Metabolomics of carotenoid accumulation in *Dunaliella salina*

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Thesis

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Chapter 1

General introduction

Introduction

The finite aspect of our natural resources creates a need for production processes based on renewable resources and/or waste streams. Microalgae are ideally suited to meet such demands, since these microorganisms are able to convert carbon dioxide and water into valuable chemicals using sunlight as the primary energy source. Although plants are equally capable of this, microalgae have become the focal point of recent commercial and academic activities in the field of sustainable production processes. This is mainly because microalgae do not require arable land and have much higher areal productivities than agricultural crops (Chisti 2007; Chisti 2008; Nielsen et al. 1996). These high productivities come at the expense of extra costs though, since they can only be reached in (semi-)controlled cultivation systems designed to prevent any limitation but light. Because of these high costs, commercial large-scale cultivation of microalgae is currently limited to the production of high-value metabolites such as carotenoids and omega-3 fatty acids (Wijffels and Barbosa 2010).

Although these processes typically still use large amounts of energy, and do not yet contribute to the sustainability of our society, they do have high potential to develop into sustainable and economic production processes for microalgal low-value bulk chemicals and biofuels. It has been estimated that cost-effective production requires a 10-fold reduction in biomass production costs, which should follow from a multidisciplinary approach aimed at realizing breakthroughs in all areas of microalgal biotechnology such as molecular biology, physiology, photobioreactor design and downstream processing (Wijffels and Barbosa 2010).

Therefore, several years ago, the project 'Photosynthetic Cell Factories' was initiated at the Bioprocess Engineering group of Wageningen University. The objective of this project was to develop the expertise to make high-efficiency cultivation methods, control of primary and secondary metabolism, strain selection and selective extraction methods possible. For this, the production of carotenoids by *Dunaliella salina* was used as a model system. This thesis

describes part of the results from this project: accumulation of carotenoids in *Dunaliella salina.*

Carotenoids

Carotenoids are naturally occurring pigments ranging in color from yellow to red. Most of them are xanthophylls, which consist of 40 carbon atoms and one or more oxygen moieties. Hydrocarbons, called carotenes, constitute less than 10% of all carotenoid species (Britton et al. 2004). Carotenoids are synthesized by all photosynthetic organisms, in which they play a key role in photosystem assembly, light-harvesting and photoprotection (Britton et al. 1999; Li et al. 2009; Pogson et al. 2006). Carotenoids are also accumulating in many animals that use these pigments as precursors of vitamin A, to boost their immune system and to advertise health (Baron et al. 2008; McGraw et al. 2006). Animals usually acquire these pigments via their diet, although recently the plant louse *Acyrthosiphon pisum* was reported to produce carotenoids as a result of lateral gene transfer from fungi (Moran and Jarvik 2010). Finally, carotenoids have substantial commercial value owing to their color and antioxidant properties. Typical markets of application include food, feed, cosmetics and nutraceuticals.

Dunaliella salina

A particularly rich source of natural carotenoids, especially β-carotene, is *D. salina* (which includes the recently reclassified *D. bardawil*; Borowitzka and Siva 2007; www.algaebase.org). This green alga is a unicellular ovoid biflagellate (Figure 1.1). It lacks a rigid cell wall, which enables the alga to rapidly change its cell volume upon a sudden change in the extracellular osmotic pressure. This, together with the ability to produce glycerol as an intracellular osmoregulating agent, results in an extreme halotolerance enabling *D. salina* to cope with sodium chloride concentrations ranging from only 0.5% (w/w) up to a saturated

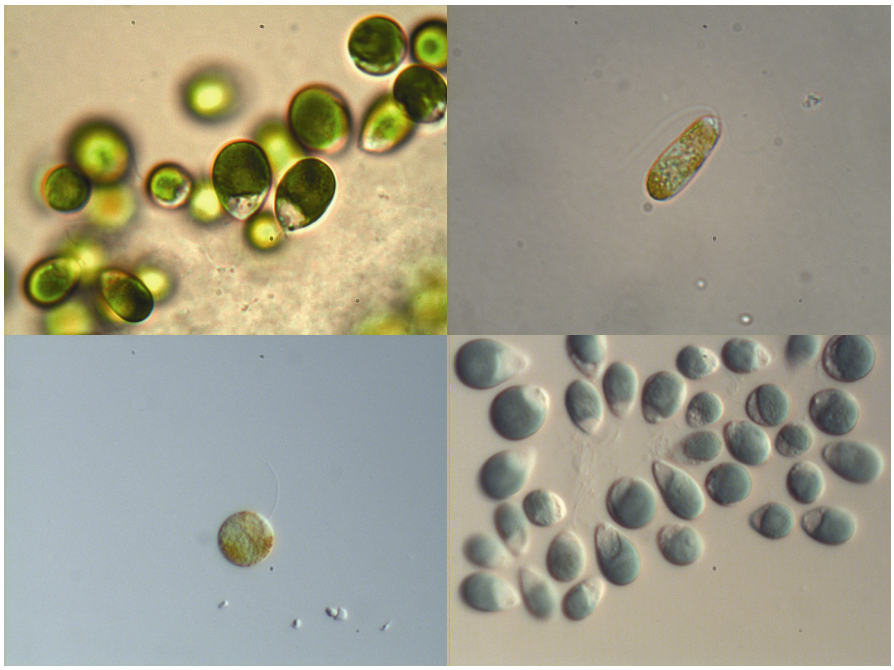

Figure 1.1 *Light microscopy photos of Dunaliella salina cells (courtesy of Marjon van Es).*

salt solution of about 30% (w/w) (Borowitzka et al. 1990). When *D. salina* is exposed to such high salinity, or to other environmental stress conditions like high light intensity, nutrient deprivation or extreme temperature, it is capable of accumulating β-carotene up to 10% of its dry weight (Ben-Amotz 1996; Ben-Amotz and Avron 1983; Ben-Amotz et al. 1982; Borowitzka et al. 1990; Kleinegris et al. 2009; Krol et al. 1997; Shaish et al. 1993). This high productivity has led to the large-scale application of *D. salina* for the commercial production of natural β-carotene (Del Campo et al. 2007).

Aim and thesis outline

In *D. salina*, the accumulated β-carotene molecules are stored in intracellular lipid globules that are formed at the onset of adverse growth conditions (Ben-

Amotz et al. 1982) and it has been suggested that the regulation of β-carotene and fatty acid synthesis are interrelated (Rabbani et al. 1998). Even though *D. salina*'s capacity for accumulating massive amounts of β-carotene has been studied and commercially exploited for many years now (Oren 2005), the regulatory mechanisms underlying carotenoid accumulation are not yet unraveled (see Chapter 2 for a review).

The aim of this thesis is to gain more insight into the regulation of β-carotene overproduction and how *D. salina* senses and responds to changes in culture conditions. The ultimate goal is defining optimal strategies for β-carotene production, and possibly also for other stress-inducible metabolites in *D. salina* and other microalgae.

First, the cell-factory potential of *D. salina* and the state of knowledge at the start of the project are reviewed in **Chapter 2**. The potential roles of reactive oxygen species and the plastoquinone redox state in signal sensing are discussed, together with available evidence on transcriptional and (post) translational regulation of β-carotene accumulation. In addition, the need for comprehensive metabolite profiling techniques and for experiments with defined light regimes is explained.

In **Chapter 3**, the response of *D. salina* to a sudden increase in light intensity is evaluated. It describes the use of flat panel photobioreactors that were run in turbidostat mode to ensure stable experimentalist-defined light regimes throughout the experiments. Using such turbidostats, the relationship between carotenoid and fatty acid metabolism during high-light stress was investigated.

The metabolic response of *D. salina* to nitrogen starvation is the topic of **Chapter 4**. The experimental approach is analogous to that of chapter 3, and hence a detailed discussion on the differences and similarities between *D. salina*'s responses to either nitrogen starvation or high-light stress is included.

Whereas Chapter 3 and 4 only focused on the apolar fraction of *D. salina* biomass, which contains the high-value compound β-carotene, **Chapter 5** describes a comparative polar metabolite analysis of high-light stressed and nitrogen-starved *D. salina* cells. The observed stress-induced changes in polar

metabolite profiles have been correlated to the stress-induced changes in apolar compounds, with the aim of identifying what cellular mechanisms are associated with β-carotene accumulation.

Finally, in **Chapter 6** a discussion towards the uncovering of the stress-sensing machinery in *D. salina* is presented. This is followed by a hypothesis on the potential stress-response mechanisms of *D. salina* based on the findings presented in this thesis. In addition, future directions for research on microalgal metabolite production are given, as well as some suggestions for efficient and continuous large-scale production of microalgal metabolites.

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Chapter 2

Exploring and exploiting carotenoid accumulation in *Dunaliella salina* for cell-factory applications

The contents of this chapter have been published as:
Lamers PP, Janssen M, De Vos RCH, Bino RJ, Wijffels RH. 2008. Exploring and exploiting carotenoid accumulation in *Dunaliella salina* for cell-factory applications. Trends Biotechnol 26(11):631-638.

Abstract

The unicellular alga *Dunaliella salina* is the most interesting cell factory for the commercial production of β-carotene because this species accumulates carotenoids to high concentrations. Nevertheless, little is known about the underlying mechanisms of carotenoid accumulation. Here, we review the regulatory mechanisms involved in β-carotene overproduction in *D. salina*. The potential roles of reactive oxygen species and the plastoquinone redox state in signal sensing are discussed, together with available evidence on transcriptional and (post)translational regulation. Moreover, future directions, that might further our knowledge in this area are given. Ultimately, a better understanding of the regulatory mechanisms involved in β-carotene overproduction will facilitate innovative production of specific carotenoids and other products in *D. salina* and in related organisms.

Introduction

Microalgae are photosynthetic microorganisms with which many added-value compounds can be produced for applications in food, feed, cosmetics and feedstock for the chemical industry, and they also have potential as sustainable energy carriers. Economically feasible production of added-value compounds with microalgae is possible because microalgae produce biomass and specific biomass ingredients directly from solar irradiation at high photosynthetic efficiencies and high volumetric and areal productivities. As an example, we focus on discussing the production of carotenoids in *Dunaliella salina*.

Carotenoids constitute one of the most widely distributed groups of naturally occurring pigments. More than 700 different carotenoid molecules have been described and they can be found in all photosynthetic organisms, in addition to several non-photosynthetic organisms (Britton et al. 2004). The colors of these pigments range from yellow to red, and some well-known examples are lycopene (found, for instance, in tomato fruit), zeaxanthin (maize corn) and β-carotene (carrots and the alga *D. salina*; see Figure 2.1 for the carotenoid biosynthetic pathway).

In photosynthetic organisms, carotenoids function as accessory light-harvesting pigments, structural components for photosystem assembly, moderators of non-photochemical quenching and as scavengers of reactive oxygen species (ROS) (reviewed in Britton et al. 1999). These antioxidant properties, in addition to their color, have resulted in important industrial applications of carotenoids. In the food, feed and cosmetic industries, carotenoids such as β-carotene are widely used as dyes. In addition, studies claiming that carotenoids can prevent several human diseases (reviewed in Elliott 2005; Giovannucci 1999; Ribaya-Mercado and Blumberg 2004) have resulted in various nutraceutical products containing carotenoids. However, contradictory studies that either demonstrate no positive effect on health or, indeed, even have a negative effect of carotene supplementations also exist (Bjelakovic et al. 2007; Kavanaugh et al. 2007). Despite the ongoing discussion of their health claims, carotenoids remain commercially interesting for industrial applications, mainly as colorants.

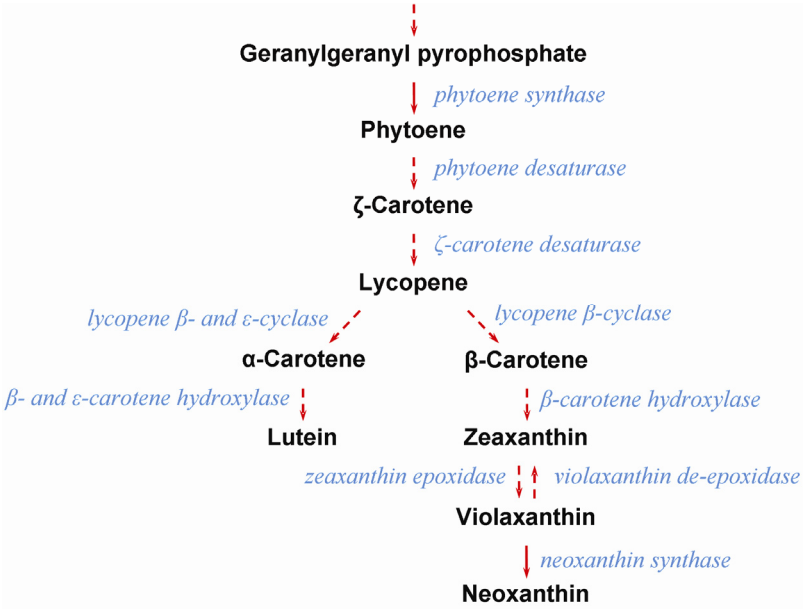

Figure 2.1 *Widely accepted pathway of carotenoid biosynthesis in microalgae. The first committed step of the carotenoid pathway is the condensation of two geranylgeranyl pyrophosphate molecules by phytoene synthase (enzyme names are written in blue italics in figure) and is considered to have an important regulatory role in carotenoid biosynthesis in several plant species (Sandmann et al. 2006). The role of transcriptional regulation of the carotenoid pathway in D. salina is not clear at present because contradicting results have been reported for the transcriptional regulation of both phytoene synthase and phytoene desaturase (Coesel et al. 2008; Rabbani et al. 1998; Sanchez-Estudillo et al. 2006).*

Cellular localization and function of β-carotene in *D. salina*

Some of the richest sources of natural β-carotene are the unicellular green microalgae *D. salina* (Chlorophyta, Dunaliellales) and *Dunaliella bardawil*, which is a close relative or, as has been suggested recently, possibly a subspecies of *D. salina* (Borowitzka and Siva 2007; Gonzalez et al. 2001). When exposed to specific extreme environmental conditions, such as high light intensity, high salinity, extreme temperatures and/or nutrient deprivation, *D. salina* can accumulate an extremely large amount of β-carotene (up to ~10% of the dry

algal biomass; Ben-Amotz et al. 1982) resulting in orange-colored microalgae. This high carotene productivity has led to the large-scale application of *D. salina* and *D. bardawil* for the commercial production of natural β-carotene (Del Campo et al. 2007).

