considerations are taken into account, we think that milking is a powerful tool to obtain secondary carotenoids from *D. salina* in a constant process.

**Conclusions**

Our first aim to produce different carotenoid profiles with three strains was successfully obtained. The extraction profiles, however, were quite similar for all circumstances. Mainly, secondary carotenoids were extracted. Only the amounts varied for the different strains. The highest amounts of carotenoids were extracted from *D. salina* 19/18. *D. salina* 19/25 seemed not to be a suitable strain for milking, since no carotenoids were extracted.

In a second set-up with more vigorous stirring, chlorophylls and primary carotenoids were also extracted next to secondary carotenoids. Thus, milking decreased in selectivity. Our results suggest that chlorophylls and primary carotenoids were extracted from cells that were killed due to shear stress because of the vigorous aeration. If one is only interested in high extraction rates and not too much in the selectivity of the process and whether (small) amounts of chlorophyll are milked as well, it is better to create a large surface between cell suspension and the organic phase, for example, by vigorous aeration. This results in a much higher extraction rate than when gently stirred. At low stirring rates, the milking process can be selective for secondary carotenoids only.

**Acknowledgements**

The authors would like to thank Dr. Ami Ben-Amotz for kindly providing *D. bardawil* and would like to thank Ric de Vos for his help with the interpretation of the HPLC results. This research was financially supported by the technology foundation STW (www.stw.nl; project WLM.6622), which is part of the Netherlands Organisation for Scientific Research (www.nwo.nl).
Chapter 4

Carotenoid fluorescence in *Dunaliella salina*

Abstract

*Dunaliella salina* is a halotolerant green alga that is well known for its carotenoid producing capacity. The produced carotenoids are mainly stored in lipid globules. For various research purposes, such as production and extraction kinetics we would like to determine and/or localize the carotenoid globules *in vivo*. Here we show that the carotenoid rich globules emit clear green fluorescence, which can be used in for example fluorescence microscopy (e.g. CLSM) to obtain pictures of the cells and their carotenoid content.

Introduction

*Dunaliella salina* Teodoresco is a green alga, capable of producing high concentrations of carotenoids, i.e. more than 8% of the dry weight (Ben-Amotz et al. 1982) when stressed. These carotenoids are stored in lipid globules. Already good HPLC-based methods exist for the separation, detection and quantification of a wide variety of carotenoids (Fraser et al. 2000). However, these methods do not give information about the intracellular localization of the carotenoids.

To gain more insight in for example the milking process (Hejazi and Wijffels 2004c) it would be advantageous to have an imaging technique at our disposal that can visualize these carotenoid containing globules. Pictures of *Dunaliella* cells made with electron microscopy show small globules, labelled as carotenoid containing lipid droplets (Ben-Amotz *et al.* 1982; Hejazi *et al.* 2004b; Hejazi and Wijffels 2004c; Borowitzka and Siva 2007). However, a disadvantage of electron microscopy is that it cannot be used on living cells.

Fluorescence microscopy is a non-invasive imaging technique and can be used to study cellular processes *in vivo*. The detection of endogenous fluorescence from cell components or exogenous fluorescence from added dyes can be used to show and localize cellular components *in vivo*. An example of an endogenous fluorophore is chlorophyll, which is widely known for its autofluorescence (Papageorgiou and Govindjee 2004). Also, the fluorescence of carotenoids in solution is proven to be existent (Gillbro and Cogdell 1989; Bondarev 1997). However, intracellular carotenoids as part of the photosystem are known to mainly quench fluorescence emitted by other cell components (Lichtenthaler 1987; Chapelle *et al.* 1991).

Here we show that the carotenoid containing globules produced by the alga *D. salina* emit green fluorescence that can be detected with confocal laser scanning microscopy (CLSM). Absorbance and fluorescence measurements show that the fluorescence most likely originates from the β-carotene in these globules.
Materials and Methods

Strain and culture medium
*Dunaliella salina* CCAP 19/18 was obtained from CCAP (Culture Collection of Algae and Protozoa, Oban, UK). Cells were grown and stressed in a flat-panel photobioreactor with a working volume of 2500 mL and a light path of 3 cm. The medium used was as described by Kleinegris et al. (2010c). The cell suspension was mixed by gassing (0.6 L N₂ min⁻¹) and the pH was controlled at 7.5 by adding CO₂ pulses. The temperature was kept at 30°C by circulating temperature-controlled water through the reactor water-jacket. For the growth period the reactor was illuminated from one side with an average incoming light intensity of 206 μmol photons m⁻² s⁻¹ (high-pressure sodium lamp). For the stress period the light intensity was raised to 1672 μmol photons m⁻² s⁻¹ by moving the lamp closer to the reactor. Small amounts (1-5 mL) of green cells harvested during the growth period and orange cells harvested during the stress period were used for microscopy purposes. At the end of the cultivation all remaining biomass was used for isolation of β-carotene globules.

Isolation of β-carotene globules
Isolation of β-carotene globules was performed as described by Ben-Amotz et al. (1982) with minor adaptations. From a stressed culture 8*40 mL cell suspension was centrifuged at 1000 g for 15 min. Each pellet was re-suspended in 40 mL of 30 mM NaCl and centrifuged at 12,000 g for 10 min. To break the cells each pellet was re-suspended in 4 mL demineralised water and again centrifuged at 12,000 g for 10 min. The supernatant was mixed with a solution of 2 mL of 50% sucrose in 10 mM Tris-HCl (pH 8.0). On top of this mixture 0.5 mL 10 mM Tris-HCl (pH 8.0) was layered. This mixture was centrifuged for 2 h at 48,000 g to separate the globules from the chloroplast membranes. The globules were collected from the top layer and pooled. All steps were performed at approximately 4°C. The pooled samples were stored on ice in a refrigerator at 4°C.

Absorbance and fluorescence determination
The absorbance and autofluorescence of the globules were measured using a spectrophotometer (Avaspec-2048-USB, Avantes, The Netherlands) and a
spectrofluorimeter (Jobin Yvon Fluorolog FL3-22, Horiba) respectively. As reference sample 10 mM Tris-HCl (pH 8.0) was used. The excitation wavelengths for the emission spectra of the autofluorescence were 450, 488 and 510 nm. For emission the bandwidth was 0.6 nm and the step size was 0.2 nm.

**Microscopy**

Bright field microscopy pictures from cells and globule suspensions were made with a Microphot fluorescence microscope (Nikon) or a CK 40 bright field microscope (Olympus), equipped with an Olympus AX 70 camera. Fluorescence microscopy pictures of cells and globule suspensions were made with a confocal laser scanning microscope (CLSM, Zeiss LSM 510-META 18). The CLSM was connected to an inverted microscope (Axiovert 200 M) with differential interference contrast (DIC). For excitation the Argon diode laser (30 mW, 488 nm) was used. For the detection of emitted fluorescence we applied the BP505-530/LP650 filter combination. A Zeiss 639 α-Plan Fluar oil objective (NA 1.45) was used for all imaging experiments. The obtained CLSM pictures were a combination picture of images from three channels, namely a DIC microscopy image from the cell, an image from the green fluorescence between 505 and 530 nm, and an image from the red fluorescence of 650 nm and higher.

**Results**

When *Dunaliella* cells are stressed they start to produce carotenoids. The green cell which is dominated by the chloroplast starts to turn orange. The chloroplast shrinks, chloroplast membranes decrease in size and carotenoid containing lipid globules are formed. Pictures obtained by light microscopy clearly showed this difference between non-stressed and stressed cells (Figure 4.1a and b). Pictures obtained by fluorescence microscopy also showed that a distinct difference in fluorescence pattern arose after stress (Figure 4.1c and d). The red fluorescence emitted by chlorophyll decreased and green fluorescence appeared. To prove that this green fluorescence was emitted by the carotenoid containing globules we isolated the globules (Figure 4.1e-h). Light microscopy showed clear orange globules and with the CLSM again the green fluorescence was detected. This fluorescence was emitted by the globules and not by the solution as can be
Figure 4.1: a and b: Bright field microscopy picture of (a) non-stressed and (b) stressed D. salina cells. Scale bars = 20 μm. c and d: Fluorescence microscopy pictures (CLSM) of (c) non-stressed and (d) stressed D. salina cells. Scale bars = 10 μm. The CLSM pictures are a combination image of three channels: DIC microscopy, green fluorescence (505-530 nm) and red fluorescence (>650 nm) e - h: Isolated carotenoid containing globules. (e) Isolated globules in Tris-HCl buffer (pH 8.0). f: Bright field picture of the carotenoid containing globules. g and h: CLSM pictures of the carotenoid containing globules. Scale bars = 10 μm (g) and 2 μm (h). Green fluorescence comes from the globules, red fluorescence from chlorophyll remains.
seen in Figure 4.1h. Some red fluorescence indicated chlorophyll contamination. The absorbance and emitted fluorescence from the globule suspension were measured with a spectrophotometer and spectrofluorimeter, respectively, and are shown in Figures 4.2 and 4.3.

Figure 4.2: Absorbance spectrum of carotenoid containing globules. Peak from 400 – 550 nm was carotenoid absorbance. The peak at 670 nm was absorbance from chlorophyll.

In the absorbance curve the carotenoid peak is clearly visible (350 – 550 nm). Next to this peak a very small peak at 670 nm was visible, originating from some chlorophyll contamination still left in the solution. In the fluorescence emission graph (Figure 4.3) this chlorophyll contamination showed a very high emission peak at 670 nm. At 560 nm a second, much smaller peak was visible, which must have originated from another component of the suspension, most likely the carotenoid containing globules. With different excitation wavelengths this emission peak did not shift its position with respect to the wavelength.

To determine whether the fluorescence peak at 560 nm was emitted by the β-
carotene in the carotenoid rich globules, we compared the relative values for the quantum yields of the chlorophyll peak and the 560 nm peak, to see whether these compare to values found in literature for chlorophyll and carotenoid fluorescence.

Figure 4.3: Emission spectra of carotenoid containing globules, for three different excitation wavelengths (450 nm, 488 nm and 510 nm).

Discussion

Dunaliella salina stores secondary carotenoids (predominantly β-carotene) in lipid globules when cultivated under stress conditions. Non-stressed cells are dominated by the chloroplast, emitting red fluorescence originating from the chlorophyll packed on the thylakoid membranes. When the cells are stressed, the red fluorescence from chlorophyll partly disappears as the membranes in the chloroplast are broken down. At the same time the cells start to produce carotenoid globules. Simultaneously green fluorescence appears. Dunaliella salina
cells show this distinct difference in red and green fluorescence pattern between non-stressed and stressed cells. We determined the relation between the carotenoid containing globules and the green fluorescence and studied the possibility to visualize and localize these lipid globules inside the cells in vivo using this autofluorescence.

First, we isolated the carotenoid containing globules. The purified globules, as determined by Ben-Amotz et al. (1982), are composed almost entirely of lipids. Over 90% of the lipids are neutral lipids, with β-carotene as the dominant fraction (65% of dry weight).

The carotenoid peak in the absorbance curve (350 – 550 nm) showed great similarity to the absorption spectrum of an 80% acetone extract of the purified β-carotene globules from D. bardawil, found by Ben-Amotz et al. (1982) and resembles the absorbance peak of pure β-carotene in solution (Kandori et al. 1994). The peak originated mainly from β-carotene, but other carotenoids might have contributed as well, just as some chlorophyll contamination. This originated probably from some chloroplast membrane remainders that were connected to the lipid globules. Chlorophyll has absorbance peaks between 400 – 500 nm and >600 nm (Lichtenthaler 1987). The small peak visible at 670 nm in the absorbance graph therefore must originate from chlorophyll still left in the solution.

The fact that with different excitation wavelengths the emission peak did not shift its position with respect to the wavelength proves that the peak is indeed fluorescence and can not be ascribed as a Raman peak (Shanker and Bane 2008).

The high relative intensity of the emitted fluorescence from the relatively small chlorophyll contamination coheres with its high quantum yield.

The ratio between the absorbance peaks and the emission peaks can be used to determine the ratios in quantum yields (Shanker and Bane 2008). The quantum yields for chlorophyll vary from 0.02 to 0.33, depending on whether measured in various solutions or in the cell (Forster and Livingston 1952; Latimer et al. 1956). According to Kandori et al. (1994) the quantum yield for β-carotene is 1.7·10⁻⁴. This results in a ratio between the quantum yields of β-carotene and chlorophyll varying from approximately 100 – 2000. The ratio in fluorescence between the both peaks lies between 0.07 and 0.11, when we divide the maximum height of both peaks (e.g. 3.0·10⁴ / 2.7·10⁵ for 488 nm ex. wavelength). The ratio between the absorbance peaks is around 14, when we divide the maximum height of both
Carotenoid fluorescence

peaks (0.64/0.05). Consequently the ratio between the relative values for the quantum yields for both peaks in this research is somewhere between 100 – 200. Comparing these to literature shows that the emission peak at 560 nm most likely originates from the β-carotene in the lipid globules.