In *D. bardawil*, β-carotene is accumulated in oil-containing globules, which are located in the interthylakoid space of the chloroplasts (Ben-Amotz et al. 1982). It has been suggested that most of the β-carotene-containing globules are localized peripherally in close vicinity to the plasma membrane (*i.e.* close to the outside of the cell; Hejazi et al. 2004). This cellular location of the β-carotene-containing globules is consistent with the widely accepted hypothesis that *D. salina* accumulates β-carotene to absorb a substantial part of the excess light (in the range of 330 to 500 nm) before this light would be able to damage the photosynthetic machinery of the cells. In further support of this hypothesis, it was observed that *D. bardawil* cells containing high levels of β-carotene were particularly protected against photoinhibition mediated by high-intensity blue light or by UV-A radiation (Ben-Amotz et al. 1989; White and Jahnke 2002).

Regulation of β-carotene accumulation

To date, many studies have addressed the effect of different culture conditions on the β-carotene content in *D. salina*. The accumulation of β-carotene can be induced by high light intensities and cultivation conditions that lead to reduced growth rates, including extreme temperatures, high salinity and nitrogen limitation. However, it remains difficult to compare the exact results of these studies, mainly owing to the fact that various light regimes have been applied and, furthermore, different carotenogenic subspecies of occasionally unknown genetic background are used, such as *D. salina* (Teodoresco 1905) and *D. bardawil* (Ben-Amotz et al. 1982).

Apart from these factors, progress in deciphering the physiological response of the algae to stress stimuli is also hindered by a lack of understanding of the regulatory mechanism that underlies the stress response in *D. salina*.

Information on the regulation of carotenogenesis at the molecular level in *D. salina* is still incomplete. Here, we give an overview of the current state of knowledge on the regulatory mechanism of carotenoid overproduction in *D. salina* and also point out possible ways to improve our knowledge in this area. For clarity, the regulatory mechanism is divided into separate steps of: (i) sensing of the induction signal, (ii) signal transduction, and (iii) regulation of carotenoid biosynthesis (Figure 2.2).

Signal sensing and signal transduction

To respond to changes in their environment, *D. salina* cells must be able to detect any relevant changes. It is well known that light intensity is a key induction parameter for β-carotene overproduction in *D. salina*, and the existence of a photoreceptor that would function as the signal-sensing mechanism, therefore, seems a plausible option. In algae, various photoreceptors have been described and have shown to be responsible for a large diversity of responses to light stimuli (reviewed in Hegemann et al. 2001), including the transcriptional regulation of carotenoid-pathway genes in *Chlamydomonas reinhardtii* (Im et al. 2006). For *D. bardawil*, however, it was shown that β-carotene accumulation was independent of light quality, although an increase in β-carotene accumulation was observed for cultivation under white light combined with UV-A, as compared with cultivation under white light alone at equal light intensities (Ben-Amotz and Avron 1989; Jahnke 1999).

Therefore, it seems unlikely that a specific photoreceptor is involved in directly sensing the signal for β-carotene overproduction in *D. bardawil*. However, the presence of a yet-unknown UV-A-specific photoreceptor, which would be capable of mediating a higher degree of overproduction as compared with that of white light alone, cannot be excluded at this point.

The positive effect of UV-A radiation on β-carotene accumulation might, however, also be explained by another mechanism. In *D. bardawil*, high β-carotene concentration protects the cell against UV-A-mediated photoinhibition through absorption of UV-A rays (White and Jahnke 2002). In addition, it has

been suggested that this UV-A-mediated photoinhibition is the effect of ROS that have been generated by the UV-A light (White and Jahnke 2002). A role for ROS as a direct trigger for the synthesis of photoprotective β-carotene, therefore, does not seem unlikely.

Indeed, it has been shown for *D. bardawil* that the addition of inducers of ROS promoted β-carotene biosynthesis under growth conditions that otherwise did not lead to carotenoid overproduction (Shaish et al. 1993). The addition of azide, an inhibitor of two ROS-scavenging enzymes (superoxide dismutase and catalase), rendered similar results (Shaish et al. 1993). Thus, ROS are likely to be involved in the signaling process that eventually leads to accumulation of β-carotene, although the mechanisms by which ROS are sensed remain poorly understood in both algae and plants (Pitzschke and Hirt 2006).

In addition to being generated by UV-A radiation, ROS are likely to also be produced under many other environmental stress conditions that are known to induce β-carotene overproduction. The levels of β-carotene in *D. bardawil* are positively correlated to the overall amount of irradiation perceived during the cell-division cycle (Ben-Amotz and Avron 1983). In other words, the slower the growth rate and/or the higher the light intensity, the more β-carotene is produced per cell. This observation can explain the increased β-carotene levels in response to stimuli other than light, such as extreme temperatures, nutrient limitation and high salinity, because these conditions inevitably lead to reduced growth rates.

The imbalance between light harvesting and energy utilization during conditions of high light intensity and/or low growth rate will lead to the production of singlet oxygen, a potent ROS, owing to overexcitation of chlorophyll molecules in the cellular photosystems (Noguchi 2002). For this reason, and because singlet oxygen has been shown to be capable of inducing β-carotene accumulation in *D. bardawil* (Shaish et al. 1993), singlet oxygen might be the signaling molecule responsible for β-carotene overproduction under all carotenogenic cultivation conditions. However, a singlet-oxygen sensor that is able to mediate carotenoid accumulation has not yet been identified in *D. salina* or in other algal species. If

such a sensor molecule existed, it would probably be located close to the site of singlet-oxygen generation [*i.e.* photosystem II (PSII)] because singlet oxygen is highly reactive and, hence, has a very short lifetime.

Alternatively, singlet oxygen might react with secondary messengers, initiating a signal-transduction cascade that would connect the initial stimulus with a sensor molecule. Possible candidates for such messenger molecules are the lipids of the chloroplast membrane as singlet-oxygen-derived lipid-oxidation products have been shown to be involved in activation of gene expression in human cells (Grether-Beck et al. 2000). However, no information is currently available on the existence and possible nature of a signal-transduction pathway involved in β-carotene overproduction in *D. salina*.

Recently, however, a gene encoding a mitogen-activated protein (MAP) kinase was isolated from *D. salina*. Kinases, and particularly MAP kinases, have gained a lot of attention for their essential role in signaling pathways present in plants and animal cells (Avruch 2007; Mishra et al. 2006). The expression level of the MAP kinase in *D. salina* was found to be affected by changes in temperature and salinity (Lei et al. 2008), conditions that also influence β-carotene accumulation. Unfortunately, β-carotene production was not addressed in this study (Lei et al. 2008), and future studies will have to establish the precise role of this MAP kinase, or other kinases, in the carotenogenic response mechanism of *D. salina*.

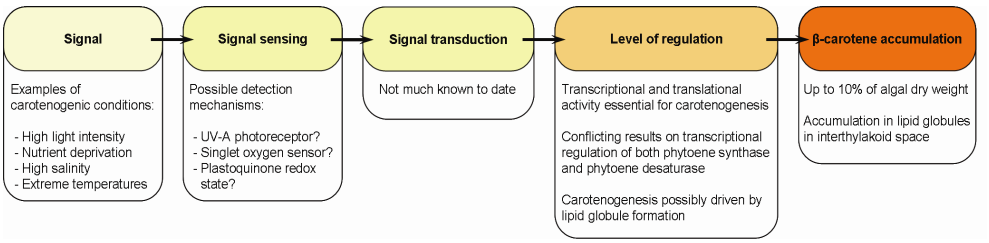

Figure 2.2 *Current knowledge of the regulatory mechanism for the induction of* β*-carotene overproduction in D. salina. The events between the actual signal and* β*-carotene accumulation can be divided into 'signal sensing', 'signal transduction' and 'level of regulation', and currently available information for each step is shown.*

Level of regulation of carotenoid biosynthesis

The fluxes through metabolic pathways can be regulated at different hierarchical levels, for instance at the level of transcription, translation or enzyme activity. At what level is the induced accumulation of β-carotene in *D. salina* regulated? By adding transcriptional and translational inhibitors to cultures of *D. bardawil* and subsequently exposing these cultures to high light intensities, it was shown that gene activation was essential for high-light-induced carotenoid production (Lers et al. 1990). Unfortunately, the lack of genomic information on *D. salina* or *D. bardawil* has, thus far, prevented extensive gene-expression studies with DNA microarrays to identify pathways that are either upregulated or downregulated under β-carotene-inducing conditions. In addition, only limited, and mostly contradictory, information is available on carotenoid-biosynthesis enzymes and gene transcripts in *D. salina* and *D. bardawil*.

The enzyme phytoene synthase catalyzes the first committed step of the carotenoid pathway (Figure 2.1) and is regarded as an important regulatory step for carotenoid biosynthesis in various plant species (Sandmann et al. 2006). In *D. salina,* however, the regulatory role of phytoene synthase is unclear because three different studies on the transcriptional and translational regulation of phytoene synthase attained contradicting conclusions. Specifically, under carotenogenic conditions, no upregulation of phytoene synthase was observed for *D. bardawil* in one report (Rabbani et al. 1998), but another study of the same species observed increased gene-expression levels (Coesel et al. 2008). By contrast, phytoene-synthase-transcript levels remained unchanged in *D. salina* (Sanchez-Estudillo et al. 2006).

Similar contradictory results exist for the transcriptional and translational regulation of phytoene desaturase, which catalyzes the second step of the carotenoid pathway (Figure 2.1). It was found that, under carotenogenic conditions, the amount of phytoene desaturase did not change in *D. bardawil* and transcript levels remained stable (Rabbani et al. 1998), whereas another study reported elevated transcript levels of the phytoene-desaturase gene in this alga (Coesel et al. 2008).

The transcriptional regulation of lycopene β-cyclase, which catalyzes the conversion of lycopene to β-carotene (Figure 2.1), has only been addressed in one study, which demonstrated upregulated lycopene β-cyclase transcript levels in *D. bardawil* under various carotenogenic conditions (Ramos et al. 2007). These inconclusive results clearly indicate that more work is necessary to establish which steps, if any, of the carotenoid pathway (Figure 2.1) are transcriptionally or translationally regulated during carotenogenesis.

However, a mechanism that is independent of a direct regulation of the carotenoid pathway has also been suggested. In *D. salina*, overproduced β-carotene is accumulated in lipid globules that are associated with both triacylglycerol (TAG) and peripherally located carotene globule proteins (Cgps). Cgp has been suggested to be involved in the stabilization of the membrane-free lipid globules (Katz et al. 1995) and is overproduced concomitantly with β-carotene.

In addition to β-carotene, TAG is one of the main constituents of the globules (Ben-Amotz et al. 1982; Fried et al. 1982; Rabbani et al. 1998). The relationship between TAG biosynthesis and β-carotene accumulation has been studied, and it was suggested that both pathways were interdependent (*i.e.* the overproduction of β-carotene was inhibited upon chemical inhibition of TAG biosynthesis; Rabbani et al. 1998).

The authors also observed a high-light-induced increase in enzymatic activity of acetyl-coenzyme A carboxylase, which is a key step in plant acyl-lipid biosynthesis (Ohlrogge and Jaworski 1997; Sasaki and Nagano 2004). This led to the suggestion that the production of β-carotene-sequestering structures (*i.e.* lipid globules) increases upon environmental stress conditions, thereby creating a plastid-localized sink for β-carotene (Rabbani et al. 1998). Considering that, in this study, no transcriptional and translational upregulation of the first two steps in the carotenoid pathway occurred under carotenogenic conditions, the authors further hypothesized that the carotenoid-pathway enzymes are not maximally active under non-inducing conditions and that the β-carotene sink might stimulate their activity by sequestering β-carotene, thus, avoiding end-product

inhibition of the carotenoid pathway (Rabbani et al. 1998).
A similar metabolic-sink effect has been observed when cauliflower and potato were transformed with a gene encoding a protein involved in the differentiation of proplastids and/or non-colored plastids into carotenoid-sequestering chromoplasts (Li and van Eck 2007). Taken together, these observations provide some evidence for the metabolic-sink hypothesis, which deserves to be studied in more detail.

Furthering our knowledge on carotenoid metabolism

First of all, it needs to be stressed that the available data on overproduction of carotenoids in *D. salina*, as reported in various scientific papers, are difficult to compare. This is mainly owing to the different illumination techniques and, thus, to the different light regimes applied. We, therefore, strongly advocate the use of photobioreactors that enable well-defined light regimes that remain constant throughout experiments (Box 2.1).
Despite the commercial profits that are already being made from selling algal carotenoids, many unanswered questions remain regarding the regulatory

Box 2.1 Importance of well-defined light regimes for light-responsive microorganisms.