Conclusions

*Dunaliella salina* stores secondary carotenoids in lipid globules when cultivated under stress conditions. To visualize these carotenoid containing globules, confocal laser scanning microscopy (CSLM) can be used, in combination with the autofluorescence of cell components. This autofluorescence of the cells is emitted by chlorophyll in the red part of the spectrum and by the carotenoid globules in the green part of the spectrum. When comparing the relative quantum yields obtained from the absorbance and fluorescence emission spectra with data from literature, we can conclude that the green fluorescence most likely is emitted by the β-carotene that comprises the main part of the carotenoid rich globules.

Acknowledgements

The authors would like to thank André van Lammeren and Norbert de Ruijter for all help regarding confocal laser scanning microscopy. Furthermore the authors would like to thank Herbert van Amerongen for his help with interpreting data and for useful discussions. This research was financially supported by the technology foundation STW (www.stw.nl; project WLM.6622), which is part of the Netherlands Organisation for Scientific Research (www.nwo.nl).
Abstract

In the so-called milking process of *D. salina* carotenoids are extracted and simultaneously produced by the culture, whilst the biomass concentration remains constant. Different theories exist about the extraction mechanisms although none have been proven yet. In this research direct contact between dodecane and cells during the extraction process was observed microscopically and effects of direct contact were determined during *in situ* extraction experiments. Our results showed that water-solvent interphase contact resulted in cell death. This cell death and consequent cell rupture resulted in the release and concomitant extraction of the carotenoids.

Furthermore, it has been suggested to add a small amount of dichloromethane to the biocompatible dodecane to create an organic phase with more extraction capacity. Our results showed that the addition of dichloromethane resulted in increased cell death and consequently the extraction rate increased. The improved solubility of carotenoids in an organic phase with dichloromethane did not significantly increase the extraction rate.
**Introduction**

*Dunaliella salina* is the main natural producer of β-carotene. This unicellular, halotolerant microalga produces this carotenoid up to 10% of its dry weight when stressed. Commercial production of β-carotene with algae is done by growing and stressing the algae in open ponds (Ben-Amotz 1995; Borowitzka 1999). During this process, the algae are first grown to obtain a high biomass. Next, the cells are stressed to produce β-carotene. Stress is implied by increasing the light intensity or allowing the water of the ponds to evaporate to increase the salt concentration. When high levels of β-carotene are reached, the biomass is harvested and further processed.

Milking the algae is a newly introduced method to continuously produce and simultaneously extract carotenoids from *D. salina* in closed reactor systems. The extraction is performed by adding a biocompatible organic solvent (dodecane) to the bioreactor. During the milking process the production of β-carotene by the cells is prolonged while it is extracted *in situ* at the same time. As a result the production step in which biomass is grown, does not need to be continuously repeated, increasing the overall volumetric productivity of carotenoids. The milking process can last for several weeks, with constant biomass levels and increasing amounts of extracted β-carotene (Hejazi et al. 2004a).

Though the process was shown to be successful, the mechanism behind it is unclear. It was hypothesized that β-carotene was extracted from the cells while the cells stayed viable and kept producing carotenoids under the stress conditions applied. It is unclear whether carotenoids are released from the cells into the water phase after which they are transferred into the dodecane phase, or whether direct contact between cells and the dodecane phase is required, and what the effect of direct contact between dodecane and cells is on the viability of the biomass. The mechanism of β-carotene transport across the cell membrane is also unknown.

In previous research two hypotheses concerning the carotenoid extraction were proposed (Hejazi *et al.* 2004b). The first hypothesis suggested an active role for endocytosis and exocytosis. The other hypothesis was based on diffusion of carotenoid molecules. Neither of these were proven true or false thus far.

Therefore a combination of microscopic extraction experiments and macroscopic
experiments in small reactors was used to reveal the mechanisms behind the extraction process. In previous studies it was shown that adding a more polar organic solvent to the biocompatible solvent improved the extraction, because of a more favorable product distribution coefficient between the water and solvent phase (Mojaat et al. 2008a; Bruce and Daugulis 1991; Evans and Wang 1988). Very small amounts of these polar, normally more toxic, components should not be toxic to the cells in a two-phase system, as long as the critical aqueous concentration is not reached. This is the concentration of toxic component in the water phase corresponding to the concentration of the toxic compound in the cell membrane that is lethal to the cell (Osborne et al. 1990).

In this research we added two different volume fractions of dichloromethane to the biocompatible dodecane to determine the effect on the extraction process. Mojaat et al. (2008a) showed that the solubility of β-carotene in a solvent mixture of decane and 190 mM dichloromethane increased approximately 1.5 times compared to pure decane. Next to that they showed a six times improved extraction of β-carotene from D. salina. This improved extraction was suggested to result from both the improved solubility of β-carotene and from the permeability effect of mixed solvents on the cell membrane (Mojaat et al. 2008a). We wanted to test whether addition of dichloromethane had a positive effect on the in situ extraction process.

Materials and Methods

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. (2010c). The medium consisted of 1.50 M NaCl, 9.95·10⁻¹ M Na₂H₂PO₄.2H₂O, 3.78·10⁻² M KNO₃, 2.25·10⁻² M Na₂SO₄, 1.00·10⁻² M NaHCO₃, 4.87·10⁻³ M K₂SO₄, 3.68·10⁻⁴ M MgCl₂.6H₂O, 1.89·10⁻⁵ M CaCl₂.2H₂O and 1.13·10⁻⁵ M FeEDTA. In addition, the medium contained micronutrients: 1.94·10⁻⁵ M Na₂EDTA.2H₂O, 1.89·10⁻⁶ M MnCl₂, 1.48·10⁻⁶ M ZnSO₄, 6.65·10⁻⁷ M CuSO₄, 1.10·10⁻⁸ M Na₂MoO₄, 9.95·10⁻⁹ M CoCl₂. The solution was buffered with 4.00·10⁻² M HEPES acid and adjusted to pH 7.5 with 3 M NaOH.

The medium was sterilized at 121°C for 30 minutes. The phosphate, magnesium,
Calcium, and carbonate salts were separately sterilized and added after autoclaving to avoid precipitation. All strains were maintained as suspended culture in 250 mL Erlenmeyer flasks containing 100 mL of medium. Cultures were kept in a growth cabinet at 25°C, under continuous light with an intensity of 70±10 μmol photons m⁻² s⁻¹ and were continuously shaken. The headspace consisted of an air/CO₂ ratio of 95/5%. Every three weeks 10 mL of a culture was transferred to a new flask containing fresh medium.

**Cultivation system**

Cells were grown and stressed in a batch process in a flat-panel photobioreactor. Stressed cells were used for microscopy and extraction purposes. The bioreactor consisted of two compartments, a culture compartment and a water jacket for temperature regulation, as described in more detail by Lamers et al. (2010). The culture chamber had a depth of 3 cm. The reactor contained 2500 mL of culture medium (as described above, but without the addition of HEPES buffer). Mixing and oxygen removal was provided by gassing the cell suspension with 0.6 L min⁻¹ of dinitrogen. The pH was controlled at 7.5 by automatically dosing short pulses of carbon dioxide to the cell suspension. Temperature was maintained at 30°C. The reactor was illuminated from one side with a high-pressure sodium lamp (Philips 400W Master SON-T PIA Green Power) with an average light intensity of 200 μmol photons m⁻² s⁻¹ for the growth period. During stress, light intensity was increased to 1665 μmol photons m⁻² s⁻¹ moving the lamp closer to the reactor. The average light intensity was measured using a PAR 2p quantum sensor (SA-190, Li-cor Biosciences, Lincoln, Nebraska, USA).

For each cultivation the reactor was inoculated with a stock culture to a concentration of 7·10⁵ cells per mL. When a cell number of more than 1·10⁶ cells mL⁻¹ was reached in the reactor, stress conditions were applied. Cells were stressed for at least 24 hours during which significant amounts of β-carotene were produced (2.2 – 2.5 % w/w).

**Analytical procedures**

Dry weight of the cell suspension was determined at the end of the cultivation, as previously described by Kleinegris et al. (2010c). Cell number and volume were measured with a Beckman Coulter Multisizer 3 (100 μm orifice; Beckman Coulter
Mechanism of extraction

Inc., Fullerton, CA, USA). Diameter of the cells was calculated from their volume. During the extraction experiments cell numbers were also determined by counting with a Bürker-Türk counting chamber. This was done because during the extraction experiments a dodecane emulsion was formed in the mixed two-phase reactors. As the droplets interfered with the Multisizer measurement, it was necessary to perform cell counting by microscopy. In this manner it was possible to distinguish between droplets and cells. For every sample, a volume of $2.56 \cdot 10^{-4}$ mL (4*16 squares) was counted in duplicate. Lugol solution was added to each sample to stop cell movement. Carotenoid levels of biomass and dodecane were determined spectrophotometrically. One mL aliquots of cell suspension were centrifuged at 4000 rpm for 10 min (SX 4250 rotor, Allegra X-22R, Beckman Coulter). After centrifugation, the supernatant was discarded and 3 mL dodecane was added. The sample was shaken vigorously to re-suspend the pellet. Then 9 mL of methanol was added to completely break up the cells and the tube was shaken vigorously again and centrifuged for 3 min at 4000 rpm. The dodecane containing lipophilic carotenoids (upper layer) was measured with a spectrophotometer (Ultrospec 2000, Pharma Biotech) at 453 nm and 665 nm and dodecane as reference.

From a previous experiment biomass samples were used for carotenoid determination by HPLC as described by Kleinegris et al. (2010c). These samples were used to obtain a calibration curve for the spectrophotometer for β-carotene, the main carotenoid present in stressed *D. salina* cells (Ben-Amotz et al. 1982), see equation.

$$C_{\beta-car} = \frac{(ABS_{453} - ABS_{665})}{3.91} \cdot 3.657 \cdot 3 \cdot X \quad [\text{mg/L}]$$

with:
- $\frac{(ABS_{453} - ABS_{665})}{3.91}$: Absorbance of β-carotene corrected for chlorophyll contamination
- 3.657: calibration factor derived from HPLC analysis of β-carotene concentration
- 3: amount of mL 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 (though of course without the factor 3).

**Microscopy**

Bright field microscopy pictures were made with a CK 40 bright field microscope (Olympus), equipped with an Olympus AX 70 camera. Fluorescence microscopy pictures were made with a confocal laser scanning microscope (CLSM, Zeiss LSM 510-META 18) that consisted of an inverted microscope (Axiovert 200 M) equipped with differential interference contrast (DIC) and a range of excitation laser lines. For the excitation we used the Argon diode laser (30 mW) with 488 nm excitation wavelength. For the detection of emitted fluorescence the BP505-530/LP650 filter combination was used. A Zeiss 639 α-Plan Fluar oil objective (NA 1.45) was used for all imaging experiments. The observed fluorescence was emitted in green (505-530 nm) by the carotenoid rich globules in the cell and in red (> 650 nm) by the chlorophyll (Kleinegris et al. 2010a).

**In situ extraction experiments**

The extraction experiments were carried out in 1 L glass bottles, containing 500 mL of culture medium. The bottles (diameter 9 cm) were equipped with one magnetic stirrer fixed on an axis, stirring at a rate of 100 rpm. The stirrer (diameter 6.0 cm, width 1.0 cm) was placed 0.5 cm from the bottom in the water phase (Figure 5.1).

In the first experiment dodecane was solely used as the organic phase. In the second experiment a mixture of dodecane and dichloromethane was used. Dichloromethane was added in two concentrations, 1.2% v/v of the total amount of organic phase and 5.0% v/v. This corresponds to concentrations of 190 mM and 781 mM of dichloromethane in the organic phase. The total volume of organic phase in each system was 200 mL. Four reactor flasks were used for each run, two as blank and two as sparged systems. Sparging was done by pumping organic phase from the top layer to the inlet at the bottom of the reactor. Two gearpumps (VG-015.5, Verdergear), viton tubing and stainless steel connectors were used for the pumping. *D. salina* cells frequently rupture and fall apart when they die. Consequently, it is difficult to count the total amount of dead cells (both intact and lysed cells) and as a result death rate is very difficult to measure. By stopping the growth, the
remaining cells could be counted and thus cell death could be calculated. To ensure growth arrest hydroxyurea (Zachleder 1994) was added to the cell suspension at the start of the extraction experiments, with a final concentration of 1.28 mM. This concentration was based on a set of experiments in which a range of concentrations (20 μM – 1.5 mM) of hydroxyurea was tested for effectiveness.