Light intensity is (one of) the most important factor(s) determining the amount of β*-carotene accumulated in D. salina. A well-defined light regime is, therefore, essential when the effects of light intensity, or other growth conditions, on* β*-carotene productivity are studied. Unfortunately, studies described in the literature are often performed in photobioreactors of various geometrical shapes with poorly defined and/or described light profiles, making the comparison of their results extremely difficult. In addition, so far, most studies have been performed with batch-grown algal cultures in which the increasing biomass causes a gradual decrease of the average light intensity perceived per cell surface during the experiment. This decrease in light perception will probably influence the outcome of the experiment. Hence, we encourage the use of photobioreactors with well-defined and controlled light regimes, such as flat-panel or tubular reactors that are operated as chemostats or turbidostats (Myers and Clark 1944). Clearly, this reasoning does not only apply to studies with D. salina but also to studies with other light-responsive microorganisms, in which the effect of a changing light regime could influence the outcome of the study.*

mechanism of carotenoid overproduction in *D. salina.* For instance, do the various environmental stress conditions (e.g. high light intensity, high salinity, extreme temperatures and nitrogen deprivation) all give rise to the same oxidative stress in *D. salina*, and is singlet oxygen the key signaling molecule for initiation of carotenoid accumulation under these conditions? The recently developed luciferase reporter system in *C. reinhardtii*, enabling the *in vivo* detection of singlet oxygen and hydrogen peroxide, is a promising tool that, if shown to be applicable in *D. salina*, could help elucidate the aforementioned questions (Shao et al. 2007).

Another candidate for the 'stress' signal is the redox state of the plastoquinone pool in the photosynthetic electron-transport chain. This so-called redox hypothesis is based on the discovery of a photon-sensing mechanism able to regulate the amount of light harvesting complex II (LHCII) protein in both *D. tertiolecta* (Escoubas et al. 1995) and *D. salina* (Maxwell et al. 1995).

When PSII is excited by light, it transfers electrons via a cascade of redox reactions to plastoquinone, which is subsequently reduced to plastoquinol (Figure 2.3). The subsequent oxidation back to plastoquinone is widely assumed to be the rate-limiting step in the entire photosynthetic electron-transport chain (Cramer and Crofts 1982; Junge 1977). When the light intensity increases or the growth rate (and, hence, the need for NADPH) decreases, the rate of electron production by PSII might exceed the rate of oxidation of plastoquinol. As a consequence, the plastquinone pool becomes highly reduced. This change in the redox state of plastoquinone is, thus, the effect of an imbalance in the supply and demand of photosynthetic products in the cell (Durnford and Falkowski 1997; Wilson et al. 2003).

It has been shown that, in *D. tertiolecta*, changes in the plastoquinone redox state are responsible for the altered expression of a nuclear *cab* gene encoding a LHCII protein (Escoubas et al. 1995). Considering the reasoning noted before, it was suggested that this redox state, thus, might act as a light-sensing mechanism and is an underlying factor in the light-intensity-dependent regulation of the *cab* gene.

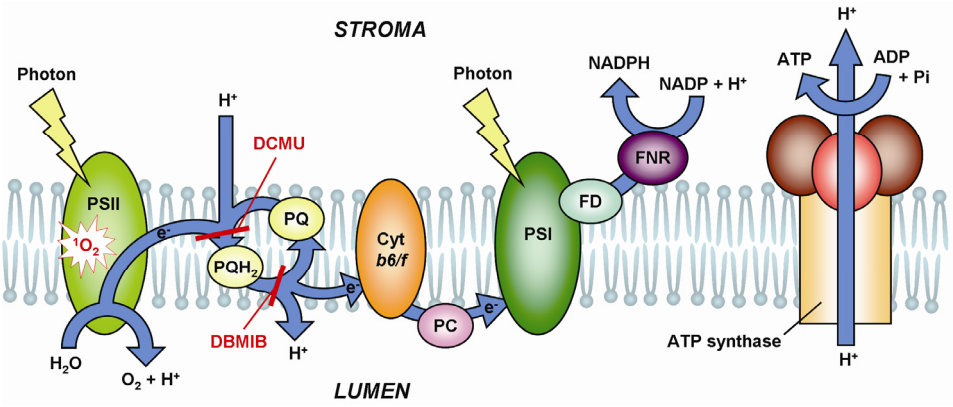

Figure 2.3 *Schematic overview of the photosynthetic electron-transport system at the thylakoid membrane. Carotenoid accumulation in D. salina is induced under conditions of high light intensity or when the growth rate is reduced. Possible signaling mechanisms for the detection of such an imbalance between supply and demand of photosynthetic products are the production of singlet oxygen and/or a reduction of the plastoquinone redox pool. The latter hypothesis could be evaluated by using inhibitors, such as DCMU and DBMIB, which block the reduction and the oxidation of plastoquinone, respectively. Abbreviations: $^1O_2$ – singlet oxygen; Cyt b6/f – cytochrome b6/f complex; DBMIB – 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FD – ferredoxin; FNR – ferredoxin-NADP reductase; PC – plastocyanin; PQ – plastoquinone; PQH_2 – plastoquinol; PSI – photosystem I; PSII – photosystem II.*

It would be worthwhile to test this redox hypothesis for β-carotene accumulation in *D. salina*, for instance by using photosynthetic electron-transport-chain inhibitors such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB). These inhibitors block either reduction or oxidation of the plastoquinone pool (Figure 2.3) and, therefore, provide ways to alter the plastoquinone redox state independently of the environmental conditions. Experiments in the presence and absence of these inhibitors under both inductive and non-inductive conditions could provide evidence to determine the validity of this redox hypothesis. Indeed, in *Haematococcus pluvialis*, an astaxanthin-accumulating green alga, comparable experiments have already indicated that the plastoquinone pool might function as a redox sensor and regulate the expression

of carotenoid biosynthesis genes (Steinbrenner and Linden 2003). With regard to the underlying mechanisms of the signal sensing, it remains to be elucidated whether and how a change in the redox state of plastoquinone is transmitted into the induction of β-carotene overproduction in *D. salina*.

Another important issue to be resolved is the role of transcriptional regulation of carotenoid biosynthesis genes and their response to stress conditions. cDNAs or expressed sequence tag databases (Park et al. 2006) can be used to identify *D. salina* genes that are homologous to plant and/or algal genes known to be involved in carotenoid biosynthesis. Any novel genes, in addition to the genes that have already been characterized but with contradictory conclusions, such as those encoding phytoene synthase and phytoene desaturase, could then be screened for a differential expression under carotenogenic conditions, which might help to elucidate the role of transcriptional regulation. The genome sequencing program for *D. salina*, which is currently ongoing at the US Department of Energy Joint Genome Institute (www.jgi.doe.gov), will certainly facilitate the identification and cloning of genes involved in the carotenoid pathway.

Apart from focusing on specific genes known to be involved in regulating carotenoid biosynthesis, large-scale '-omics' approaches, including transcriptomics (Hu and Polyak 2006), proteomics (Liska et al. 2004; Walker 2005) and metabolomics (De Vos et al. 2007; Lisec et al. 2006) could also be employed. These comprehensive profiling techniques might provide global clues and novel insights into the algal response to stress stimuli and into the cellular regulation of their response. Metabolic profiling will be particularly helpful in understanding the metabolite alterations taking place inside the cell upon induction of carotenoid overproduction and in elucidating any precursors or key biosynthetic steps that determine and limit carotenoid overproduction.

Mutants can also play an important part in unraveling the regulatory mechanisms of carotenoid overproduction. For example, the *high pigment-2*^{dg} mutant of tomato, which is hypersensitive to light, is characterized by elevated levels of carotenoids, in addition to other antioxidants, present throughout all

stages of fruit development (Bino et al. 2005). In these light-sensitive tomato mutants, complementary metabolomics (Bino et al. 2005) and transcriptomics (Kolotilin et al. 2007) studies have identified chloroplast biogenesis as an important determinant of carotenoid accumulation and, moreover, indicated that the transcription of structural carotenogenesis genes was not tightly linked with carotenoid overproduction.

To date, several mutants of *D. salina* and *D. bardawil* have been obtained (Jin et al. 2003; Shaish et al. 1991) but, to our knowledge, none of these mutants have been used to study the regulation of carotenoid accumulation in detail. The recent development of genetic transformation strategies for *D. salina* (Geng et al. 2003; Tan et al. 2005) will enable a more widespread use of specifically designed mutants to address the regulation of carotenoid metabolism.

Microalgae as cell factories

Microbes and microalgae can be used as cell factories for the industrial production of carotenoids. Such cell factories aim to produce either naturally occurring carotenoid species or novel and tailor-made carotenoid species with improved industrial applications or health benefits. In recent years, much progress has been made in the construction of the carotenoid biosynthesis pathway in *E. coli*, which lacks a natural carotenoid biosynthesis pathway, to produce novel carotenoid species (Wang et al. 2007).

In most cases, however, the carotenoid productivity in microbes is too low to be commercially exploited. The advantage of *D. salina* is that both production and accumulation of carotenoids in oil globules can be enhanced. Considering the recently developed transformation strategies for *D. salina* (Geng et al. 2003; Tan et al. 2005) and its high carotenoid-accumulation capacity, this algal species might be a particularly suitable cell factory for the production of novel carotenoids.

The successful development of algal cell factories will certainly be facilitated by a better understanding of the regulation of carotenoid accumulation in *D. salina*.

In addition, the improved knowledge on the exceptionally high carotenoid accumulation in *D. salina* might also provide new leads for the optimization of carotenoid production in other algae (an overview of the carotenoid contents of various carotenoid-rich microalgae is given in Table 2.1). As outlined before, sequestration of carotenoids can be an important factor in reaching high carotenoid levels, and a simultaneous overexpression of 'metabolic-sink genes' together with carotenoid-pathway genes might prove to be particularly successful (Giuliano et al. 2008).

'Milking' processes have been described for the simultaneous production and extraction of β-carotene (Hejazi et al. 2004) and phytoene (León et al. 2005) from *D. salina* and *D. bardawil* without damaging the cells. Because β-carotene and TAG are both present in the same lipid globules, such a milking process might also enable the extraction of TAGs for their use in industrial applications, for example as biofuels. To our knowledge however, the milking of TAG from *Dunaliella* sp. has never been studied, and thus it is yet unknown whether such a process might be cost effective. The advent of genetic transformation methods for *D. salina* (Geng et al. 2003; Tan et al. 2005) will enable the milking process in

Table 2.1 *Carotenoid content of different carotenoid-rich microalgae.*

Strain	Carotenoid	Content[a]	References
Dunaliella salina	β-Carotene	~ 10 %	(Ben-Amotz et al. 1982)
Haematococcus pluvialis	Astaxanthin	~ 7.7 %	(Kang et al. 2007)
Muriellopsis sp.	Lutein	~ 0.8 %	(Blanco et al. 2007)
Scenedesmus almeriensis	Lutein	~ 0.6 %	(Sánchez et al. 2008)
Dunaliella salina mutant	Zeaxanthin	~ 0.6 %	(Jin et al. 2003)
Coelastrella striolata var. *multistriata*	Canthaxanthin	~ 4.8 %	(Abe et al. 2007)

[a] *Carotenoid content expressed as percentage of algal dry weight.*

cell-factory strains that have been genetically engineered to accumulate other lipophilic molecules in their oil-containing globules, such as tailor-made carotenoids, tocopherols, lipophilic proteins and other biologically active or economically important compounds.

Conclusion and prospects

The carotenoid production by *D. salina* is an example of the great potential of microalgae for the production of added-value compounds. However, to fully exploit the economic potential of microalgal cell factories, combined efforts in the fields of photobioreactor development, strain selection and optimization, in addition to elucidating metabolic regulation, are needed. The current status and perspectives of photobioreactor development and strain improvement have been reviewed elsewhere (Chisti 2007; Chisti 2008; León-Bañares et al. 2004; Wijffels 2008). Detailed knowledge of microalgal metabolism is unquestionably a prerequisite for the maximization and control of metabolite production in microalgae, as exemplified here for β-carotene production in *D. salina*.

Although the environmental conditions that lead to carotenoid accumulation in *D. salina* have become clear, our understanding of the regulatory mechanism that underlies this response is far from complete. Important issues that need resolving are the roles of transcriptional and translational regulation and the identity of the signaling molecules that initiate carotenoid overproduction under various environmental stress conditions.

Clarification of the role of transcriptional regulation has, thus far, been hindered by the lack of the genome sequence of *D. salina*, but this obstacle will shortly be overcome because the genome is currently being sequenced. The identification of the signaling molecules will be a complex task because there are several candidates, in addition to the multitude of environmental conditions that can lead to carotenoid production, all of which will require complex measurements. Experiments with inhibitors of the photosynthetic electron-transport chain, as proposed here, should be able to provide necessary information that will help to

advance our knowledge in this important area.
Finally, a better understanding of the regulation of carotenoid metabolism, lipid-globule formation and the milking process in *D. salina* is expected to enable and further improve the overproduction of specialized carotenoids and other lipophilic products in *D. salina*. Moreover, it can be anticipated that this knowledge will be applicable to many other microalgae, which should considerably increase their cell-factory potential.

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Chapter 3

Carotenoid and fatty acid metabolism in light-stressed *Dunaliella salina*

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Abstract

β-Carotene is overproduced in the alga *Dunaliella salina* in response to high light intensities. We have studied the effects of a sudden light increase on carotenoid and fatty acid metabolism using a flat panel photobioreactor that was run in turbidostat mode to ensure a constant light regime throughout the experiments. Upon the shift to an increased light intensity, β-carotene production commenced immediately. The first 4 hours after induction were marked by constant intracellular levels of β-carotene (2.2 $g \cdot LCV^{-1}$), which resulted from identical increases in the production rates of cell volume and β-carotene.

Following this initial phase, β-carotene productivity continued to increase while the cell volume productivity dropped. As a result, the intracellular β-carotene concentration increased reaching a maximum of 17 $g \cdot LCV^{-1}$ after 2 days of light stress. Approximately 1 day before that, the maximum β-carotene productivity of 30 $pg \cdot cell^{-1} \cdot d^{-1}$ (equivalent to 37 $mg \cdot LRV^{-1} \cdot d^{-1}$) was obtained, which was about one order of magnitude larger than the average productivity reported for a commercial β-carotene production facility, indicating a vast potential for improvement.

Furthermore, by studying the light-induced changes in both β-carotene and fatty acid metabolism, it appeared that carotenoid overproduction was associated with oil globule formation and a decrease in the degree of fatty acid unsaturation. Our results indicate that cellular β-carotene accumulation in *D. salina* correlates with accumulation of specific fatty acid species (C16:0 and C18:1) rather than with total fatty acid content.

Introduction

There is a high interest in the cultivation of microalgae as a source of high value, naturally synthesized products such as β-carotene (Bhosale 2004; Cardozo et al. 2007; Spolaore et al. 2006). β-Carotene is a lipid-soluble orange pigment and antioxidant that is mainly used in cosmetics and as a colorant for feed and food.

A rich source of natural β-carotene is the unicellular green microalga *Dunaliella salina* (including a strain previously known as *Dunaliella bardawil*; Borowitzka and Siva 2007; www.algaebase.org), which can accumulate β-carotene to as much as 10% of the cellular dry weight under certain extreme environmental conditions, such as high light intensity, nutrient deprivation, high salinity and extreme temperatures (Ben-Amotz 1996; Ben-Amotz and Avron 1983; Ben-Amotz et al. 1982; Borowitzka et al. 1990; Kleinegris et al. 2009; Krol et al. 1997; Shaish et al. 1993).

Even though the overproduction of β-carotene in *D. salina* has been studied extensively (Oren 2005), little is known about its regulatory mechanisms (Lamers et al. 2008, Jin and Polle 2009). Despite the knowledge that high-light-induced β-carotene overproduction requires both transcriptional and translational activity (Lers et al. 1990), it remains unclear what genes and enzymes are involved in the regulation of the overproduction. Various studies (Coesel et al. 2008; Rabbani et al. 1998; Ramos et al. 2007; Ramos et al. 2009; Sanchez-Estudillo et al. 2006) have been directed towards elucidating the role of structural genes that encode enzymes of the carotenoid biosynthetic pathway, but an unambiguous consensus is still lacking (Lamers et al. 2008).

Alternative regulatory mechanisms have been proposed as well, including the hypothesis that β-carotene overproduction is driven by the formation of lipid globules (Rabbani et al. 1998). These lipid globules can sequester the apolar carotenoids (Ben-Amotz et al. 1982) and may thereby serve as a metabolic sink, transporting β-carotene away from its biosynthetic machinery and consequently avoiding end-product inhibition of the carotenoid biosynthetic enzymes (Rabbani et al. 1998). The positive correlation between oleic acid (C18:1) and β-carotene levels in *D. salina* cells grown at various light intensities (Mendoza et al. 1999) is

in support of this metabolic sink theory, as are the concomitant β-carotene and total fatty acid increases observed for high-light-treated (Rabbani et al. 1998) and nitrogen-deprived, high-light-treated *Dunaliella salina* cells (Mendoza et al. 1999).

Furthermore, the observation that carotenogenesis can be induced by high light intensities as well as by environmental conditions that limit cell growth, has led several authors to suggest that β-carotene levels in *D. salina* are actually regulated by integration of the effects of all growth variables through modulation of either photosystem II excitation pressure (Krol et al. 1997) or the overall amount of irradiation perceived during the cell-division cycle (Ben-Amotz and Avron 1983). This, together with the widely accepted hypothesis that overproduced β-carotene mediates the protection of *D. salina* cells against photoinhibition (Ben-Amotz et al. 1989), suggests that light intensity is likely one of the most important factors that control β-carotene accumulation in *Dunaliella salina*.

A well-defined and controlled light regime is therefore indispensable in experimental studies on *D. salina*'s response to stress conditions. However, in most studies on carotenogenesis by *D. salina*, batch cultivations have been applied. The algal cells cultivated in such systems will experience changing light regimes throughout the experiment as a result of increased absorption by light-induced biomass and pigment accumulation in the reactor (Lamers et al. 2008). Suitable alternatives to batch cultivations are turbidostat cultivations, which enable defined light regimes by maintaining the light transmission through the reactor at a fixed, experimentalist-defined level via constant adjustment of the dilution rate of the culture (Myers and Clark 1944).

To further elucidate the effect of light and the possible role of fatty acid metabolism in carotenoid production in *D. salina*, we studied the effects of stepwise and large sudden increases in light intensity on both carotenoid and fatty acid levels in *D. salina*, using turbidostat cultivations in a panel photobioreactor with a short light path. The metabolic capacity of *D. salina* for β-carotene production and the possible relation between carotenoid and fatty acid metabolism in this species are discussed.

Materials and methods

Algae, growth medium and pre-cultivation conditions

Dunaliella salina (CCAP 19/18) cells were grown in a liquid medium (pH = 7.5) of which the composition was based on a stoichiometric analysis of *Dunaliella tertiolecta* (Ho et al. 2003). It contained: 1.50 M NaCl, 37.75 mM KNO_3, 22.50 mM Na_2SO_4, 4.87 mM K_2SO_4, 1.00 mM NaH_2PO_4, 0.37 mM $MgCl_2$, 19.35 µM Na_2EDTA, 18.9 µM $CaCl_2$, 11.25 µM NaFeEDTA, 1.89 µM $MnCl_2$, 1.48 µM $ZnSO_4$, 0.67 µM $CuSO_4$, 10.95 nM Na_2MoO_4 and 9.95 nM $CoCl_2$. Pre-cultures were grown in 250 mL Erlenmeyer shake flasks with 100 mL medium containing 40 mM of HEPES buffer. The pre-cultures were placed in an orbital shaker incubator. The agitation rate was 100 rpm, temperature 25°C, concentration of CO_2 in gas phase 2.5% and incident light intensity was ~70 µmol photons$\cdot m^{-2}\cdot s^{-1}$.

Cultivation conditions of batch experiments

To ensure highly reproducible inoculi for the batch experiments, algae were pre-cultivated to steady state in a 2 L chemostat operated at 27 °C, pH = 7.5, D = 0.5 d^{-1}, an average light intensity of ~100 µmol photons$\cdot m^{-2}\cdot s^{-1}$, and a gas mixture of air (98%) and CO_2 (2%), which was led into the reactor through a microsparger. The algae were then inoculated in Plexiglas flat panel photobioreactors to an optical density of 1 measured at 530 nm (OD_{530}). The reactors had a volume of 800 mL and an optical path of 4 cm and further details are described by Bosma and Wijffels (2003). Temperature was controlled at 27°C, pH was controlled at 7.5 and a gas mixture of air (98%) and CO_2 (2%) was led through a sparger to mix the culture, supply CO_2 and remove oxygen. Tungsten-halogen lamps of 100 W and 300W were used for the illumination of the cultures. At all conditions tested (see Table 3.1), the algae were cultivated in two independent replicate experiments.

Cultivation conditions of turbidostat experiments

The algae were cultivated in an aseptic panel photobioreactor (see Figure 3.1) operated as a turbidostat to maintain a steady light regime throughout the

Table 3.1 *Cultivation conditions of batch experiments.*

Condition code[a]	Light (µmol·m^{-2}·s^{-1})	Temp. (°C)	N-depletion?	NaCl (M)
LL	100	27	No	1.5
HL	2 x 1000[b]	27	No	1.5
LL – ND	100	27	Yes	1.5
HL – ND	2 x 1000[b]	27	Yes	1.5
LL – HS	100	27	No	4
HL – HS	2 x 1000[b]	27	No	4
LL – LT	100	15	No	1.5
HL – LT	2 x 1000[b]	15	No	1.5

[a] *Codes used in Figure 3.2.*
[b] *The flat panel bioreactor was illuminated on both sides with 1000 µmol·m^{-2}·s^{-1}.*

entire experiment. The culture volume of the reactor was 2.275 L (height, width and optical light path were 0.38 m, 0.2 m and 0.03 m, respectively). Temperature was controlled at 30°C and pH was controlled at 7.5 by on-demand CO_2 supply. Mixing of the culture and oxygen removal were established by sparging with nitrogen gas, controlled by a mass flow controller (Brooks Instrument, Ede, the Netherlands) at 0.6 L min^{-1}, through needles in the bottom of the reactor.

The reactor was illuminated with a high-pressure sodium plant growth lamp (Philips 400W MASTER SON-T PIA Green Power). The incident light intensity was set by adjusting the distance between the lamp and the reactor. The light intensity leaving the culture was detected with a PAR photon flux density sensor that was connected to an ADAM-5000 data acquisition card and controlled via a LabView virtual instrument running on a PC (LabView 7.1, National Instruments Corp., Austin, TX, USA). The light transmission through the culture was evaluated every minute and compared to the set-point which was set at 13% transmission to allow for a biomass density that was high enough to enable frequent sampling of small aliquots for reliable metabolite analysis whilst maintaining a sufficient light intensity throughout the photobioreactor. Following each light transmission

assessment, the feed medium pump was automatically switched on or off in order to maintain culture turbidity at the defined level. The culture volume was maintained at a constant level by continuous removal of culture broth through an outlet positioned at the top of the culture.

The turbidostat experiment, in which the incident light intensity was increased in small steps, consisted of two separate runs. In these runs, four different incident light intensities were tested: 150, 250 and 400 µmol photons·m^{-2}·s^{-1} in the first run, and 400 and 650 µmol photons·m^{-2}·s^{-1} in the second run. The light intensity was increased after every third volume change of the reactor. The effects of an immediate increase of the incident light intensity from 200 to 1400 µmol photons·m^{-2}·s^{-1} were also studied using turbidostat cultivation.

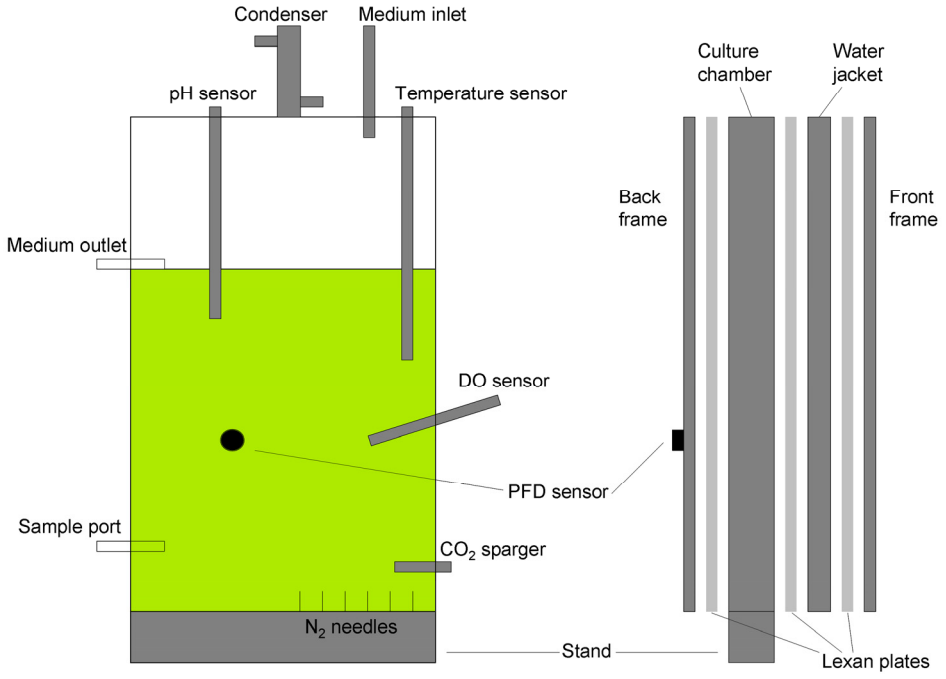


Figure 3.1 *Schematic illustration of the flat panel turbidostat. Left: front view. Right: side view.*

Determination of cell number and size

A Multisizer 3 (Beckman Coulter Inc., Brea, CA, USA) with a 100 µm aperture tube was used to determine both the number and diameter of the cells in triplicate. The cell suspension was diluted 60x to obtain a cell count of approximately $3 \cdot 10^4$ cells·mL^{-1}. The dilution buffer (pH = 7.5) was equiosmolar to the cultivation medium and contained 10 mM Hepes buffer, 1.58 M NaCl, and Na_2SO_4, K_2SO_4, $MgCl_2$, $CaCl_2$ at concentrations identical to the cultivation medium. The running liquid was identical to the dilution buffer, except that for practical reasons 1 M NaCl and no Hepes buffer was used. We found that this difference in composition and osmolarity does not affect the determined cell number and size.