Furthermore the experiments were performed in the dark in order to prevent β-carotene from being broken down.

Figure 5.1: Schematic overview of sparged set-up. 1) sampling tubes for biomass phase and dodecane phase. 2) dodecane phase. 3) biomass phase. 4) gear pump. 5) sparged dodecane bubbles. 6) magnetic stirrer.
Chapter 5

Results and discussion

The effect of direct contact between cells and dodecane

The effect of direct contact between cells and dodecane was studied using light microscopy. Figure 5.2 shows cells in water droplets surrounded by dodecane. When *D. salina* cells were exposed to a dodecane phase, the cells actively moved away from dodecane phase and rather stayed in the medium droplets. By adding more dodecane to the sample, medium droplets divided into smaller droplets. When the medium-droplets became too small for all cells they pushed each other out and cells that came into direct contact with the dodecane fell apart, as can be seen in Figure 5.2a and b. Figure 5.2c and d show fluorescence microscopy pictures of surviving cells in the water phase and a cell that did not survive contact with the dodecane. The living cells were nicely round and were actively moving with their flagella, while the dead cell showed a very rough surface indicating a distorted membrane. Most dead cells decomposed completely as can be seen in Figure 5.2e and f. Small red and green fluorescent algal parts (red and green fluorescence are emitted by chlorophyll and carotenoid globules, respectively) remained in the water phase or at the interphase between the dodecane droplets and the water. Moreover, the colorless dodecane phase started to show green fluorescence, indicating the presence of dissolved β-carotene.

There are various reasons why the presence of a solvent phase can be toxic to a cell, such as nutrient extraction from the aqueous phase to the organic phase, disruption of cell membranes and limited access to nutrients due to emulsion formation or coating of the cell with solvent (Bar 1987). The duration of this experiment will not have been long enough for any deprivation of nutrients to take effect. Disruption of the cellular membrane is the most obvious reason for *D. salina* cells to die. This effect has been mentioned before in various cases as main toxic effect upon addition of apolar solvents to various microorganisms (Bruce and Daugulis 1991, Osborne *et al.* 1990; Hocknull and Lilly 1987; Vermuë *et al.* 1993).

From these experiments we can conclude that *Dunaliella* cells died when in direct contact with the solvent phase but remained viable in the water phase. So dodecane does exert phase toxicity but does not exert molecular toxicity. Thus far phase toxicity of dodecane on *Dunaliella* cells was mainly determined by e.g.
Figure 5.2: Light microscopy pictures of *D. salina* cells in aqueous phase surrounded by dodecane. a and b: bright field microscopy pictures. Size bars indicate 20 μm. c - d: CLSM pictures of surviving cells (c) and a dead cell (d). Size bars indicate 10 μm. e - f: CLSM pictures of cell remains in the presence of dodecane. Size bars indicate 20 μm. Red fluorescence is emitted by chlorophyll, green fluorescence by β-carotene.
oxygen evolution rate for a total culture and not for single cells with direct contact (Mojaat et al. 2008a; Hejazi et al. 2002). This can be the reason why this phase toxicity was not found before.

**The role of dodecane in the extraction process**

The effect of direct dodecane-cell contact during the extraction process was examined. Dodecane was sparged through the medium with different velocities; at higher dodecane sparging rates the contact area between both phases is enlarged. Four reactor flasks were used; one reactor flask was a blank without organic phase, to determine the effect of stirring and dark incubation on cell death. One reactor flask had a stagnant layer of dodecane (200 mL) on top with no interphasial mixing, as a blank. And two reactor flasks were sparged with dodecane, at 300 mL L\(^{-1}\) min\(^{-1}\) and at 400 mL L\(^{-1}\) min\(^{-1}\) (Figure 5.1).

As shown microscopically *D. salina* cells often rupture when they die. During *in situ* extraction in a reactor experiment, however, the number of ruptured cells is difficult to measure. The method described by Hejazi et al. (2003a) to determine the viability of the cells by means of flow cytometry could only distinguish between live and dead ‘intact’ cells but it could not account for ruptured cells. If the number of cells stay constant during extraction cell death cannot be excluded as cell growth could have occurred at the same time. For that reason we arrested cell growth by addition of hydroxyurea to the aqueous phase. By stopping cell growth, cell death can be quantified by measuring the number of intact cells during the extraction experiment.

Hydroxyurea acts as DNA synthesis inhibitor. It does not prohibit cell proliferation immediately after addition. Zachleder (1994) showed that the reproductive processes that the cells had already commenced were still completed after the addition of hydroxyurea. As can be seen from Figure 5.3 some cell growth indeed still occurred during the first hours of the experiment, depicted as negative cell death in Figure 5.3c. However, after these first hours growth was completely stopped and cell death could be demonstrated, confirming the microscopic observations.

From Figure 5.3 follows that the faster dodecane was sparged, the more cell death occurred. At higher sparging rates the cell-dodecane contact area is enlarged resulting in a higher death rate. In Figure 5.3 it is also shown that as the death rate increased, the rate of extraction increased as well. It is clearly
Figure 5.3: Cell numbers and carotenoid extraction in two-phase (dodecane/aqueous phase) stirred reactor systems in time. One reactor without organic phase, one with stagnant layer of dodecane on top and two systems with dodecane sparged through (300 and 400 mL L$^{-1}$ min$^{-1}$). A: Cell concentration in time. Error bars represent standard deviation, $n = 2$. B: Extracted $\beta$-carotene in percentage of total $\beta$-carotene present versus time. C: Cell death as % calculated from cell number at the start of the experiment in time.
shown that there is a direct relation between death rate and extraction rate (Figure 5.3b and c).

Some cell death was observed in the blank experiment as well, due to stirring and prolonged incubation without light (Figure 5.3a). The presence of a stagnant dodecane phase did not increase cell death in comparison to the blanks, nor did a very low sparging rate (14 mL L$^{-1}$ min$^{-1}$, results not shown). A sparging rate of 100 mL L$^{-1}$ min$^{-1}$ showed a slight increase in cell death but due to the inherent noise on the cell counting measurements it was not significant compared to the blanks (results not shown). The extraction of β-carotene in the reactor with a stagnant dodecane phase or the low sparging rates was likewise to cell death very low.

In the above experiments, extraction of β-carotene was mainly attributed to cell death. However, no evidence was found that living cells cannot loose at least a small part of their carotenoids. Fluorescence microscopy pictures of cells from a two-phase culture mixed and kept in the dark for 48 h show that these cells did not loose a significant amount of their carotenoids as shown in Figure 5.4.

The fact that cells die during the extraction process seems contradictory to the milking process with constant biomass levels reported earlier (Hejazi et al. 2004a;

![Figure 5.4: CLSM pictures of D. salina cells from a two-phase system. The cells still had significant amounts of carotenoids after being exposed to dodecane in a mixed two-phase system for 48 hours. Red fluorescence is emitted by chlorophyll, green by the carotenoid globules.](image)
Leon et al. (2003). However, the results found in our study do not have to conflict with those results. If cell growth and cell death cancel each other out, a net constant cell level remains. These antagonistic processes of simultaneous cell growth and destruction have been previously described by Bar (1988) in a dodecanol two-phase system for the yeast *Saccharomyces cerevisiae*. This would also explain the frequent observed biocompatibility of dodecane for *D. salina* cells; single cells die because of direct phase contact, but the culture as a whole remains viable.

From Figure 5.3 follows that it took approximately three days to reduce the cell number by half. In order to keep biomass levels constant the cell division rate should equal this death rate. Under stress conditions cell growth is limited but a doubling rate of a few days is certainly possible as observed by Ben-Amotz et al. (1995) and Lamers et al. (2010).

**Role of dichloromethane in extraction process**

The addition of very small amounts of dichloromethane has been suggested to improve carotenoid extraction from *Dunaliella salina* due to both a higher affinity of the extracted β-carotene with the organic phase and to the permeability effect of mixed solvents on the cell membrane (Mojaat et al. 2008a).

As we showed in this study, the extraction of β-carotene was found to be correlated to cell death. The question remains whether the improved extraction reported with dichloromethane addition could also be a result of increased cell death.

An organic solvent can both exert molecular toxicity, when the solvent is present in levels below saturation, and physical toxicity when there is enough solvent present to form a separate phase (Bar 1987). As discussed before, phase contact between cells and dodecane is toxic to the cells. However, dodecane does not show molecular toxicity. Dichloromethane is toxic to cells already on a molecular level. Osborne et al. (1990) found that total loss of activity of cells corresponded to a constant solvent concentration in the membrane, irrespective of the solvent type used, the so-called critical membrane concentration.

As long as the critical aqueous concentration of dichloromethane, which is the concentration in the water phase corresponding to the critical membrane concentration, is not reached, dichloromethane should not be toxic to a cell. Therefore we set our aqueous dichloromethane concentrations to be well below
the critical aqueous concentration, namely 16 mM (1.2% v/v) and 66 mM (5.0% v/v) with corresponding membrane concentrations of 35 mM and 148 mM respectively (see appendix for calculations). Mojaat et al. (2008a) determined the critical membrane concentration of dichloromethane to be 271 mM.

The experiment was performed in two subsequent runs, each with a different dichloromethane concentration (1.2% v/v and 5.0% v/v) and starting from a new carotenoid-rich cell suspension grown in the panel photobioreactor. For every run, four reactor flasks were used: one as a blank without organic phase, and one with a stagnant layer of organic phase on top to examine the possible molecular toxic effect of the organic phase. The other two reactors were sparged with organic phase (duplicate) at 300 mL L⁻¹ min⁻¹.

As can be seen in Figure 5.5, the influence of dichloromethane on cell death varies with the concentration. At a concentration of 1.2% v/v no increase in cell

![Figure 5.5: Cell numbers in a two-phase system (aqueous phase/dodecane) in time. The influence of different concentrations of dichloromethane in dodecane (0% v/v, 1.2% v/v, 5% v/v) were determined. The organic phase was sparged through the medium with 300 mL L⁻¹ min⁻¹.](image)
death was observed compared to a culture without dichloromethane added to the organic phase. At a concentration of 5% v/v cell death occurred much faster and approximately all cells were dead after 1.5 days, whereas at a dichloromethane concentration of 0% v/v or 1.2% v/v after more than five days still more than 20% of the cells was alive. Apparently at around 5.0% dichloromethane there is molecular toxicity. This can also be observed in the reactor where only a stagnant layer of organic phase was present (Figure 5.6). Also in this reactor the cell number drastically decreased when 5.0% v/v dcm was present in the organic (dodecane) phase, in comparison to 0% v/v and 1.2% v/v dichloromethane present. A reason for this discrepancy in toxicity compared to the value for the critical membrane concentration obtained by Mojaat et al. (2008a) could be the type of measurement determining viability of the culture, namely oxygen evolution for a short period of time versus cell counting.

Figure 5.6: Cell numbers in a two-phase system (aqueous phase/dodecane) in time. The influence of different concentrations of dichloromethane in dodecane (0% v/v, 1.2% v/v, 5% v/v) were determined. The organic phase was present as a stagnant layer on top of the aqueous phase.
Chapter 5

The extraction of β-carotene by the organic phase appeared to parallel the degree of cell death. In Figure 5.7a – c the extraction of β-carotene in the absence and presence of dichloromethane is shown. In the first period of the experiment the extraction goes somewhat faster than the cell death, especially in the case of 1.2% dichloromethane. This can be either due to the increased distribution coefficient of β-carotene for the solvent mixture compared to pure dodecane, or just to more dead cell material already present in the cell suspension at the start of the experiment. We conclude therefore that cell death is the most important factor determining the β-carotene extraction rate.

Conclusions

Contact between cells and the water-dodecane interphase resulted in cell death, as was observed microscopically. In two-phase systems the cell death rate increases with increasing sparging rates of the dodecane. The amount of β-carotene extraction was proportional to the cell death rate: therefore we conclude that extraction is mainly based on cell death.

The influence of dichloromethane on the extraction rate in the extraction process is mainly caused by its influence on cell death. Small amounts of dichloromethane (≤ 1.2 % v/v in organic phase, dodecane) do not result in increased cell death when compared to absence of dichloromethane. However, when the dichloromethane concentration is increased cell death increases as well. The mass transfer rate of carotenoids to the organic phase might be enhanced by the presence of dichloromethane, however the most important and limiting step in extraction is the cell death rate.