Dry weight determination

Triplicate dry weight determinations were essentially performed as described by Zhu and Lee (1997). Glass fiber GF/F filters (Whatman plc, Maidstone, UK) were used instead and 1.5 M filtered ammonium formate was used as a washing buffer.

Extraction of fatty acids and pigments

Samples of 2-3.5 mL cell suspension were taken from the reactors and transferred to 10 mL tubes, after which the algae were immediately precipitated by centrifugation at 4000g for 4 min. The supernatant was discarded and the tubes with cell pellets were immediately frozen and stored at -80°C. Apolar compounds, including carotenoids and acyl lipids, were extracted based on the extraction method for lipid-soluble isoprenoids described by Fraser et al. (2000), which was optimized for our tissue type.

First, 4 mL of a chloroform/methanol mixture (2:2.5) was added to each algal pellet. In the case of lipid extraction for fatty acid determination, 66 µg·mL^{-1} glyceryl-tri-nonadecanoate was added to this mixture as an internal standard. In the case of pigment analysis, 0.1 % (w/v) butylated hydroxytoluene (BHT) was added as an antioxidant. After vortexing (10 s) and sonication (10 min), 2.5 mL of

a 50 mM Tris-buffer (pH = 7.5) containing 1 M NaCl was added to the suspension. The samples were vortexed and sonicated once more and subsequently centrifuged to separate the polar and apolar phase. The chloroform phase was transferred to a fresh tube and the polar phase and debris were re-extracted twice with 1 mL chloroform (containing 0.1% BHT for samples extracted for pigment analysis). About 88-94% of all pigments and fatty acids were extracted in the first extraction, and after all three extractions ~99.8 % of all lipids and pigments were collected. Finally, the pooled chloroform fractions were dried by evaporation under a stream of nitrogen gas and stored at -80°C until analysis.

Pigment analysis

The dried chloroform fractions were taken up in 1 mL ethylacetate containing 0.1% BHT. The compounds were separated on a reverse-phase C_{30} column (250 mm x 4.6 mm; 5 µm) and analyzed by HPLC coupled to a photodiode array (HPLC-PDA) as described previously (Bino et al. 2005; Moco et al. 2007). Lutein, β-carotene, lycopene, chlorophyll *a*, and chlorophyll *b* were identified based on comparison of their retention time and absorption spectra (240 to 750 nm) with authentic standards (Sigma Aldrich, St. Louis, MO, USA; Apin Chemicals, Abingdon, UK; CaroteNature, Heysingen, Switzerland). Calibration curves with pure standards were constructed to enable quantification of the pigments. Duplicate samples were analyzed for each time point of each experiment.

Fatty acid analysis

The fatty acids present in the lipids of the dried chloroform extracts were converted into fatty acid methyl esters (FAMEs) using a 3 mL solution of 5% (v/v) H_2SO_4 in methanol. After extraction with 3 mL hexane, the FAMEs were analyzed by GC-MS, essentially as described by Bouwmeester et al. (1999). A ZB-1 column (Phenomenex, Toorance, CA, USA, 30 m x 0.25 mm internal diameter and 0.25 µm film thickness) was used and the column oven was programmed at an initial temperature of 45°C for 1 min, followed by a ramp of 10°C·min^{-1} to 310°C

and a final step of 7.5 min at 310°C. The FAMEs were quantified by comparing the total ion count of detected peaks to the total ion count of the internal standard. Duplicate samples were analyzed for each time point of each experiment.

Freeze fracture scanning electron micrographs

Algae in suspension were rapidly frozen in liquid propane. The frozen samples were placed in a dedicated cryopreparation chamber (Oxford Instruments CT 1500 HF, Eynsham, England), fractured and sputter-coated with a layer of 10 nm Pt at -90°C. The samples were cryotransferred into a field emission scanning microscope (JEOL 6300F, Tokyo, Japan) on a sample stage at -180°C. The analyses were performed with SE detection at 5 kV. All images were recorded digitally and optimized for publication by Adobe Photoshop CS.

Results and discussion

Induction of β-carotene accumulation

Batch experiments were performed to determine which stress factors induce β-carotene accumulation in *D. salina*. Eight combinations of stress factors were studied (Table 3.1) of which five combinations resulted in increased β-carotene levels (Figure 3.2). All conditions with high light intensities induced β-carotene production, whereas at a low light intensity only nitrogen depletion led to an elevated β-carotene content. We did not observe β-carotene overproduction in the cultures where low light intensities were combined with either high salinity or low temperature, even though this was expected based upon previous studies (Ben-Amotz and Avron 1983; Borowitzka et al. 1990; Krol et al. 1997). Possibly, the differences between these studies and our results can be explained by the lower light intensity used in our study. In any case, high light intensity and nitrogen depletion are clearly the most potent inducers of β-carotene accumulation in *D. salina*.

One of the problems associated with batch experiments is a significant reduction

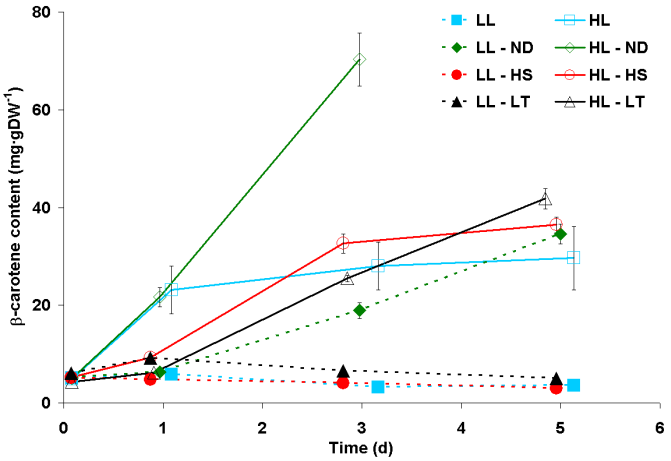


Figure 3.2 *Effect of various cultivation conditions (codes explained in Table 3.1) on the β-carotene content of D. salina cells cultivated in batch reactors. Lines are drawn to guide the eye.*

of the average light intensity inside the reactor. Such reduction was most notable (data not shown) for the high-light conditions and was the result of enhanced light absorption due to accumulation of biomass and β-carotene. To circumvent this problem, the effect of light intensity on carotenogenesis was studied in more detail using turbidostat cultivations, which enable a better control of the light regime.

Response to successive stepwise increases in light intensity

Turbidostat cultivations in which the incident light intensity was increased stepwise were performed. At each light step (150, 250, 400, and 650 µmol photons·m^{-2}·s^{-1}), the cell size and intracellular concentrations of β-carotene and total chlorophyll were determined. The cell size increased within approximately 1 day after each increase in light intensity and remained constant after that (Figure 3.3). A similar response pattern was observed for the β-carotene content of *D. salina* cells (Figure 3.3). Upon each shift in light intensity, the intracellular

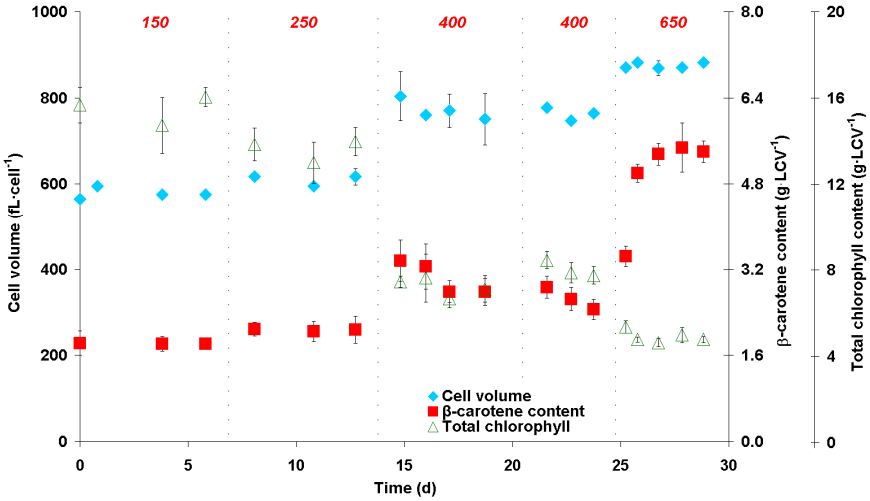


Figure 3.3 *Effect of stepwise increases in light intensity on the cell size and intracellular concentrations of β-carotene and total chlorophyll of D. salina cells cultivated in a turbidostat. The dotted lines indicate the shifts to higher light intensities, which are indicated in italics (in µmol photons·m^{-2}·s^{-1}). The dotted line between the two intensities of 400 µmol photons·m^{-2}·s^{-1} splits two separate runs.*

β-carotene concentration increased within approximately 1 day and did not significantly change afterwards. A different response pattern was found for the total chlorophyll (*a* + *b*) content, which decreased within 1 day upon light increase and subsequently remained unaltered (Figure 3.3).

The changes in cell size as well as β-carotene and chlorophyll content suggest that *D. salina* is able to respond within approximately 1 day to increases in incident light intensity with step sizes ranging from 100 to 250 µmol photons·m^{-2}·s^{-1}. This result indicates that it was necessary to study the light-induced carotenogenesis in time-intervals much shorter than 1 day.

Low to high light shift: effects on cell division and size

To further analyze the kinetics of the initial change in β-carotene content, we

applied a second turbidostat cultivation strategy, in which the response of *D. salina* to a large stepwise increase in light intensity was followed at short time intervals of about 90 minutes. After reaching steady cell numbers, cell volume, and intracellular pigment concentrations at 200 µmol photons·m^{-2}·s^{-1}, the incident light intensity was increased to 1400 µmol photons·m^{-2}·s^{-1} whilst maintaining a light transmission of 13%. Upon this shift to high light, the dilution rate increased approximately threefold within 1 hour and subsequently decreased to zero in about 4 days (Figure 3.4). The sudden input of extra light energy apparently elicited a nearly instantaneous response in *D. salina*, which would have led to an undesirable enhancement of culture turbidity in standard batch cultivations.

The initial increase in dilution rate was mainly the result of an increase in cell size rather than cell division. In fact, whereas the cells started swelling immediately after the shift to high light (first sample of induced cells was taken within 1 hour after the shift to high light; Figure 3.5B), cell division was arrested during the first 7.5 hours following induction by high light (based upon a calculation using the

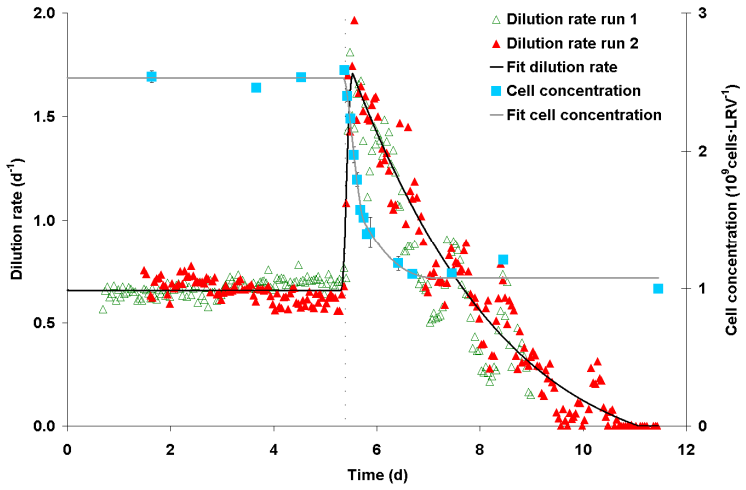
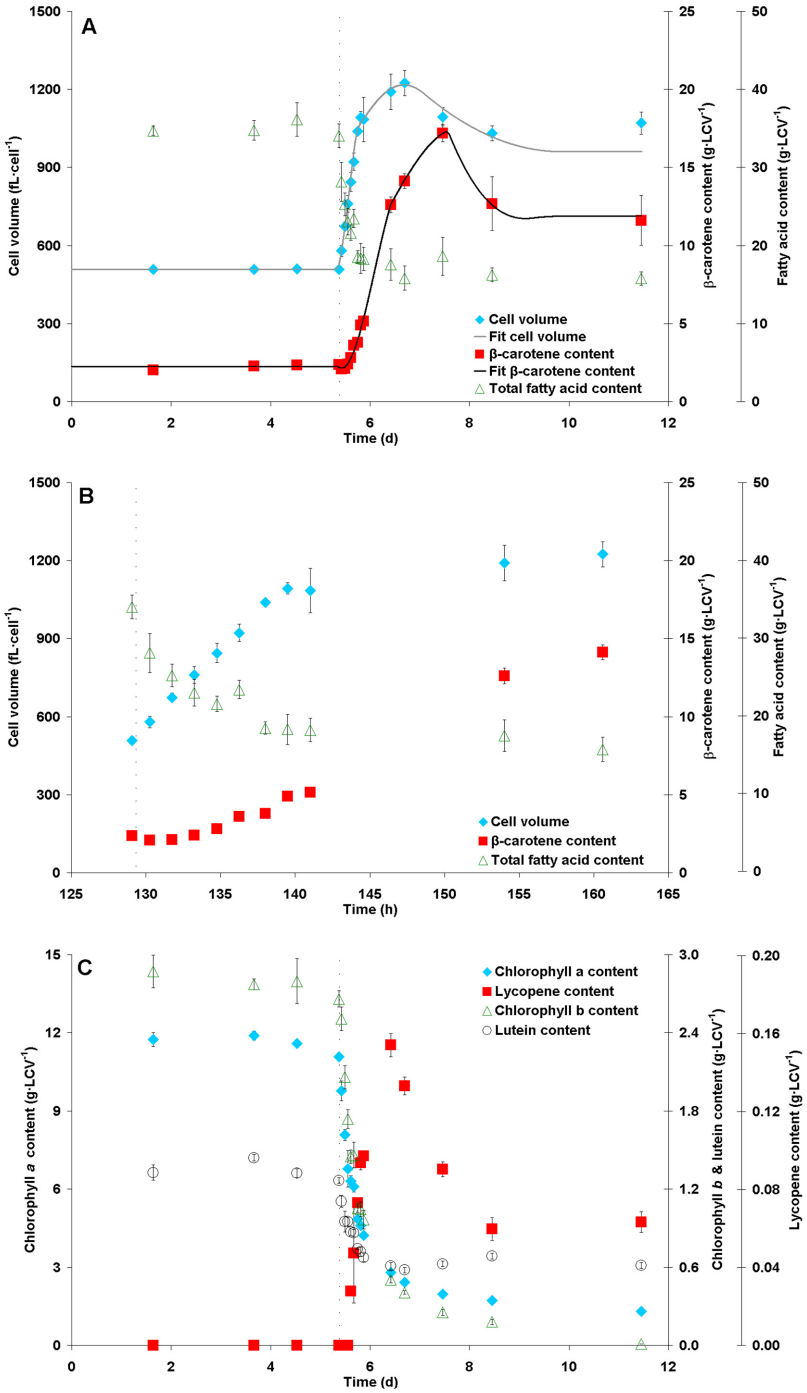


Figure 3.4 *Effect of a shift to high light intensity (dotted line) on the dilution rate and cell concentration of duplicate turbidostat cultivations of D. salina cells.*


A
Cell volume (fL·cell⁻¹)
β-carotene content (g·LCV⁻¹)
Fatty acid content (g·LCV⁻¹)
Time (d)
Cell volume
Fit cell volume
β-carotene content
Fit β-carotene content
Total fatty acid content
B
Cell volume (fL·cell⁻¹)
β-carotene content (g·LCV⁻¹)
Fatty acid content (g·LCV⁻¹)
Time (h)
Cell volume
β-carotene content
Total fatty acid content
C
Chlorophyll a content (g·LCV⁻¹)
Chlorophyll b & lutein content (g·LCV⁻¹)
Lycopene content (g·LCV⁻¹)
Time (d)
Chlorophyll a content
Lycopene content
Chlorophyll b content
Lutein content

changes in measured cell concentrations and dilution rate; Figure 3.6A). Similar swelling has been observed for another *D. salina* strain that was exposed to an increased light intensity (Cowan et al. 1995) and high-light-induced cell-cycle arrest was as well found in a previous study on *D. salina* (Lers et al. 1990). Finally, the combination of these growth responses resulted in a sharp decrease in cell concentration upon induction (Figure 3.4).

Low to high light shift: effects on β*-carotene accumulation*

Immediately after the shift to high light, *D. salina* cells started to produce β-carotene (Figures 3.5A, 3.5B and 3.6A). During the first 4 hours however, the intracellular β-carotene concentration remained stable at 2.2 $g \cdot LCV^{-1}$, which resulted from equiproportional increases in the production rates of cell volume and β-carotene (Figure 3.6B). Thus, the lag phase in β-carotene accumulation (Figure 3.5B) does not indicate a lag in the initiation of β-carotene synthesis upon induction.

Following this initial phase, the β-carotene productivity ($g \cdot LRV^{-1} \cdot d^{-1}$) continued to increase, whereas the cell volume productivity ($LCV \cdot LRV^{-1} \cdot d^{-1}$) suddenly dropped leading to a 7.6-fold enhanced intracellular β-carotene concentration after 2 days of stress (Figures 3.5A and 3.6B). The maximum observed β-carotene level was 17 $g \cdot LCV^{-1}$ (equivalent to 31 $mg \cdot gDW^{-1}$ or 19 $pg \cdot cell^{-1}$), which is significantly lower than the 10% of dry weight reported for *D. salina* by Ben-Amotz et al. (1982) and the final concentration of 80 $pg \cdot cell^{-1}$ found in *D. salina* by Lers et al. (1990). However, the first and possibly also the second study applied combinations of stress factors together (high salinity combined with high light intensity and nitrogen depletion combined with high light intensity, respectively).

Figure 3.5 *Effect of a shift to high light intensity (dotted lines) on D. salina cells cultivated in a turbidostat. A: Cell size and intracellular concentrations of* β*-carotene and total fatty acids. B: Zoom-in of (A) with time given in hours. C: Intracellular concentrations of chlorophyll a, chlorophyll b, lutein and lycopene.*

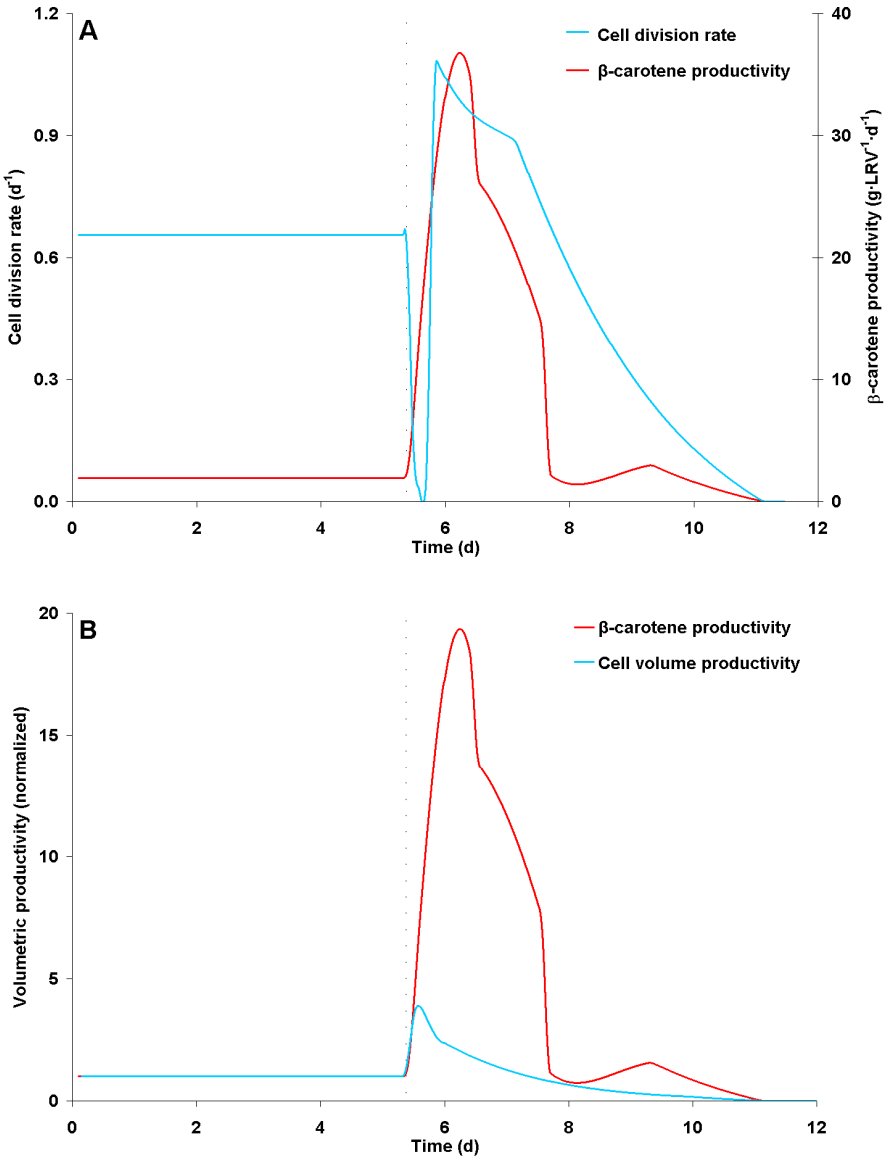


Figure 3.6 *Effect of a shift to high light intensity (dotted lines) on D. salina cells cultivated in a turbidostat. A: Cell division rate and the volumetric* β*-carotene productivity. B: Normalized volumetric productivity rates of* β*-carotene and cell volume.*

Lers et al. actually observed two periods of enhanced productivity upon induction with light, the second phase starting about 5 days after induction (Lers et al. 1990). We did not observe such a second phase and propose that the second accumulation phase was the result of nitrogen depletion as can be calculated from the data provided by the authors (Lers et al. 1990), an assumed cellular nitrogen content of 10% (dry weight basis) and an assumed cellular dry weight of 700 pg/cell, which values are both based on our own studies with *D. salina*. In addition, in the studies of Lers et al. (1990), the β-carotene content after the first phase was 20 pg·cell^{-1}, which is similar to the value we obtained in our study.

These results emphasize the importance of combined stress regimes for reaching highest cellular β-carotene contents. High cellular contents, however, do not necessarily mean high productivity. The maximum β-carotene productivity observed in our study was 37 mg·LRV^{-1}·d^{-1} (equivalent to 25 g·LCV^{-1}·d^{-1}, 41 mg·gDW^{-1}·d^{-1} or 30 pg·cell^{-1}·d^{-1}) which was reached 21 hours after induction (Figure 3.6A). Batch experiments with high-light-induced *D. salina* cells at low initial biomass concentrations showed a similar productivity plot with a maximum after 18 hours of induction (Lers et al. 1990). The maximum itself, however, was significantly lower than that found in the present study (approximately 20 pg·cell^{-1}·d^{-1} vs. 30 pg·cell^{-1}·d^{-1}).

The observed maximum volumetric β-carotene productivity of 37 mg·LRV^{-1}·d^{-1} is 370 times higher than the average productivity of large open ponds (Borowitzka 1999) and ~25 times higher than found in smaller, more productive, ponds (Ben-Amotz 1995).

On the cell side of the production process, a similar gap is evident. In the present study a maximum productivity of 30 pg·cell^{-1}·d^{-1} was observed, compared to an average productivity of 3 pg·cell^{-1}·d^{-1} found in the small open ponds (Ben-Amotz 1995). These gaps between the maximum and average productivities point to a large potential for improvement. A potential which could possibly be exploited when milking of *D. salina*, that is, the online extraction of β-carotene with organic solvents without destroying the cells (Hejazi et al. 2004), could be applied at industrial scale.

Low to high light shift: effects on lutein, lycopene and chlorophyll levels

Besides β-carotene, we also quantified the cellular contents of lutein, lycopene, chlorophyll *a*, and chlorophyll *b* during the shift from 200 to 1400 µmol photons·m^{-2}·s^{-1} (Figure 3.5C). Similarly to what happens in most high-light-stressed green algae (Neale 1987), chlorophyll degradation quickly started after light induction. The intracellular concentrations of chlorophyll *a* and chlorophyll *b* decreased rapidly and stabilized at a total chlorophyll content that was reduced by ~85%. Lutein concentrations decreased sharply upon light-stress and featured a dilution curve directly opposite to that of cell size (Figure 3.5A and 3.5C).

An increase in intracellular concentration was observed for lycopene, which only became detectable at the onset of the second β-carotene accumulation phase, that is, 4 hours after the shift to high light (Figure 3.5C). The intracellular lycopene concentration already started to decrease after 1 day of stress and 1 day before the decrease of β-carotene commenced. Nevertheless, the positive correlation between lycopene and β-carotene overproduction suggests that these metabolites might be under the control of the same regulatory process, which does not seem unlikely as lycopene is the biosynthetic precursor of β-carotene (Figure 3.7).

The accumulation of lycopene at high light intensities also suggests that the activity of the enzyme lycopene β-cyclase may become a limiting step in the production of β-carotene.

Low to high light shift: effects on fatty acid accumulation

D. salina cells contained five major fatty acids (C16:0, C16:4, C18:1, C18:2, and C18:3) which accounted for about 95% of the total fatty acid content (based on the total ion counts in GC-MS chromatograms). Immediately after the increase in light intensity, the intracellular concentration of all fatty acids decreased, with the final total fatty acid content (expressed as intracellular concentration) being a factor 2.2 lower than under non-inducing conditions (Figure 3.5A).