Acknowledgements

The authors would like to thank André van Lammeren and Norbert de Ruijter of Laboratory of Plant Cell Biology, Wageningen University for stimulating discussions and great help with the microscopy experiments. Furthermore they would like to thank Arjen Rinzema of Bioprocess Engineering, Wageningen University for his help with the calculations of critical membrane concentrations.
Figure 5.7: Cell death and carotenoid extraction in time for the reactors sparged (300 mL L^{-1} min^{-1}) with organic phase with three different dichloromethane concentrations in the organic phase (dodecane): A, 0% v/v; B, 1.2% v/v; C, 5.0% v/v.
This research was financially supported by the technology foundation STW (www.stw.nl; project WLM.6622), which is part of the Netherlands Organisation for Scientific Research (www.nwo.nl).
Appendix I: Calculation dichloromethane concentration in cell membrane

Determination of the partition coefficient of dichloromethane in a dodecane/water mixture ($P_{dcm,d/w}$) with ProSimPlus 3 (ProSim SA, Cedex, France), based on UNIFAC data.

In a system with 500 mL water, 197.57 mL dodecane and 2.43 mL dichloromethane (1.2% $v_{dcm} v_{org.fase}$), the dichloromethane partitions between the dodecane phase and the water phase. We calculated the dichloromethane concentration in the water phase ($C_{dcm,w}$) to be $1.67 \cdot 10^{-2}$ mol L$^{-1}$ and the dichloromethane concentration in the dodecane ($C_{dcm,d}$) to be $1.58 \cdot 10^{-1}$ mol L$^{-1}$.

In the system with 500 mL water, 190 mL dodecane and 10 mL dichloromethane (5.0% $v_{dcm} v_{org.fase}$), we calculated the dichloromethane concentration in the water phase ($C_{dcm,w}$) to be $6.12 \cdot 10^{-2}$ mol L$^{-1}$ and the dichloromethane concentration in the dodecane ($C_{dcm,d}$) to be $6.32 \cdot 10^{-1}$ mol L$^{-1}$.

This gave a partition coefficient of dichloromethane in a dodecane/water mixture ($P_{dcm,d/w}$) of (averaged for both situations):

$$P_{dcm,d/w} = \frac{C_{dcm,d}}{C_{dcm,w}} = 9.9$$

Calculation volume of cell membrane present in water phase:

Assumptions:
- Number of cells present: $1.0 \cdot 10^9$ cells L$^{-1}$
- Thickness cell membrane: $1.0 \cdot 10^{-8}$ m
- Diameter of cells: $1.0 \cdot 10^{-5}$ m

Volume cell membrane:

$$V_{mem} = 4 \cdot \pi \cdot r^2 \cdot h = 3.14 \cdot 10^{-18} \text{ m}^3 \text{ cell}^{-1}$$

Total volume membrane:

$$V_{total} = 3.14 \cdot 10^{-6} \text{ L}_{mem} \text{ L}_{w}^{-1}$$

Calculation of the dichloromethane concentration in the cell membrane ($C_{dcm,mem}$) in a system with 500 mL water, 197.57 mL dodecane, 2.43 mL dichloromethane (1.2% $v_{dcm} v_{org.fase}$) and $1.0 \cdot 10^9$ cells L$^{-1}$ present, the dichloromethane partitions between the water phase, the dodecane and the cell membrane.
Table 5.1: Data for calculation of dichloromethane concentration in aqueous phase and membrane. With $M_{dcm}$: mass dichloromethane, $V_w$: volume water, $V_d$: volume dodecane, $V_{mem}$: volume cell membrane and $P_{dcm\_mem/w}$: partition coefficient of dichloromethane in cell membrane/water.

<table>
<thead>
<tr>
<th>Data</th>
<th>1.2% v/v</th>
<th>5.0% v/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_{dcm}$ [mol]</td>
<td>0.038</td>
<td>0.156</td>
</tr>
<tr>
<td>$V_w$ [L]</td>
<td>0.500</td>
<td>0.500</td>
</tr>
<tr>
<td>$V_d$ [L]</td>
<td>0.197</td>
<td>0.190</td>
</tr>
<tr>
<td>$V_{mem}$ [L]</td>
<td>$0.5 \cdot 3.14 \cdot 10^{-6} \cdot 1.57 \cdot 10^{-6}$</td>
<td>$0.5 \cdot 3.14 \cdot 10^{-6} \cdot 1.57 \cdot 10^{-6}$</td>
</tr>
<tr>
<td>$P_{dcm_d/w}$ [-]</td>
<td>9.9$^a$</td>
<td>9.9$^a$</td>
</tr>
<tr>
<td>$P_{dcm_mem/w}$ [-]</td>
<td>2.26$^b$</td>
<td>2.26$^b$</td>
</tr>
</tbody>
</table>

$^a$: see calculation above. $^b$: data from Mojaat et al. (2008a)

Equations:

\[
M_{dcm} = V_w \cdot C_{dcm\_w} + V_d \cdot C_{dcm\_d} + V_{mem} \cdot C_{dcm\_mem}
\]

\[
C_{dcm\_d} = P_{dcm\_d/w} \cdot C_{dcm\_w}
\]

\[
C_{dcm\_mem} = P_{dcm\_mem/w} \cdot C_{dcm\_w}
\]

With three equations and three unknowns ($C_{dcm\_w}$, $C_{dcm\_d}$, $C_{dcm\_mem}$) we can calculate the dichloromethane concentration in the aqueous phase and in the cell membrane (Table 5.2)

Table 5.2: Dichloromethane concentrations in aqueous phase and in cell membrane.

<table>
<thead>
<tr>
<th>Data</th>
<th>1.2% v/v</th>
<th>5.0% v/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{dcm_w}$ [mM]</td>
<td>16</td>
<td>66</td>
</tr>
<tr>
<td>$C_{dcm_mem}$ [mM]</td>
<td>35</td>
<td>148</td>
</tr>
</tbody>
</table>
Chapter 6
Continuous production of carotenoids from *Dunaliella salina*

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 0.286 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.

This chapter has been submitted for publication: Kleinegris DMM, Janssen M, Brandenburg WA, Wijffels RH. Continuous production of carotenoids from *Dunaliella salina*.
Introduction

Carotenoids from *Dunaliella salina* are produced on a commercial scale in open ponds in e.g. Australia and Israel (Borowitzka 1999). Hejazi *et al.* (2004a) 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 (Hejazi *et al.* 2004b). However, we recently found that cell-dodecane contact resulted in cell death and carotenoids were only extracted from dead cells (Kleinegris *et al.* 2010b). Dead cells fall apart and consequently lose their carotenoids. The lipid globules containing the carotenoids dissolve easily in the lipophilic dodecane. 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.* (2008; 2010) showed that this approach is very suitable to study the effect of light stress on *Dunaliella salina*. In the turbidostat the 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.* (2004a). 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.
Materials and Methods

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. (2010c) with increased sodium chloride concentration. The HEPES-buffered medium (pH 7.5; 4.00·10^-2 M HEPES) consisted of 1.50 M NaCl, 9.95·10^-3 M Na,H,PO_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_3MoO_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.

Turbidostat system
The turbidostat-operated system with combined in situ extraction was performed in a flat-panel photobioreactor as described by Lamers et al. (2010) with modifications as shown in Figure 6.1.

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 2p 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 \(7 \cdot 10^8\) cells L\(^{-1}\). During the growth phase an average incoming light intensity was used of 200 \(\mu\text{mol photons m}^{-2} \text{ s}^{-1}\). 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 \(1.6 \cdot 10^9\) cells L\(^{-1}\) was reached, the culture was diluted to reach \(0.8 \cdot 10^9\) 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 \(\mu\text{mol photons m}^{-2} \text{ s}^{-1}\). The inflicted light stress resulted in carotenoid production by the cells. As shown by Lamers et al. (2010) 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.

Figure 6.1: Schematic drawing of the flat-panel turbidostat and gear pump for dodecane sparging. Left: front view. Right: side view (without gear pump).
The light intensity at the back side 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 \( L_{\text{dod}} \)) 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 \( L_{\text{dod}} \) \( L_{\text{RV}}^{-1} \) min\(^{-1} \)) was calculated based on the volumetric sparging rate applied by Hejazi et al. (2004a) of 0.200 \( L_{\text{dod}} \) 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. (2010) with minor modifications and set-points for temperature, aeration, pH as described above. The reactor was inoculated at a lower cell concentration of \( 7 \times 10^7 \) 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 (\( L_{\text{RV}} \)).

**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 (100 μm orifice; Beckman Coulter, Fullerton, CA, USA). 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. (2010c) 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. (2010b). One mL aliquots of cell suspension were centrifuged at 3220 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 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. (2010b).

\[
C_{\beta-car} = \frac{(ABS_{453} - ABS_{665})}{3.91} \times 3.657 \times 3 \times X \quad [\text{mg/L}]
\]

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 mL 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.

**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.
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. (2010) the cells use the first four hours 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 Figure 6.2. This rate is comparable to values obtained by Hejazi et al. (2004a) 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 whilst keeping the total aqueous volume constant via an overflow tube. As can be seen in Figure 6.3 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 (Kleinegris et al. 2010b). 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 carotenoids 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 the first run and
shows that cell growth was considerably higher than cell death. In Table 6.1 all steady state data for the experiments performed are summarized. With a cell concentration of $1.6 \cdot 10^9$ cells LRV$^{-1}$ and a cellular $\beta$-carotene content of $9$ pg $\beta$-carotene cell$^{-1}$, combined with the aforementioned dilution rate of $0.55$ d$^{-1}$, a daily volumetric production rate of $7.6$ mg $\beta$-carotene LRV$^{-1}$ d$^{-1}$ is obtained. As a comparison, $\beta$-carotene extraction by the dodecane yielded $0.7$ mg Ldod$^{-1}$ d$^{-1}$, which was ten times less than the $7.6$ mg of $\beta$-carotene removed from the reactor via the biomass overflow. Consequently, we can conclude that a much higher productivity 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 $\beta$-carotene into account the total volumetric productivity of the system was $8.3$ mg $\beta$-carotene LRV$^{-1}$ 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...
Continuous production

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 Figure 6.4. The small dodecane 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 (Figure 6.4d).

With these experiments we showed that it was possible to shift the equilibrium between cell growth and cell death obtained by Hejazi et al. (2004a) towards a higher cell growth rate (Table 6.1). 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 6.1.

Figure 6.3: 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.
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 Figure 6.5 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 \(1.2 \times 10^9\) cells L\(_{\text{LRV}}^{-1}\) and a cellular \(\beta\)-carotene content of 15 pg \(\beta\)-carotene cell\(^{-1}\), combined with the aforementioned dilution rate of 0.75 d\(^{-1}\) a daily volumetric carotenoid production of 13.5 mg \(\beta\)-carotene L\(_{\text{LRV}}^{-1}\) d\(^{-1}\) was reached, which was more than half as much as the daily volumetric carotenoid production of 8.3 mg \(\beta\)-carotene L\(_{\text{LRV}}^{-1}\) d\(^{-1}\) of the first run, 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
Continuous production

Table 6.1: Comparison of steady state values of the \textit{in situ} extraction experiment of Hejazi \textit{et al.} (2004a), the \textit{in situ} extraction turbidostat experiment of this article, and the turbidostat without \textit{in situ} extraction.

<table>
<thead>
<tr>
<th></th>
<th>Hejazi \textit{et al.} (2004a)</th>
<th>With \textit{in situ} extraction</th>
<th>Without \textit{in situ} extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light intensity</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
</tr>
<tr>
<td>([\mu \text{mol} \text{ m}^{-2} \text{ s}^{-1}])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light path</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>[m]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transmission</td>
<td>-</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>[%]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sparging rate</td>
<td>0.286</td>
<td>0.286</td>
<td>0.286</td>
</tr>
<tr>
<td>([\text{lrod LRV}^{-1} \text{ min}^{-1}])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reactor volume (aqueous phase)</td>
<td>0.7</td>
<td>1.9</td>
<td>2.5</td>
</tr>
<tr>
<td>([\text{LRV}])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell concentration</td>
<td>(1.2\cdot10^9)</td>
<td>(1.6\cdot10^9)</td>
<td>(1.2\cdot10^9)</td>
</tr>
<tr>
<td>([\text{cells LRV}^{-1}])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cell volume</td>
<td>-</td>
<td>(1.1\cdot10^{-3})</td>
<td>(1.5\cdot10^{-3})</td>
</tr>
<tr>
<td>([\text{LCV LRV}^{-1}])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average cell volume</td>
<td>-</td>
<td>(7.4\cdot10^{2})</td>
<td>(1.2\cdot10^{3})</td>
</tr>
<tr>
<td>([\text{fl cell}^{-1}])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intracellular carotenoid concentration</td>
<td>-</td>
<td>12.0</td>
<td>12.2</td>
</tr>
<tr>
<td>([\text{g }\beta\text{-carotene LCV}^{-1}])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular carotenoid content (^a)</td>
<td>4-51</td>
<td>8.9</td>
<td>15.0</td>
</tr>
<tr>
<td>([\text{pg }\beta\text{-carotene cell}^{-1}])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotenoid content per dry weight</td>
<td>-</td>
<td>28.1</td>
<td>19.0</td>
</tr>
<tr>
<td>([\text{mg }\beta\text{-carotene g}_{\text{DW}}^{-1}])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Net specific growth rate</td>
<td>0</td>
<td>0.55</td>
<td>0.75</td>
</tr>
<tr>
<td>([\text{d}^{-1}])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Produced (\beta)-carotene in biomass overflow</td>
<td>0</td>
<td>7.6</td>
<td>13.5</td>
</tr>
<tr>
<td>([\text{mg }\beta\text{-carotene LRV}^{-1} \text{d}^{-1}])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracted (\beta)-carotene in dodecane</td>
<td>2.5</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>([\text{mg }\beta\text{-carotene LRV}^{-1} \text{d}^{-1}])</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\): the cellular carotenoid content increased in time from 4 to 51 pg cell\(^{-1}\).
first run (with in situ extraction) the cell concentration was higher than for the second run (without in situ extraction), namely $1.6 \times 10^9$ cells LRV$^{-1}$ and $1.2 \times 10^9$ cells LRV$^{-1}$ respectively. The intracellular carotenoid concentration was for both runs comparable (12 g LCV$^{-1}$), 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$^{-1}$.

![Figure 6.5: Cell concentration in the reactor [\# LRV$^{-1}$], 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.](image)

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 $1.0 \times 10^9$ cells LRV$^{-1}$ 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 photo-oxidative damage, this will have had a diminishing effect on the growth rate (Foyer *et al.* 2009; Ledford and Niyogi 2005).

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 (Hejazi *et al.* 2003a; Lin and Chen 2005). 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 (Ben-Amotz *et al.* 1982; Borowitzka and Borowitzka 1988). 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 (Ben-Amotz 1995; Lamers *et al.* 2010; Mojaat *et al.* 2008b; García-González *et al.* 2005). Leonardi and Cáceres (1997) 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 Figure 6.6. (Lerche 1937; Oren 2005; Borowitzka and Siva 2007). Most cells were clearly orange, containing a high intracellular carotenoid concentration, although some green cells remained present as well.

Figure 6.6: 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.

Hejazi et al. (2004a) found a volumetric productivity of 2.5 mg β-carotene LRV⁻¹ d⁻¹ for a similar system (Table 1). In the research of Hejazi et al. (2004a) 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
Continuous production

concentration of the biomass increased in time from 3 to 60 mg β-carotene LRV⁻¹ in 46 days. If we take this accumulation of carotenoids into account we can deduce an additional productivity of 1 mg β-carotene LRV⁻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 LRV⁻¹ d⁻¹ with in situ extraction and 13.5 mg β-carotene LRV⁻¹ d⁻¹ without the combination with in situ extraction. 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. (2010) increasing the light intensity to 1400 μmol photons 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 LRV⁻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.

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 LRV⁻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 LRV⁻¹ d⁻¹ was reached. In both systems the carotenoids from the produced biomass still have to be extracted. For the first system, 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. 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.

**Acknowledgements**

This research was financially supported by the technology foundation STW (www.stw.nl; project WLM.6622), which is part of the Netherlands Organisation for Scientific Research (www.nwo.nl).
Chapter 7

Two-phase systems: potential for in situ extraction of microalgal products

Abstract

Algae are already being used for production of niche products and become more and more interesting for the production of bulk products, as biodiesel. Before the production of these bulk products will be economically feasible the production costs will have to be lowered with one order of magnitude. The application of two-phase systems might be used to lower production costs, as the costly step of cell harvesting can be circumvented, whilst the product is extracted and ready for down-stream processing. The mechanism of extraction is a fundamental aspect of the practical question whether two-phase systems can be applied for in situ extraction, viz, simultaneous fermentation and extraction, or as a separate down-stream processing step. Three possible mechanisms are discussed; 1) products excretion and extraction, 2) cell permeabilization, product leakage and extraction, and 3) cell death and product extraction. It was shown that in case of product excretion, the application of two-phase systems for in situ extraction can be very valuable. In case of permeabilization and cell death, in situ extraction is not preferred, but the application of two-phase systems as down-stream extraction step can be part of a well-designed biorefinery process. This way processing costs can be decreased while the product is mildly and selectively extracted.

Thus far all algal strains used in two-phase systems have shown not to excrete their product, but cell death was necessary to obtain the product of interest. Two-phase systems can be a good approach for these species as down-stream processing step. For future applications of two-phase in situ extraction in algal production processes, either new species that show product excretion should be discovered, or existing species should be modified to induce product excretion.

This chapter has been submitted for publication in combination with chapter 1: Kleinegris DMM, Janssen M, Brandenburg WA, Wijffels RH. Two-phase systems: potential for in situ extraction of microalgal products.
Introduction

Algae are used for commercial production of high value products for niche markets, such as e.g. carotenoids with *Dunaliella salina* or *Haematococcus*, health food with *Chlorella* and *Spirulina* and several species for aquaculture (Borowitzka 1999). In these cases the algae are usually cultivated in large open ponds and after harvesting the cells are either used as whole-cell product or the interesting compounds are extracted in a separate down-stream process.

Momentarily, there is an increased worldwide attention for the production of microalgae because of their potential as biodiesel feedstock producers, as many species are well known for their high content of lipids and some species excrete hydrocarbons. However, only if algal biomass can be produced at a cost below 0.50 € kg\(^{-1}\) it becomes feasible to compete with fossil fuels, whereas at this moment, with present knowledge and systems, it seems possible to produce biomass in a reactor of 100 ha at a cost price of 4 € kg\(^{-1}\) (Wijffels *et al.* 2010).

In the production process of algae and their products, one of the most expensive steps is harvesting as the biomass concentrations reached are not that high and separation of the cells from the water costs large amounts of energy (Lardon *et al.* 2009; Schlipalius 1991; Tafreshi and Shariati 2009; Wijffels *et al.* 2010). The harvesting process encompasses first the separation of the cells from the liquid phase by e.g. centrifugation, filtration, gravity sedimentation or flocculation. Next the biomass can be processed further by drying it (spray-drying, drum-drying, freeze-drying or sun-drying) or the product can be extracted from the wet biomass paste by extraction with e.g. edible oils, solvents, liquid or supercritical CO\(_2\), or it can be crystallized (Del Campo *et al.* 2007; Tafreshi and Shariati 2009; Ben-Amotz 1995; Molina Grima *et al.* 2003). As can be imagined, every step has its own advantages depending on time, costs and product recovery. Moreover, each algal strain has its own characteristics (size, shape, buoyancy, cell wall thickness and robustness) and therefore not every step is appropriate for each algal strain.

Over the years, extraction of water-insoluble products in two-phase systems has been applied to a wide variety of biocatalysts, ranging from enzymes to bacteria, yeasts, plant cell cultures and algae. *In situ* two-phase extraction is the combination of fermentation and production with simultaneous product
extraction. This process is used to increase productivities by e.g. alleviating feedback inhibition of the product on cell growth or product formation, or prevention of product degradation by extracellular enzymes, and can be used to circumvent cell harvesting steps (Stark et al. 2002; Anvari and Khayati 2009; Sim and Chang 1993). Furthermore two-phase systems are applied as down-stream process to selectively extract and concentrate the product of interest and thus ease further down-stream processes.

The choice for two-phase extraction systems, either combined with the fermentation step or not, depends on the choice of microorganism, the product, the solvent and the mechanism of extraction. In the next paragraphs we will discuss possible mechanisms and in relation to that the potential of two-phase microalgal production processes.

**Mechanisms of extraction**

**Excreted products**

The storage location of compound of interest is of importance in the extraction process. For example, for most plant cells secondary metabolites are stored in the vacuole. Secretion of the accumulated product by the cell into the medium is an important parameter for a successful extraction process. When a second, organic, phase is applied, the hydrophobic excreted product will be extracted to the second phase.

Small particles such as inorganic ions and small water-soluble organic molecules can leave the cell via specialized transmembrane carrier or channel proteins. Larger particles can be transferred over the cell membrane via exocytosis (Figure 7.1). Usually the compound of interest produced by the biocatalyst will be a large molecule, and excretion by the cell will take place via exocytosis. Excretion of products can appear spontaneously or can be induced. For example during the production of the secondary metabolite anthraquinone from *Cruciata glabra* cell cultures, where excretion of anthraquinone is induced. Normally these cells retain the secondary metabolite anthraquinone intracellularly and only release very small amounts under physiological conditions. Dörnenburg and Knorr (1996) have shown that the addition of the triglyceride Miglyol to a culture of *C. glabra* cells increased anthraquinone synthesis and induced the release with
increasing Miglyol concentration. Another successful example of induced release of the product from the cells is the production of shikonin with *Echium italicum* L. cells described by Zare et al. (2010). The addition of liquid paraffin to the medium in which the *E. italicum* cells were grown induced production of shikonin, whereas without the liquid paraffin no shikonin was produced.

In most cases, direct cell-solvent contact is not necessary to extract the product. The excreted product is released into the medium and freely available for extraction. This does not hold for the extraction of hydrocarbons from *Botryococcus braunii*. The excreted hydrocarbons are stored in the outer matrix of the cell colonies and direct contact between the colonies and the solvent phase is necessary for extraction (Frenz et al. 1989a, 1989b; Sim et al. 2001). Direct contact generated several adverse effects, such as biomass aggregation. Moreover the choice of solvent was difficult. Biocompatible solvents did not show good extractability as the aqueous concentrations did not become high enough. More polar solvents showed good extractability but had a negative effect on cell viability (Frenz et al. 1989b). This process could be much more successful if the hydrocarbons would be released into the medium and consequently direct cell-solvent contact related problems could be avoided.

Systems without direct contact between cells and the solvent phase have advantages, such as easy phase separation, prevention of emulsion formation, and a widened choice of solvents. Emulsion formation can give rise to problems.
in subsequent down-stream processing steps as the solvent phase and the aqueous phase are difficult to separate. In many cases, biocompatibility of a solvent is reversely related to extractive power. In a system without direct contact between cells and organic phase only molecular solvent toxicity will play a role and not phase toxicity. Therefore, solvents can be chosen that show increased extractability without affecting cell viability.

In order to prevent direct contact between the cells and the solvent phase systems with external circulation, the use of membranes or encapsulation of either phase can be used.

For example, during the production of solavetivone from hairy root cultures of *Hyoscyamus muticus* the application of an external loop circulation yielded a doubled productivity as it allowed for the use of a solvent with lower biocompatibility but higher distribution coefficient (Corry et al. 1993). The structure of the hairy root cultures of *Hyoscyamus muticus* resembled immobilized tissue. Consequently, it was possible to re-circulate cell-free medium through a trickle-bed bioreactor using the volatile organic solvents hexane and pentane to recover the product. Hexane and pentane are toxic when directly added to a culture of *H. muticus*, but by applying an external circulation loop the cells were only exposed to very low concentrations due to their low solubility in water.

The use of membranes to separate the organic solvent from the biomass is another method to circumvent certain process constraints of two-phase systems. Easy phase separation, prevention of emulsion formation, and a widened choice of solvents as there is no phase toxicity and no need for a significant density difference between aqueous phase and solvent. Furthermore, there is a wide variety of membranes and set-ups possible, depending on the product and solvent used.

For example, in the biotransformation process of acetophenone to S-phenylethanol by *Rhodotorula glutinis* the use of a membrane bioreactor combined with organic solvent nanofiltration yielded acceptable reaction rates and high overall S-phenylethanol formation (Valadez-Blanco and Livingston 2009). Direct contact between cells and solvent proved unsuitable for this process as the used solvents either showed phase-toxicity to the cells or low product partitioning.

Doig *et al.* (1998) and Jeon and Lee (1989) describe the use of silicone rubber
membranes for the reduction process of geraniol to citronellol by baker’s yeast and the acetone-butanol fermentation by Clostridium acetobutilicum respectively. Doig et al. (1998) pumped the organic solvent hexadecane through the silicone tubing that was coiled around a cylindrical mesh cage in the bioreactor, whereas Jeon and Lee (1989) pumped the broth through the silicone tubing from the fermentor to a membrane compared separator containing the organic phase. Both systems prevented emulsion formation and alleviated feedback inhibition.