It has been reported that triacylglycerols are present in high quantities in the

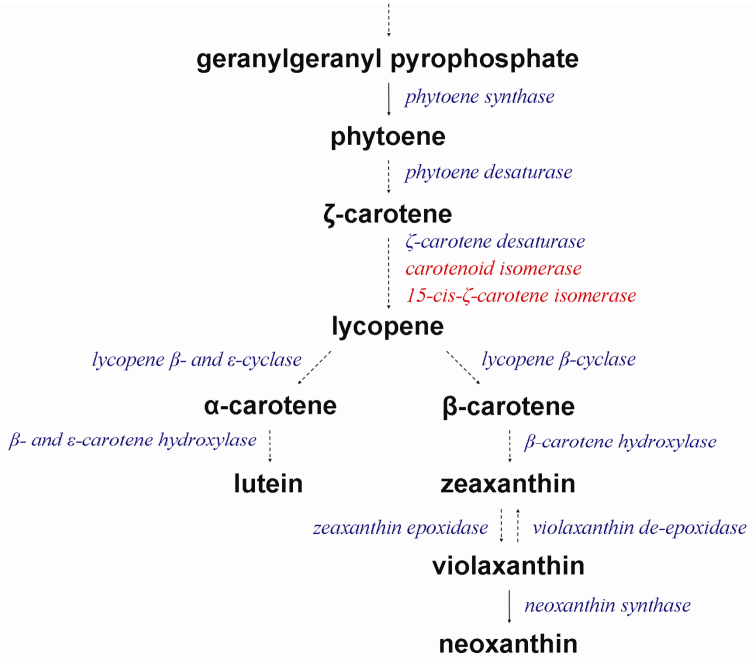


Figure 3.7 *Schematic representation of the carotenoid pathway found in green algae. Metabolites written in bold black characters; enzymes written in blue italics except for enzymes putatively involved in carotenoid isomerization (red italics); dotted arrows indicate non-shown intermediate steps.*

lipid globules of *D. salina* (Rabbani et al. 1998) and that these globules are formed under carotenogenic conditions (Ben-Amotz et al. 1982). A parallel increase of fatty acids and β-carotene has been reported for both high-light-stressed *D. salina* (Rabbani et al. 1998) and nitrogen depleted *D. salina* (Mendoza et al. 1999). In our experiments, however, both the batch cultivations (data not shown) and the turbidostat cultivations (Figure 3.5A) did not show evidence that the total fatty acid level and β-carotene content are positively correlated. On the contrary, in the turbidostat experiments we found a decrease in the intracellular total fatty acid concentration coinciding with an increase in β-carotene concentration upon the shift to high light intensity (Figure 3.5A).

When the total fatty acid concentration was expressed per cell (pg·cell^{-1}), no

Table 3.2 *Cellular concentrations of individual fatty acids of D. salina cells before and after induction by high light intensity in a turbidostat.*

Fatty acid	SS[a]	*S.D.*[b]	HL[c]	*S.D.*[b]
C16:0	3.83	*0.18*	6.10	*0.33*
C16:4	3.17	*0.15*	0.96	*0.14*
C18:1	1.97	*0.18*	4.15	*0.28*
C18:2	2.16	*0.49*	3.67	*0.48*
C18:3	6.63	*0.32*	4.18	*0.59*
TFA[d]	17.8	*0.65*	19.0	*0.89*

[a] *Steady state value (pg·cell^{-1}) before induction.*
[b] *Standard deviation (pg·cell^{-1}).*
[c] *High-light value (pg·cell^{-1}) after 31 hours of induction by high light.*
[d] *Total fatty acids.*

significant change in fatty acid content was observed after the switch to high light (Table 3.2). The pattern, however, was different for the individual fatty acid species: C16:0, C18:1, and C18:2 increased, while C18:3 and C16:4 decreased upon induction by light (Table 3.2). As a result, the degree of unsaturation, which is expressed as the average number of double bonds per fatty acid molecule, dropped from 2.2 ± 0.1 (mean ± standard deviation, n = 8) under low-light conditions to 1.6 ± 0.2 (n = 10), 1 day after the shift to high light, after which it remained stable.

Unsaturated fatty acids are particularly sensitive to oxidation and the observed specific loss of unsaturated fatty acids upon the shift to high light thus suggests photo-oxidative damage. As both the fatty acid species C16:4 and C18:3 are tightly associated with the galactolipids of the thylakoid membranes (Cho and Thompson 1987; Fried et al. 1982), their decrease may indicate that specifically the photosynthetic membranes experience oxidative stress upon increases in light intensity (Niyogi 1999).

Using scanning electron microscopy we analyzed induced and non-induced cells

of *D. salina* (Figure 3.8). After 31 hours of induction, we observed the appearance of lipid globules and the disappearance of most thylakoid membranes. This could indicate that the amount of thylakoid-associated fatty acids decreased to the same extent as the globule-associated fatty acids increased, since the observed total fatty acid concentration per cell did not

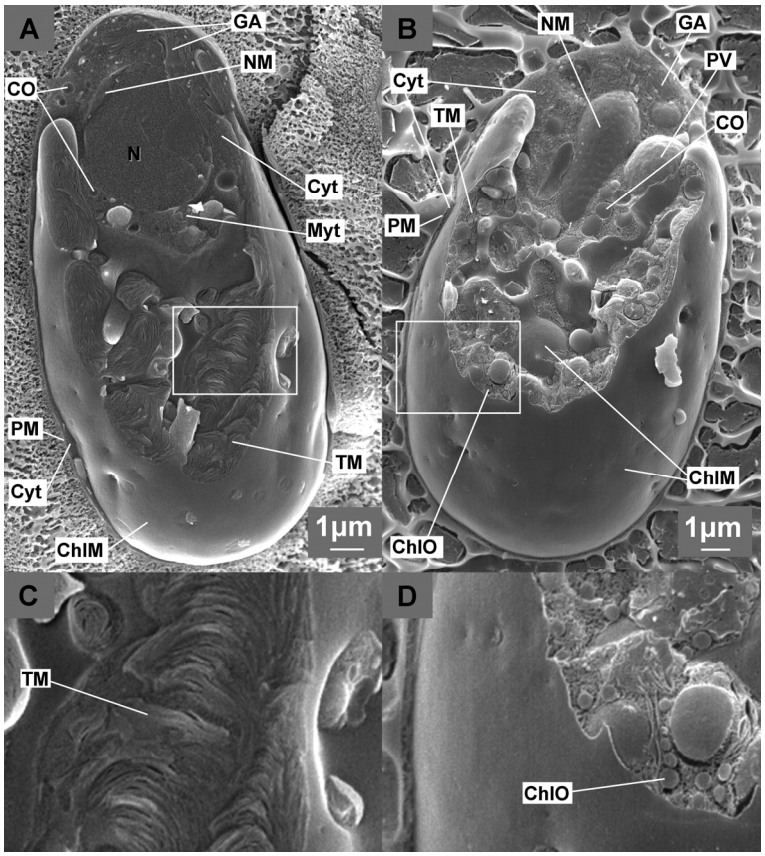


Figure 3.8 *Freeze fracture scanning electron micrographs of non-induced (A: total cell, C: enlargement of chloroplast) and induced (B: total cell, D: enlargement of chloroplast) D. salina cells. Abbreviations: ChlM – chloroplast membrane, ChlO – chloroplastid located oil droplet, CO – cytoplasm located oil droplet, Cyt – cytoplasm, GA – Golgi apparatus, NM – nuclear membrane, Myt – mitochondrion, PM – plasma membrane, PV – putative vacuole, TM – thylakoid membrane.*

significantly change upon induction (Table 3.2). In support of this, we found that the fatty acid species C16:4 and C18:3, which both are major constituents of the thylakoid membrane lipids (Cho and Thompson 1987; Fried et al. 1982), decreased upon a shift to high light (Table 3.2), whereas the amount of fatty acid species putatively typical for lipid globules (Klyachko-Gurvich et al. 1997; Mendoza et al. 1999), that is, C16:0 and C18:1, increased upon induction (Table 3.2).

From these results we conclude that high-light-intensity induction of *D. salina* cells leads to loss of unsaturated fatty acids and that β-carotene accumulation does not correlate with total fatty acid accumulation, but does positively correlate with the C18:1 and C16:0 fatty acids.

Conclusions

We have confirmed that light intensity is one of the most important factors influencing β-carotene accumulation in *D. salina*. Light-induced β-carotene production occurred rapidly and in two distinct phases. In the first 4 hours after induction, intracellular β-carotene levels remained stable, after which a massive increase was observed. The observed maximum production rates of β-carotene, defined either per cell or per reactor volume, were one or two orders of magnitude larger than the average production rates obtained at commercial β-carotene production plants, signifying an extensive potential for improvement of the production process. Furthermore, high-light-induced carotenoid accumulation appeared to be associated with oil globule formation and our results indicate that β-carotene accumulation in *D. salina* is correlating with increased accumulation of specific fatty acid species rather than with total fatty acid content.

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Nomenclature

DW: dry weight
LCV: liter cell volume
LRV: liter reactor volume
PAR: photosynthetic active radiation (400-700 nm)

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Chapter 4

Carotenoid and fatty acid metabolism in nitrogen-starved *Dunaliella salina*

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Abstract

Nitrogen availability and light intensity affect β-carotene overproduction in the green alga *Dunaliella salina*. Following a previous study on high-light stress, we here report on the effect of nitrogen depletion on the growth characteristics and β-carotene as well as fatty acid metabolism of *D. salina* under a constant light regime in a turbidostat. Upon nitrogen depletion, the biomass yield on absorbed light approximately doubled, due to a transient increase in cell division rate, swelling of the cells and a linear increase of the cell-specific density.

Simultaneously, β-carotene started to accumulate up to a final intracellular concentration of 14 mg·LCV^{-1} (*i.e.* 2.7% of AFDW). This β-carotene production accounted for 6% of the increased cell-specific density, indicating that other biochemical constituents accumulated as well.

Since *D. salina* accumulates β-carotene in lipid globules, we also determined the fatty acid content and composition of *D. salina*. The intracellular concentration of the total fatty acid pool did not change significantly during nitrogen starvation, indicating that β-carotene and total fatty acid accumulation were unrelated, similar to what was found previously for high-light treated cells. However, for both high-light and nitrogen stress, β-carotene accumulation negatively correlated with the degree of unsaturation of the total fatty acid pool and, within the individual fatty acids, correlated positively with oleic acid biosynthesis, suggesting that oleic acid may be a key component of the lipid-globule-localized triacylglycerols and thereby in β-carotene accumulation.

Introduction

The microalga *Dunaliella salina* is one of the richest sources of natural β-carotene, which is a lipid-soluble orange pigment that is used as a colorant in food and feed. Under standard growth conditions *D. salina* contains approximately 5 to 10 mg β-carotene per gram dry weight (DW), which is similar to other green algae (Del Campo et al. 2007). This β-carotene content can rise to as much as 10% of DW when *Dunaliella salina* is subjected to stress conditions such as high salinity, high light intensity, nutrient deprivation and extreme temperatures (Ben-Amotz 1996; Ben-Amotz and Avron 1983; Ben-Amotz et al. 1982; Borowitzka et al. 1990; Kleinegris et al. 2009; Krol et al. 1997; Shaish et al. 1993).

Nitrogen deficiency is, next to high light intensity, one of the most potent environmental factors inducing β-carotene accumulation in *D. salina* (Lamers et al. 2010). However, little is known about the underlying regulatory mechanisms of the carotenogenic response to nitrogen deficiency (Lamers et al. 2008). Using the inhibitors actinomycin D, chloramphenicol and cycloheximide it was found that transcriptional and translational activity are indispensible for high-light-induced β-carotene overproduction (Lers et al. 1990), but it remains to be confirmed whether this is also the case for nitrogen deficiency.

In addition, the effect of nitrogen availability on the expression of genes directly involved in the β-carotene biosynthetic pathway requires further examination. Thus far, it has been reported for *D. salina* that nitrogen deprivation resulted in a decreased expression of 1-deoxyxylulose-5-phosphate synthase, while the expression of phytoene synthase transcripts remained constant (Sanchez-Estudillo et al. 2006). The lack of a combination of nutrients, including nitrate, was reported to activate the expression of several genes involved in carotenoid biosynthesis, *i.e.* phytoene synthase, phytoene desaturase, lycopene β-cyclase and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (Coesel et al. 2008; Ramos et al. 2007; Ramos et al. 2009).

As an alternative to activation of genes of the carotenoid biosynthetic pathway, Rabbani *et al.* proposed that the formation of lipid globules, that can sequester

β-carotene, is the driving force for β-carotene overproduction (Rabbani et al. 1998). The observations that β-carotene and total fatty acid contents simultaneously increased in *D. salina* cells upon either high-light treatment alone (Rabbani et al. 1998) or high-light-treatment in combination with nitrogen depletion (Mendoza et al. 1999), support this metabolic sink theory.

Furthermore, *de novo* fatty acid synthesis appeared to be indispensible for β-carotene accumulation, as could be concluded from the absence of both lipid globules and orange pigments when *D. salina* cells were cultivated under otherwise carotenogenic conditions in the presence of sethoxydim or cerulenin, which are both inhibitors of key steps in the fatty acid biosynthesis pathway (Rabbani et al. 1998). In addition, the cellular oleic acid (C18:1) content positively correlated at various light intensities with the cellular β-carotene content of high-light-induced, nitrogen deprived, *D. salina* cells (Mendoza et al. 1999). This could suggest that oleic acid is an important constituent of the stress-induced lipid globules. Nevertheless, it remains unclear whether nitrogen-deficiency-induced overproduction of β-carotene in *D. salina* is mediated through activation of genes involved in the carotenoid pathway itself or through some other mechanism such as lipid globule formation (Lamers et al. 2008).

In the last two studies mentioned, as well as in most other studies concerning carotenogenesis in *D. salina*, batch cultivations have been used. Batch cultivations, however, are not very suitable for studying the specific and direct effect of nitrogen depletion, as the average light intensity perceived per algal cell will unavoidably decrease during the experiment due to changes in metabolism and growth (Lamers et al. 2008). Since light intensity is one of the most important determinants for β-carotene accumulation in *D. salina* (Lamers et al. 2010), stable and accurately controlled light conditions are key for studying carotenoid metabolism in *D. salina*.

One way to meet this criterion is the use of so-called turbidostat cultures in which the turbidity of the culture, and thus the light perception of the algal cells, is maintained at a fixed and defined level by a constant and automatic adjustment of the dilution rate of the culture broth (Myers and Clark 1944).