Encapsulation is a third method of preventing cell-solvent contact. Amongst others, it is used as protection against toxic solvents. Buitelaar et al. (1990) showed that immobilization of Tagetes minuta cells in calcium alginate provided slight protection against toxic solvents. Encapsulation of the organic solvent to protect Saccharomyces cerevisiae cells from phase contact was described by Stark et al. (2003). Moreover, product extraction decreased feedback inhibitory effects on the cell. The solvent dibutyl sebacate was encapsulated in a polymeric membrane and was either placed directly in the fermentor within a mesh cylinder, or in an external fluidized bed. The product-loaded capsules can either be directly used for a specific application or continuous regeneration of the capsules is possible.

Furthermore, encapsulation can induce product formation. For example during anthraquinone production with Crucita glabra cells, where the combination of immobilization with extraction resulted in increased anthraquinone production. The use of elicitor active polysaccharides in the immobilization matrix resulted in increased anthraquinone production, and the continuous extraction of the anthraquinones to the second phase (the triglyceride Miglyol) prevented feedback regulatory mechanisms and intracellular and extracellular product degradation (Dönnenburg and Knorr 1996). Brink and Tramper (1985) showed that immobilization of Mycobacterium cells did not protect the cells from inactivation by the organic solvent. However, the gel entrapment did reduce other disadvantages of the two-phase system, such as clotting of biomass and aggregation of cells at the liquid-liquid interface.

In the abovementioned examples it was shown that in case products are excreted into the medium in situ extraction is a very good choice to improve the production process. Product yields can be increased by induced product formation (e.g. by alleviating feed-back inhibition) and decreased product degradation. Because direct cell-solvent contact is not necessary in case of excreted products,
solvents with increased extractability can be chosen as solvent toxicity is limited to phase toxicity. Moreover, other possible disadvantages of two-phase systems as emulsion formation can be circumvented by separation of the two phases.

**Permeabilization**

In case the product is not or only at very low concentrations excreted by the cells two-phase systems can be applied to enhance or initiate product release due to cell permeabilization. If cells are made permeable they will leak intracellular low-molecular components to the surroundings, whilst in most cases the morphology of the cells remains intact (Figure 7.2).

However, in most cases induced extraction of intracellular products due to permeabilization goes together with suppressed cell growth, decreased cell viability or complete cell death (Brodelius 1988; Felix 1982; Flores *et al.* 1994; Wu *et al.* 2000; Xu *et al.* 2004; Zhang and Xu 2001). This will lead to decreased productivities or problems such as emulsion formation as dead cell material will act as emulgator. Moreover, the selectivity of the extraction process will decrease as more cell components will leak out of the cells, which can also be extracted to the organic phase.

However, in some cases successful permeabilization processes have been described. For example, the permeabilization of preconditioned *Coleus blumei* cells was shown to be successful with no increase in cell death compared to cultures without DMSO treatment (Park and Martinez 1992). Brodelius and Nilsson (1983) showed that it was possible to release the intracellularly stored the indole alkaloid ajmalicine from *Catharanthus roseus* cells with 5% DMSO treatment. However, permeabilization was easier on immobilized cells as immobilization mechanically stabilized the cells and prevented essential macromolecules from leaving the permeabilized cells (Brodelius 1985).

In conclusion we can say that although there are some examples of successful permeabilization processes, in general the application of two-phase systems for cell permeabilization does not seem to have great potential for *in situ* extraction. However, it can be a very elegant approach in down-stream processing for mild, selective extraction. Wijffels *et al.* (2010) showed that biodiesel production from microalgae can only be economically feasible in case all components from the algae are used. For this the components need to be extracted and fractionated while their functionality can be maintained. Therefore, permeabilization as mild
Chapter 7

Extraction by induced cell death

In the previous chapters we have focused on *in situ* extraction of carotenoids from *Dunaliella salina* and the mechanism that underlies this extraction process. Hejazi *et al.* (2004a) developed a two phase system for the continuous production and extraction of β-carotene from *Dunaliella* cells. Dodecane was used as an organic phase. It was found that the process could be run for over six weeks with constant levels of biomass and constant carotenoid extraction. A similar process for production and extraction of carotenoids from *Dunaliella salina* was described with decane as solvent or a combination of decane and dichloromethane (Leon *et al.* 2003; Mojaat *et al.* 2008a). It was hypothesized that carotenoids were extracted from the biomass and the biomass stayed alive. Carotenoids were thought to be continuously produced in the extracted cells. Kleinegris *et al.* (2010b) recently found that in fact the mechanism behind this extraction process was cell death. As was shown, upon direct phase contact dodecane entered and altered the cell membrane (Hejazi *et al.* 2004b). This did not result in extraction through exocytosis as suggested by Hejazi *et al.* (2004b), but it weakened the cell membranes in such way that the cells were disrupted into cell fragments (Kleinegris *et al.* 2010b). Consequently the carotenoid containing lipid globules were free for extraction, as shown in Figure 7.3.

**Figure 7.2:** Schematic drawing of the extraction mechanism: permeabilization.
A similar kind of extraction process to that of *Dunaliella* was described for *Haematococcus* cells grown on carrier materials (Fiedler et al. 2007). Cell death was necessary for extraction of astaxanthin from the cells. However, by applying cell-protective additives in combination with organic solvent only a part of the algal cells was destroyed for astaxanthin extraction and part of cells remained viable for re-cultivation.

In these processes, especially in the case of carotenoid extraction from *Dunaliella salina*, good volumetric productivities can be reached. Improved extraction rates were obtained with increased mixing, however, this decreased the selectivity of the extraction process (Hejazi et al. 2004a; Kleinegris et al. 2010c). The chlorophyll that is bound to the more hydrophilic photosynthetic membranes does not easily dissolve in the organic phase, as was shown on CLSM pictures in the study of Kleinegris et al. (2010b). But increased contact due to increased mixing resulted in more chlorophyll extraction (Kleinegris et al. 2010c). Moreover, comparison between a turbidostat-operated system with and without *in situ* extraction showed that the highest volumetric productivity could be reached without *in situ* extraction. Emulsion formation, elevated oxygen concentrations limiting cell growth, and light-dependent carotenoid degradation decreased the volumetric productivity in case of *in situ* extraction (Kleinegris et al. 2010d).

Although the process might be improved on some of these aspects, it is not easy to solve all disadvantages. Extraction in a separate vessel might be one approach.
For example, a combination of cultivation and down-stream processing for *Botryococcus* is described by An *et al.* (2004) in which the biomass was again recultivated after extraction. The broth was recycled between a bubble column for growth and a two-phase separator for hydrocarbon extraction. This yielded more than 60% recovery of hydrocarbons, however, the cell growth rate was seriously decreased. Moreover, the biomass still had to come into direct contact with the organic phase, ensuring many of the aforementioned problems as aggregation of biomass and difficult phase separation. In the case of carotenoid extraction from *Dunaliella*, a separate vessel could decrease the problem of light-dependent carotenoid degradation, but accumulating dead cell material will induce problems with phase separation and give problems with contaminations as the microalgal cell debris makes a good substrate for bacterial growth.

Consequently, it can be concluded that the combination of fermentation and production with two-phase extraction does not show potential when cell death is necessary for extraction. However, the use of two-phase extraction only as a down-stream processing step might be appropriate and could decrease processing costs as the biomass does not need to be harvested. Moreover, as with permeabilization, induced cell death could be part of a biorefinery process as a relatively mild and selective extraction step.

Kang and Sim (2007; 2008) describe this approach for the extraction of astaxanthin from *Haematococcus pluvialis*. They integrated cell harvesting and extraction into one down-stream processing unit. After vigorous mixing of broth with dodecane, the red cysts were disrupted and the astaxanthin was extracted to the organic phase. After sedimentation the dodecane could easily be separated from the broth and was used for further recovery of the astaxanthin by methanol extraction.

Benavides *et al.* (2008) mentioned the practical and economic interest of aqueous two-phase systems for direct integration of cell disruption and primary recovery. Aqueous two-phase systems combined in one process step with for example bead milling or sonification to destroy cell material were successfully applied for the extraction of e.g. 3-phosphate dehydrogenase from bakers' yeast, c-phycocyanin from *Spirulina maxima* or b-phycoerythrin from *Porphyridium cruentum*, and lutein from *Chlorella protothecoides* (Rito-Palomares and Lyddiatt 2002; Cisneros *et al.* 2004; Cisneros and Rito-Palomares 2004; Benavides and Rito-Palomares 2006).
Future potential

As was shown in the above discussion, in situ extraction can be a very advantageous system for product extraction when the product of interest is excreted. However, thus far all algal strains used in two-phase systems have shown not to excrete their product. Cell death was necessary for Dunaliella, Haematococcus, Spirulina, Porphyridium and Chlorella strains to obtain the desired products. Only Botryococcus braunii produces hydrocarbons in the outer matrix where they are freely available for extraction. However, extraction rates were much less than desired, as the used solvents were either not capable enough to extract high amounts of hydrocarbons or lethal to the cells. Moreover, research on Dunaliella and Botryococcus showed that in situ extraction caused many problems related to two-phase systems, such as emulsion formation, clump formation of the biomass, decreased selectivity of the process or product degradation in the organic phase. As the product was not spontaneously excreted into the aqueous phase, these problems could not be prevented by separation between both phases and external circulation or by using membranes or encapsulation of either phase as aforementioned.

In case cell death is necessary for product extraction the combination of in situ extraction with the fermentation process is not favorable, as dead cell material might influence the process, induce emulsion formation, or stimulate bacterial growth, as described before. However, the application of the two-phase system as a down-stream processing step seems to be a very promising and successful step to decrease processing costs and ease further down-stream processing. This does not mean however, that in situ extraction can not be appropriate for algal production purposes. First of all, the biodiversity amongst algae is enormous, with more than 100.000 assumed species (Norton et al. 1996). Consequently it is very plausible that there exist interesting species that can excrete their products. In that case in situ extraction is very interesting, not only to decrease the cost price by avoiding an expensive harvesting step or decreasing down-stream processing difficulties by product concentration in the solvent phase. But also in situ extraction can be used to alleviate feedback inhibition of the product on its biosynthesis route or on cell growth, and product degradation by e.g. extracellular enzymes is circumvented.

Moreover, it is possible to modify existing species, e.g. with molecular tools, and
make them excrete their product. Microalgae become more and more popular for genomics research. The total genome of the green algae *Chlamydomonas reinhardtii, Ostreococcus lucimarinus* and *Ostreococcus tauri* is already sequenced and other algae are being processed (Merchant et al. 2007; Molnar et al. 2007, internet 2). Moreover, in the last years the development of gene manipulation technologies such as transformation with plasmid DNA and RNA-mediated gene knock down techniques have shown to be successful for algae (Tan et al. 2007; Zhao et al. 2009). An alliance between Craig Venter’s Synthetic Genomics Inc. and ExxonMobil has recently gone public with its research on biodiesel production with an algal strain that excretes its hydrocarbons (Internet 3).

Concluding, we can say that so far *in situ* extraction did not show its full glory for algal production processes. However, both *in situ* extraction (the combination of extraction with the fermentation process) and two-phase extraction systems for downstream processing should definitely not be ruled out for the future as they have already shown to be very promising production systems.
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Summary
Summary

Dunaliella salina is a green alga, capable of producing large amounts of β-carotene when stressed. This property makes the alga a widely used source of natural β-carotene for the food, pharmaceutical, and cosmetic industry. Until now the alga is commercially cultivated in large open ponds in warm climates. After a growth period and subsequent stress period during which large amounts of carotenoids are produced, the biomass is harvested and the cells are either used as carotenoid-rich biomass, or further processed to extract the carotenoids from the biomass. Another suggested process is the continuous production and simultaneous extraction of the carotenoids in a closed photo-bioreactor. A two-phase system is applied for the extraction of the β-carotene, with dodecane as biocompatible organic phase. It was hypothesized that the cells lose their carotenoids to the organic phase and then reproduce the lost carotenoids. However the exact mechanism of this process was unknown. In order to be able to optimize this process it is necessary to understand the mechanism of extraction. Moreover, when this mechanism is understood and can be controlled, it can be applied to other microalgae, for example in the production of biodiesel.

In chapter 2 a first look at the extraction mechanism of β-carotene from the cells is described. Microscopy experiments showed that the presence of dodecane resulted in alterations of the cell membrane. Furthermore, it was shown that dodecane was taken up by the cells, up to a concentration of 13 pg cell\(^{-1}\). Based on these results, two possible modes of extraction were suggested. The first suggestion is the transport of the carotenoid-containing globules from the chloroplast to the space between the cell and the chloroplast membranes and subsequently from there to the outside by exocytosis. The second suggestion for the extraction mechanism is the release of β-carotene from the globules as a result of membrane modifications induced by uptake of dodecane. The released β-carotene molecules then diffuse from the chloroplast to the space between the cell and the chloroplast membranes and from there to the medium.

In chapter 3 we studied the selectivity of the carotenoid extraction process from Dunaliella salina. Previously it was shown that it were mainly carotenoids that were extracted, with β-carotene being the main carotenoid produced, and that almost no chlorophyll was extracted. We studied whether it was possible to vary
the profile of the produced carotenoids and if it consequently was possible to influence the type of carotenoids extracted. We varied the profiles of the produced carotenoids by using three different *Dunaliella salina* strains and three different stress conditions. The profiles of the carotenoids produced did not differ remarkably between *D. bardawil* and *D. salina* 19/18, but did in case of *D. salina* 19/25. However, it was not possible to extract carotenoids from this last strain. Carotenoid extraction from *D. salina* 19/18 yielded slightly higher amounts than from *D. bardawil*. The extraction process was selective for secondary carotenoids in case gentle mixing was used. However, in aerated flat-panel photobioreactors with increased mixing the selectiveness decreased and also chlorophyll and primary carotenoids were extracted. Yet, at the same time the extraction rates increased.

In chapter 2 it is described that confocal laser scanning microscopy (CLSM) was used to study the cells and the location of the β-carotene containing globules. It was assumed that the green autofluorescence from the cells was emitted by the carotenoid containing globules. In chapter 4 we show that indeed the carotenoid containing globules emit green fluorescence. We isolated the globules and determined the absorption and emission spectra from this solution. When comparing the relative quantum yields obtained from the absorbance and fluorescence emission spectra with data from literature, we can conclude that the green fluorescence was most likely emitted by the β-carotene in the globules. Consequently, fluorescence microscopy techniques such as CLSM can be used as a non-invasive imaging technique to study the carotenoid globules in *D. salina* in vivo.

In chapter 5 we used this fluorescence microscopy technique together with macro-scale extraction experiments in small reactor flasks to study the mechanism of the extraction process. It was shown that water-solvent interphase contact resulted in cell death. This cell death and consequent cell rupture resulted in release of the carotenoid containing globules and their extraction. The addition of a small amount of dichloromethane to the dodecane was used to create an organic phase with more extraction capacity. The increased extraction capacity depended only slightly on improved solubility of the carotenoids but much more importantly on the increased cell death caused by the dichloromethane.

As was mentioned before, the mechanism for extraction is important knowledge for process optimization. It was previously shown that the extraction process
could run for a long time with constant biomass concentrations and increasing carotenoid extraction levels. As cell death is necessary for extraction, cell death and cell growth should be at equilibrium to ensure constant biomass concentrations and thus a continuous in situ extraction process. In chapter 6 we used a flat-panel photobioreactor that was operated as turbidostat to keep cell numbers of stressed cells constant. In this way it was possible to study the balance between cell death and cell growth. In the turbidostat combined with in situ extraction a volumetric productivity of 8.3 mg β-carotene LRV⁻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 relatively high growth rate of the cells and subsequent dilution of the reactor. A reference run without in situ extraction yielded a volumetric productivity of 13.5 mg β-carotene LRV⁻¹ d⁻¹, which was solely based on the continuous production of carotenoid-rich biomass. The differences between these runs were explained by the lower growth rate in the run with in situ extraction due to high oxygen concentrations and, possibly, light-dependent degradation of carotenoids in the organic phase. Moreover, emulsion formation was a major problem in the run with in situ extraction.

As was shown in chapter 6, in situ extraction has several disadvantages. However, there are many reasons why two-phase systems and in situ extraction could be interesting for algal production processes, for example to increase productivities and lower cost prices for down-stream processing. In chapter 7 we describe when application of two-phase systems is advantageous. The guideline is the actual mechanism of extraction; 1) excretion of products and subsequent extraction, 2) cell permeabilization, leakage of the product and subsequent extraction, 3) cell death followed by lysis, product liberation, and subsequent extraction. In case of excretion of products in situ extraction has many advantages, especially in those cases where direct contact between cells and solvent can be circumvented and, consequently, the accompanying difficulties during processing such as emulsion formation. Moreover, product yields can be increased due to alleviation of feedback inhibition or prevention of product degradation. Cell permeabilization is not useful for in situ extraction, nor is cell death. However, both could be applied in two-phase down-stream processing steps in a ‘biorefinery’, to create mild and selective extraction processes and decrease processing costs as cell harvesting is...
not necessary.
For future algal production processes two-phase systems will be very useful, either as down-stream processing step to lower production costs in case the product of interest is not excreted (as shown for all algal strains thus far), or as in situ extraction process in case of newly discovered species that show product excretion or modified species with induced product excretion.
Samenvatting
Samenvatting

Dunaliella salina is een groene alg die in staat is om grote hoeveelheden bètacaroteen te produceren als hij gestrest wordt. Deze eigenschap zorgt ervoor dat hij wereldwijd veel gebruikt wordt door de levensmiddelen, farmaceutische en cosmetische industrie als bron voor natuurlijk bètacaroteen. Tot op heden wordt de alg voor commerciële doeleinden gekweekt in grote open vijvers in warme klimaatzones. Na een periode van groei en de daaropvolgende stress periode, gedurende welke grote hoeveelheden carotenoiden worden geproduceerd, wordt de biomassa geoogst. De cellen worden ofwel aangewend als carotenoidrijke biomassa, ofwel worden de carotenoiden uit de biomassa geëxtraheerd om op deze manier de pure vorm te verkrijgen.

Een ander proces dat is voorgesteld, is om de algen continu te kweken in een gesloten fotobioreactor en gelijktijdig de carotenoiden uit de biomassa te extraheren. Voor deze extractie wordt een tweefase-systeem toegepast, met dodecaan als biocompatibele organische fase. Het idee hierachter was dat de cellen hun carotenoiden aan de organische fase afstaan en tegelijkertijd nieuwe carotenoiden produceren om hun voorraad op te vullen. Echter, hoe het mechanisme achter dit proces precies werkte was nog onbekend. Om het mogelijk te maken het voorgestelde tweefase systeem te optimaliseren is het nodig om het mechanisme achter de extractie precies te begrijpen. Bovendien, zodra het mechanisme bekend en beheersbaar is, kan het tweefase-sytem worden toegepast ook op andere microalgen, zoals bijvoorbeeld voor de productie van biodiesel.

In hoofdstuk 2 wordt het eerste onderzoek naar het mechanisme achter de extractie van bètacaroteen uit de cellen beschreven. Met behulp van microscopie experimenten is aangetoond dat de aanwezigheid van dodecaan ervoor zorgt dat er in het celmembraan veranderingen plaatsvinden. Bovendien is er aangetoond dat dodecaan door de cellen opgenomen wordt, tot een concentratie van 13 pg cel⁻¹. Op basis van deze resultaten zijn twee mogelijke beschrijvingen van het extractiemechanismge gegeven. De eerste hypothese beschrijft het transport van de bolletjes waarin de carotenoiden opgehoopt zitten vanuit de chloroplast naar de ruimte tussen het chloroplastmembraan en het celmembraan. Vanuit daar worden de bolletjes getransporteerd naar het medium buiten de cel door
middel van exocytose. De tweede mogelijkheid betreffende het extractiemechanisme is dat het door de cel opgenomen dodecaan het membraan zodanig verandert dat de carotenoiden uit de vetbolletjes vrijkomen. De bètacaroteen moleculen verplaatsen zich dan door middel van diffusie vanuit de chloroplast naar de ruimte tussen de chloroplast en het celmembraan en vanuit daar naar het medium buiten de cel.

In hoofdstuk 3 hebben we gekeken naar de selectiviteit van het extractieproces van carotenoiden uit *Dunaliella salina*. In vorig onderzoek is aangetoond dat voornamelijk carotenoiden worden geëxtraheerd, met bètacaroteen als meest geproduceerde carotenoide, en dat er vrijwel geen chlorofyl geëxtraheerd wordt. Wij hebben bestudeerd of het ook mogelijk was om de variatie in geproduceerde carotenoiden te vergroten en of het daardoor ook mogelijk was om te beïnvloeden welke carotenoiden er geëxtraheerd zouden worden. Door drie verschillende stammen van *Dunaliella salina* te gebruiken en drie verschillende manieren om de cellen te stressen hebben we de variatie in geproduceerde carotenoiden beïnvloed. Tussen de stammen *D. bardawil* en *D. salina* 19/18 was er weinig verschil zichtbaar in de geproduceerde carotenoiden, echter tussen voorgaande twee en *D. salina* 19/25 was er een groot verschil zichtbaar. De extractie uit *D. salina* 19/18 gaf hogere concentraties geëxtraheerde carotenoiden dan uit *D. bardawil*. Verder bleek het niet mogelijk om de door *D. salina* 19/25 geproduceerde carotenoiden te extraheren. Het extractieproces was selectief voor secundaire carotenoiden in het geval van milde menging. Echter, door beluchte vlakkeplaat bioreactoren te gebruiken met een verhoogde menging werden ook primaire carotenoiden en chlorofyl geëxtraheerd. Tevens gingen ook de extractiesnelheden omhoog.

In hoofdstuk 2 werd beschreven hoe confocale laserscan microscopie (CLSM) werd toegepast om de cellen en de locatie van de carotenoidenbevattende bolletjes te bestuderen. Er werd aangenomen dat de groene autofluorescentie afkomstig van de cellen werd geëmitteerd door de carotenoidenbevattende bolletjes. In hoofdstuk 4 laten we zien dat het bovenstaande hypothese aannemelijk is en dat het de carotenoidenbevattende bolletjes zijn die de groene fluorescentie uitstralen. We hebben de bolletjes geïsoleerd en de absorptie en emissie spectra van deze oplossing bepaald. Door de relatieve kwantum opbrengsten te vergelijken met data uit de literatuur kunnen we stellen dat het waarschijnlijk de bètacaroteen in de bolletjes is die verantwoordelijk is voor de
Samenvatting

fluorescentie. Op basis van dit resultaat kan geconcludeerd worden dat fluorescentiemicroscopie technieken zoals CLSM gebruikt kunnen worden als niet-invasieve weergavetechnieken om de carotenoidenbevattende bolletjes in de cellen van *Dunaliella salina* *in vivo* te bestuderen.

In hoofdstuk 5 hebben we de bovenstaande fluorescentietechniek gebruikt tezamen met extractie experimenten op macroschaal om het mechanisme van extractie te bestuderen. Er is aangetoond dat contact met het grensvlak tussen water en oplosmiddel ervoor zorgt dat de cellen dood gaan. Deze celsterfte en dientengevolge het breken van de cellen zorgt ervoor dat de carotenoidenbevattende bolletjes vrij komen en hierdoor geëxtraheerd kunnen worden. Door een kleine hoeveelheid dichloromethaan aan de dodecaan toe te voegen wordt een organische fase gecreëerd met een hogere extractiecapaciteit. Deze verhoogde extractiecapaciteit vloeit maar voor een klein deel voort uit de verhoogde oplosbaarheid van carotenoiden in de organische fase, en voor een veel belangrijker deel uit de verhoogde celsterfte die veroorzaakt wordt door de dichloromethaan.

Zoals eerder genoemd is, is kennis over het extractiemechanisme van groot belang voor procesoptimalisatie. In voorgaand onderzoek is aangetoond dat het extractieproces meerdere weken kan worden volgehouden met constant blijvende biomassaconcentraties en een stijgend niveau van geëxtraheerde carotenoiden. Aangezien celsterfte nodig is om de carotenoiden te kunnen extraheren, moeten celgroei en celdood in evenwicht met elkaar zijn om constant blijvende biomassaconcentraties te kunnen waarborgen en zo een continu *in situ* extractieproces. In hoofdstuk 6 hebben we een vlakkeplaat fotobioreactor gebruikt, die als turbidostaat werd gedraaid om zo het aantal gestreste cellen gelijk te houden. Op deze manier was het mogelijk om de balans tussen celgroei en celdood te bestuderen. De turbidostaat in combinatie met *in situ* extractie leverde een volumetrische productiviteit van 8.3 mg bètacaroteen L_rv⁻¹ d⁻¹. *In situ* extractie leverde maar een beperkte bijdrage aan deze productiviteit. Het grootste gedeelte werd veroorzaakt door de netto productie van biomassa rijk aan carotenoiden. Deze productie kwam door een relatief hoge groeinsnelheid van de cellen en de daaraan gerelateerde hoge verdunningssnelheid van de reactor. De referentiekweek zonder *in situ* extractie gaf een volumetrische productiviteit van 13.5 mg bètacaroteen L_rv⁻¹ d⁻¹, welke alleen gebaseerd was op de continue productie van carotenoidenrijke biomassa. De verschillen tussen beide kweken
kunnen worden verklaard door de lage groeisnelheid in de kweek met in situ extractie, veroorzaakt door de hoge zuurstofconcentraties en, mogelijkerwijs, lichtgerelateerde afbraak van de carotenoiden in de organische fase. Bovendien was emulsievorming een groot probleem in de kweek met in situ extractie. Zoals in hoofdstuk 6 was aangetoond klaven er verscheidene nadelen aan in situ extractie. Echter, er zijn ook voldoende redenen om twee fase-systemen en in situ extractie een interessante optie te laten zijn voor productieprocessen gebaseerd op algen, zoals bijvoorbeeld om de productiviteit te verhogen of de kostprijs voor downstream processen te verlagen. In hoofdstuk 7 beschrijven we in welke gevallen de toepassing van twee fase-systemen voordelig is. Als richtlijn nemen we het extractiemechanisme; 1) excretie van het product en de daaropvolgende extractie, 2) het permeabel en hierdoor lek maken van de cel en de daaropvolgende extractie en 3) celsterfte, gevolgd door lysis, het vrijkomen van het product en de daaropvolgende extractie. In het geval van productexcretie heeft in situ extractie vele voordelen, voornamelijk in die gevallen waarin direct contact tussen de cellen en de organische fase kan worden voorkomen en dus de bijbehorende problemen als emulsievorming kunnen worden omzeild. Bovendien kunnen de opbrengsten worden verhoogd doordat feedbackgerelateerde inhibitie of productafbraak voorkomen kan worden. Het permeabel maken van cellen is, net als cel dood, minder geschikt in de toepassing voor in situ extractie. Beide extractiemechanismen kunnen echter wel slagen in twee fase-systemen die gebruikt worden als downstream proces stap in een zogenaamde bioraffinaderij. Ze kunnen op deze manier als milde en selectieve extractiestappen toegepast worden. Tegelijkertijd kunnen ze voor verlaagde proces kosten zorgen, doordat het oogsten van de cellen niet meer noodzakelijk is. Kortom, voor toekomstige op algen gebaseerde productieprocessen kunnen twee fase-systemen van groot belang zijn, oftewel als downstream proces stap om de productie kosten te verlagen als het product van belang niet uigescheiden wordt (wat het geval is voor alle algenstammen tot nu toe), oftewel als in situ extractiestap in het geval van nieuwe of aangepaste stammen die wel hun product kunnen uitscheiden.
Dankwoord
Dankwoord

Dat was het dan, het zit er bijna op en ik kan alleen maar terugkijken op een geweldige periode. Het was super om met die kleine groene monstertjes te werken, maar een heel groot gedeelte van de pret kwam toch wel voort uit de geweldige begeleiding, samenwerking en hulp die ik heb mogen ontvangen tijdens de afgelopen jaren. Mijn dank is groot!

René, je was de beste baas die ik me kon wensen! Je wist me te overtuigen dat onderzoek in Wageningen echt best wel leuk kon zijn en ik heb geen moment spijt gehad van mijn keuze om terug te komen en onder jou AIO te worden. Door je vertrouwen, je kritische vragen en je manier van overzicht behouden wist je me in onze besprekingen altijd weer te stimuleren en het beste proefschrift te willen maken. Bovendien was het supergezellig een bier met je te drinken, waar dan ook ter wereld!

Marcel, superbedankt voor alle input in de afgelopen jaren! Je enorme kennis, altijd kritische blik en wil om de puntjes op de i te zetten, hebben ervoor gezorgd dat ik met veel plezier heel veel heb geleerd de afgelopen jaren.

Willem, jij maakte mijn begeleiding compleet. Je had niet alleen oog voor de wetenschappelijk kant van alles, maar zorgde ook voor de persoonlijke mens. Je niet aflatende enthousiasme en bemoedigende gesprekken heb ik zeer gewaardeerd, bedankt!

Een hoofdlijn van mijn onderzoek was gericht op de selectie van de beste carotenoidenproducerende algen met behulp van flow cytometrie. Jammer genoeg bleek het niet mogelijk dit binnen de benodigde tijd te doen en daarom zijn de resultaten hier onvermeld gelaten. Toch wil ik Jan Bergervoet graag hartelijk bedanken voor alle hulp met de flow cytometer. Jan, elke keer weer als ik vragen had of de flow cytometer weer eens kuren was je altijd bereid te helpen en dat was best vaak! Bedankt, en ook voor de gezellige zeilgesprekken! Hopelijk zie ik je nog eens op of rond het water!

Ook ‘de mannen’ van de technische dienst van het Biotechnion wil ik hier graag bedanken. Elke keer weer hebben jullie alle wijzingen aan mijn reactor die weer eens liefst gister af moesten zijn voor elkaar gekregen en vaak nog beter dan dat ik het me had voorgesteld.

Graag wil ik hier ook van de gelegenheid gebruik maken om alle leden van de gebruikerscommissie van STW te bedanken voor hun inbreng tijdens onze projectvergaderingen.

Tijdens de afgelopen jaren heb ik lang niet alle werk zelf gedaan. Tijdens het project heb ik vier dames mogen begeleiden gedurende hun afstudeervakken. Katja, Darlene, Madzia, thank you all very much for all your work! Marjon, voor jou nog een speciaal dankjewel! Ik heb enorm genoten van je aanstekelijke enthousiasme en inzet. Samen als ‘jut en jul’ over de gang lopen giebelen, maar ook in ellenlange discussies het mechanisme van het melken proberen op te helderen was me een waar genoegen!

Hier aangekomen wil ik ook graag Norbert de Ruijter en André van Lammeren bedanken voor hun begeleiding van Marjon vanuit Plantencelbiologie. Door jullie hebben wij op een andere manier naar algen leren kijken. Bedankt voor jullie enorme inzet en enthousiasme! André, ik ben vereerd dat jij mijn opponent wilt zijn.

Maria, collega, mede-algenteler, vriendin! Dankjewel dat je er bent! Ik vind het enorm speciaal dat jij mijn opponent wilt zijn.

En dan iedereen van de vakgroep. Ohh, waar te beginnen en wie niet te vergeten. Jullie zijn allemaal ongelofelijk geweldige collega’s geweest en daarvoor hartelijk dank! Superbedankt voor de mooie tijd op ‘t lab en met al koffiepauzes, borrels, diners, labuitjes, brainstormweken, AIO-reizen, roeiwedstrijden en weet ik wat al nog meer.

Graag wil ik toch nog een paar mensen specifiek bedanken. Ten eerste mijn lieve kamergenootjes, dankjulliewel voor de gezellige tijden, in voor- en tegenspoed! Annette, Elsbeth, Thanawit, we have been roommates for more than four years, it was a madhouse sometimes, sharing good and bad times and all other ‘useful’ information and laughing our heads off, thank you for that! Kim, je bent er pas net, maar je ‘hokt’ gezellig mee, tof!

Packo en Annette, mede-vici’s, ook al hebben we uiteindelijk die megacaroproducerende superalg in übercoole geoptimaliseerde reactor niet meer gerealiseerd, ‘t was toch super om met jullie ons vici-clubje te vormen. Bedankt
Dankwoord

voor alle discussies, hulp en meedenken met alle rare ‘uitdagingen’ die af en toe weer voorbij kwamen! Fred, zonder je technische ondersteuning was ik nergens geweest, maar ook je oneindige bereidheid mee te denken om de experimenten beter te laten verlopen en gewoon de gezellige gesprekken heb ik altijd ontzettend gewaardeerd! Miranda, bedankt voor alle gezelligheid, heerlijke (onzin-) gesprekken en hulp bij maffe vragen! Rouke en Pieter, altijd als ik even blij wil worden hoe ik maar bij jullie langs te lopen en ik weet zeker dat ik weer grinnikend weglopen. Geniaal, bedankt! Koen, Anja, Wouter, het organiseren van de AIO-reis samen met jullie was supertof, en ik ben trots op wat we daar neergezet hebben, bedankt!

Klaske, Marieke, Koen, Sina, Jan, Floor, jullie zijn meer dan gewoon collega’s! Ik hoop dat ondanks dat we straks niet allemaal meer bij PRE werken we elkaar toch nog regelmatig blijven zien en onze gezellige avondjes blijven houden. Koen, die AIO-reisorganisatie heeft ons een tik gegeven ben ik bang, ik hoop dat we het nog heel lang volhouden onze Japan-fascinatie om te zetten in goede gesprekken, heerlijk eten en spelletjesavonden. Klaske, lang geleden begonnen als kamergenootje en nu eindigend als paranimf! Bedankt voor alles de afgelopen jaren! Wandelen rond Antalya, of was t nou Ankara….., was onvergetelijk! Ik hoop dat we nog lang vriendinnen blijven! Marieke, ondanks alle gemopper, ‘t was stiekem wel heerlijk om alle energie, frustraties en andere gedachtekronkelingen eruit te kunnen gooien tijdens ons rondje hardlopen. Bedankt voor het pushen mee te lopen en het luisterend oor! In de toekomst zal samen lopen wel niet meer zo vaak gaan lukken (hè jammer…), maar ik hoop dat we elkaar nog vaak blijven zien. Ik ben blij dat ook jij mijn paranimf wilt zijn!

Maar het allermeest heb ik toch wel aan jullie te danken, pap, mam, Marie-Claire. Marie-Claire, lief zusje, lief en leed kan ik met je delen. Met jou heb ik de allerleukste en mafste vakanties, nooit zonder avonturen. Ik hoop dat we deze relatie voor altijd blijven houden, waar we ook rondlopen op deze aardbol. Ik ben dolgelukkig dat je naast me komt te staan op het podium als paranimf. Bedankt!

Pap en mam, bedankt voor jullie nimmer afsluiterende steun en vertrouwen, bedankt voor jullie kritische vragen en stimulans, bedankt voor jullie liefde, bedankt voor alles! Zonder jullie was ik nooit zover gekomen en ik geniet van elk moment dat ik hier ben!
Curriculum vitae

Dorinde Mechtilde Meike Kleinegris was born in Terneuzen, the Netherlands, on September 17th, 1980. After living one and a half year in Rastatt, Germany, she came back to Terneuzen in 1986 and continued primary school there. Next, she went to the Zeldenrust-Steelantcollege in Terneuzen. After receiving her VWO diploma in 1998, she started the study Food Technology at Wageningen University, specializing in industrial microbiology. Her major thesis, at the group of Process Engineering at Wageningen University, was her first acquaintance with microalgae. She studied the mechanism of carotenoid extraction from *Dunaliella salina* during the milking process. Her minor thesis was performed at the Dutch Vaccine Institute (NVI), Bilthoven, The Netherlands, studying metabolic flux analysis of *Neisseria meningitidis* during A-stat cultivation. For her two internships, Dorinde went abroad. The first internship she performed at the National Institute of Water and Atmospheric Research (NIWA), Wellington, New Zealand, where she worked on obtaining anti-oxidants from marine microorganisms. She rounded off her study with her second internship at Lonza, Visp, Switzerland, working on the production of a medical protein with recombinant bacteria and yeast. After graduating in January 2005, she returned to Lonza, Visp, for another half year to finalize the project she was working on. In September 2005, she came back to Wageningen University and the field of microalgae and started her PhD at Process Engineering. Her study focused on the milking of microalgae, and in special the mechanism of extraction. Currently she is working as a Postdoc on the innovation of education at Process Engineering, Wageningen University.
Training activities

Overview of completed training activities

Courses

Discipline specific
Cell biology and advanced imaging technologies (2005)
Bioreactor design and operation (2006)
KREM crossing borders, focussing on spatial insight (2007)
Thermodynamics in biochemical engineering (2008)

General courses
VLAG PhD week (2005)
PhD competence assessment (2005)
Techniques for writing and presenting a scientific paper (2006)
Supervising BSc and MSc thesis work (2006)
Professional communication strategies for PhD students (2006)
Project and time management (2006)
Career perspectives (2009)

Conferences
Society for industrial microbiology Annual Meeting, Baltimore, USA (2006)
International Marine Biotechnology Conference, Eilat, Israel (2007)
Algae Congress, Amsterdam, The Netherlands (2008)

Optionals
Brainstormweek Process Engineering (2005)
Process Engineering PhD study tour to Denmark and Sweden (2006)
Organizing Process Engineering study tour to Japan (2008)
Process Engineering PhD study tour to Japan (2008)
This research was financially supported by the technology foundation STW (www.stw.nl; project WLM.6622), which is part of the Netherlands Organisation for Scientific Research (www.nwo.nl).