Previously, we applied such turbidostat cultures to study the effect of light intensity and the possible role of fatty acid metabolism in carotenoid production by *D. salina* (Lamers et al. 2010). It appeared that high-light-induced carotenoid overproduction was associated with oil globule formation and a decrease in the degree of fatty acid unsaturation. Furthermore, β-carotene accumulation was found to correlate with the production of specific fatty acid species, *i.e.* C16:0 and C18:1, rather than with total fatty acid content (Lamers et al. 2010).
In this paper, we report on the specific response of *D. salina* cells to nitrogen depletion under controlled and constant light conditions. The effects of nitrogen depletion on the growth characteristics and biomass productivity, as well as on the kinetics of β-carotene accumulation and fatty acid metabolism were investigated.

Materials and methods

Algae and cultivation conditions

Dunaliella salina (CCAP 19/18) cells were (pre-)cultivated as described previously (Lamers et al. 2010). The incident light intensity and light transmission through the turbidostat were set at 200 µmol photons·m^{-2}·s^{-1} and 13%. After steady state was reached, samples were taken from the reactor on a daily basis for a period of 3 days. Subsequently, the standard feed medium was replaced with nitrogen-free feed medium, in which KNO_3 was replaced with an equivalent amount of KCl, and the sampling rate was increased to approximately once per every 2 to 5 hours.

Determination of residual nitrate

Duplicate 5 mL samples taken from the reactor were gently filtered through a 0.22-µm filter, after which the filtrate was stored at -20°C for later analysis. Residual nitrate was measured using an automated cadmium reduction method (Nitrogen, 4500-NO_3-F, Clesceri et al. 1998).

Determination of cell number, cell size and ash-free dry weight

Measurements were performed as described previously (Lamers et al. 2010) with the addition that, following the gravimetric determination of the algal dry weight, the filters were transferred to a 600°C oven for 24 hours, after which the ash content was determined gravimetrically.

Extraction and analysis of fatty acids and β-carotene

Methods and materials were essentially the same as described previously (Lamers et al. 2010) with the exception that a DB-225 column (Agilent Technologies, 30 m x 0.25 mm internal diameter and 0.25 µm film thickness) was used to separate the fatty acid methyl esters, with the column oven programmed at an initial temperature of 60°C for 2 minutes, followed by a ramp of 20°C·min^{-1} to 120°C, a second ramp of 3°C·min^{-1} to 165°C, a third ramp of 2°C·min^{-1} to 205°C and a final step of 4 min at 205°C.

Results

To study the specific effect of nitrogen starvation on *D. salina*, cells were cultivated in a turbidostat, which ensured a constant light regime during the experiment by automatically diluting the culture with fresh medium to a desired density. First, the algae were grown for 15 days at an incident light intensity of 200 µmol photons·m^{-2}·s^{-1} and supplied with nitrogen-containing medium until a steady state was reached in the turbidostat. An overview of all steady-state data is given in Table 4.1. The feed medium was then (at t = 0) replaced with nitrogen-free medium, after which the initial response to nitrogen starvation was studied.

Nitrogen consumption, dilution rate and cell growth

The specific consumption rate of nitrogen was 0.11 gN·gAFDW^{-1}·d^{-1} during nitrogen-replete steady-state conditions. Upon switching to nitrogen-free medium (at t = 0) the nitrogen consumption rate remained constant, leading to a complete depletion of residual nitrate in approximately 8 hours (Figure 4.1). The

Table 4.1 *Growth conditions and characteristics of D. salina cells during steady-state cultivation in a turbidostat under nitrogen replete and low light conditions.*

Steady state parameter	**SS-value**[a]	***St.dev.***[b]
Nitrogen content of feed medium (mM)	5.57	*0.01*
Residual nitrogen content of turbidostat (mM)	0.94	*0.01*
AFDW concentration of reactor ($gAFDW \cdot LRV^{-1}$)	0.38	*0.03*
Specific nitrogen consumption rate ($gN \cdot gAFDW^{-1} \cdot d^{-1}$)	0.11	*0.01*
Algal hold-up ($mLCV \cdot LRV^{-1}$)	1.28	*0.03*
Cell volume ($\mu m^3 \cdot cell^{-1}$)	495	*6*
Dilution rate = cell division rate (d^{-1})	0.61	*0.04*
Cell concentration (10^9 $cells \cdot LRV^{-1}$)	2.59	*0.05*
Ash content of cells (% of total dry weight)	23	*1*
Volumetric AFDW productivity ($gAFDW \cdot LRV^{-1} \cdot d^{-1}$)	0.23	*0.01*
Cell-specific density ($gAFDW \cdot LCV^{-1}$)	300	*24*
β-carotene content ($g \cdot LCV^{-1}$)	2.2	*0.1*
β-carotene content (% of AFDW)	0.75	*0.05*
Total fatty acid content ($g \cdot LCV^{-1}$)	37.0	*0.1*
Total fatty acid content (% of AFDW)	12.8	*0.4*

[a] *Steady state value.*
[b] *Standard deviation, n = 12.*

dilution rate of the turbidostats remained constant at 0.61 d^{-1} until residual nitrogen was depleted (t = 8h). Upon complete nitrogen depletion, the dilution rate increased to 0.82 d^{-1} in approximately 7 hours (Figure 4.1) and then decreased again to about 0.4 d^{-1}. These changes in dilution rate, which were observed in two independent turbidostat cultures (Figure 4.1), indicate a transient increase in the production rate of light-absorbing material in the culture.

The algal hold-up in the turbidostat (*i.e.* the fraction of the total culture volume occupied by the algae) remained constant throughout the entire experiment

(Figure 4.1), which suggests that the transient increase in dilution rate was at least partly characterized by an enhanced cell growth. Indeed, cell growth, in size as well as in number, has a large impact on light absorption by the culture and thus on the dilution rate. Following the switch to medium without nitrogen, the apparent cell division rate remained constant until residual nitrogen was depleted (at t = 8h) and thereafter increased from 0.61 d^{-1} to 0.87 d^{-1} in approximately 4 hours (Figure 4.2). The apparent cell division rate was defined as the true cell division rate minus the cell lysis rate and was calculated from the cell number measurements and the online-measured changes in dilution rate. After having increased to 0.87 d^{-1} the apparent division rate decreased and finally became negative when the true cell division rate became lower than the cell lysis rate.

Cell growth through swelling of the cells started approximately 10 hours after nitrogen was depleted from the culture (at t = 18h; Figure 4.2). This swelling continued for at least 25 hours, during which the shape of the cells changed from ovoid to round (data not shown) and the cells almost doubled in size from 495 to 927 μm^3 (Figure 4.2).

Cell-specific density and yield on light

Upon complete depletion of residual nitrogen (t = 8h), we observed a linear increase in the cell-specific density from 300 to 503 $gAFDW \cdot LCV^{-1}$ at the end of the experiments (t = 43h, Figure 4.3). This increase in cell-specific density, in combination with the swelling and the transient increase in apparent cell division rate (Figures 4.2 and 4.3), had a large impact on the volumetric biomass productivity. While the productivity at steady state was 0.23 $gAFDW \cdot LRV^{-1} \cdot d^{-1}$, it started to increase within 2 hours after nitrogen depletion (t = 10h) reaching a maximum of 0.52 $gAFDW \cdot LRV^{-1} \cdot d^{-1}$ at t = 16.5h (Figure 4.4).

Since the amount of absorbed photons in the turbidostat was, by definition, constant during the experiment, this nitrogen-deficiency-induced increase in volumetric biomass productivity implies that the algae more than doubled their yield on absorbed light energy (*i.e.* from 0.45 to 1.0 $gAFDW \cdot mol\ photons^{-1}$).

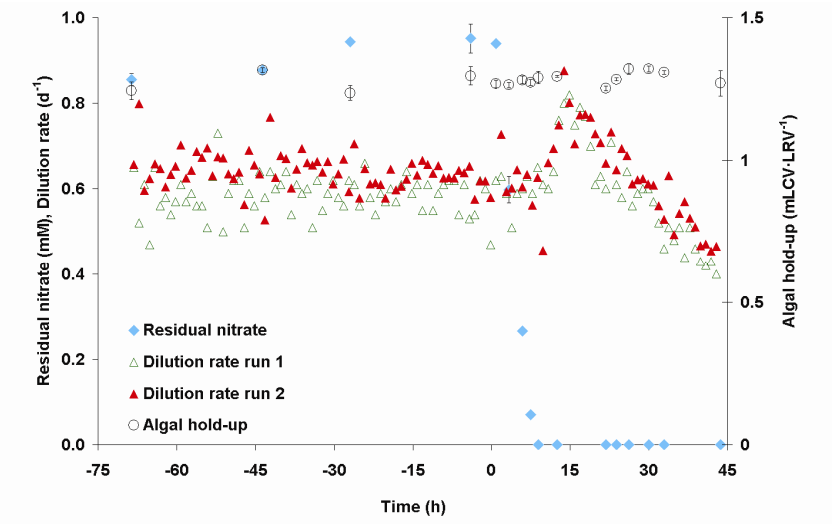

Figure 4.1 *Effect of nitrogen depletion on the dilution rate and algal hold-up of duplicate turbidostat cultivations of D. salina cells. Error bars indicate standard deviations of duplicate measurements.*

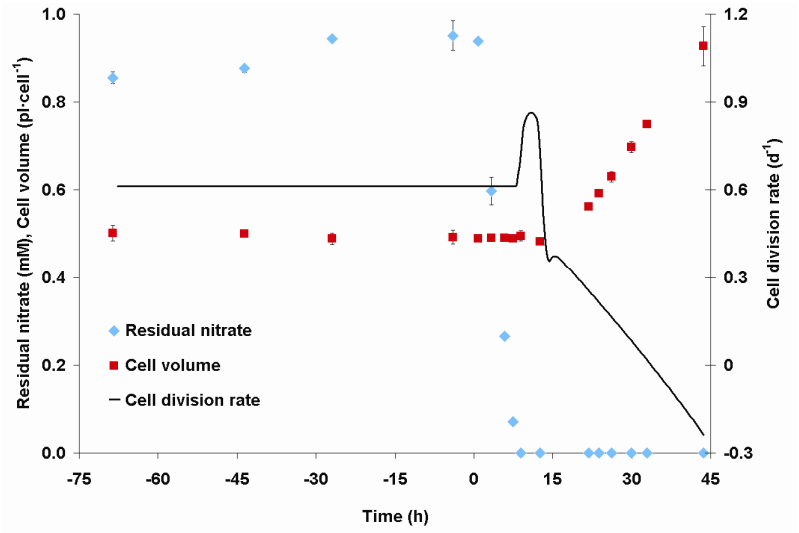

Figure 4.2 *Effect of nitrogen depletion on the cell size and apparent cell division rate of D. salina cells cultivated in a turbidostat. Error bars indicate standard deviations of duplicate measurements. The apparent division rate of algal cells in the turbidostat, defined as the true cell division rate minus the cell lysis rate, was calculated from the cell number measurements and the online-measured changes in dilution rate.*

β-carotene and fatty acids

The production of β-carotene was low at nitrogen-replete steady-state conditions, but started to increase immediately after all residual nitrogen was depleted, and after 4 hours of nitrogen depletion β-carotene production was boosted (t = 12h; Figure 4.5). During the first 4 hours after nitrogen depletion, the production rates of β-carotene and cell volume increased proportionally (Figure 4.5) leading to a stable intracellular β-carotene concentration of 2.2 g·LCV^{-1} (Figure 4.6). However, in the next 31 hours the production rate of β-carotene exceeded that of cell volume, which resulted in a linear increase of the intracellular β-carotene concentration to 13.6 g·LCV^{-1} (*i.e.* from 0.7 to 2.7% on AFDW basis). The β-carotene productivity increased from 1.28 g·LCV^{-1}·d^{-1} to a maximum of 14.4 g·LCV^{-1}·d^{-1} (*i.e.* from 1.64 to 18.5 mg·LRV^{-1}·d^{-1}) at about t = 38h (data not shown).

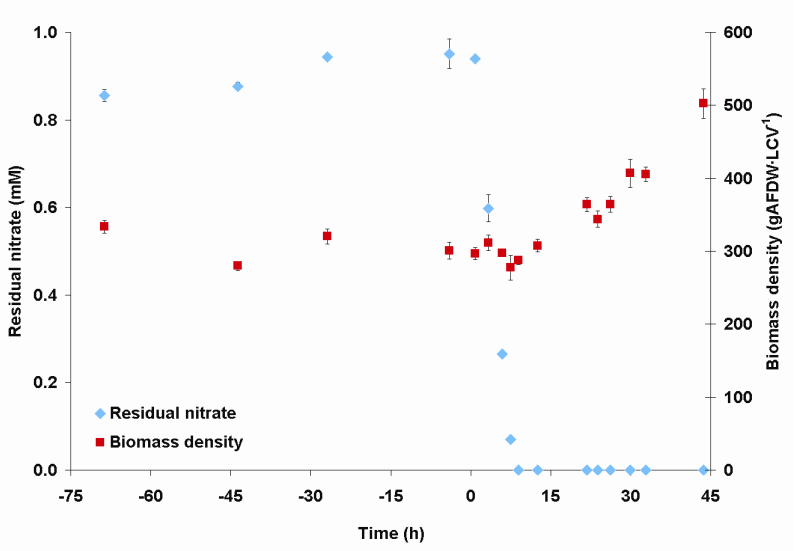

Figure 4.3 *Effect of nitrogen depletion on the cell-specific density of D. salina cells cultivated in a turbidostat. Error bars indicate standard deviations of duplicate measurements.*

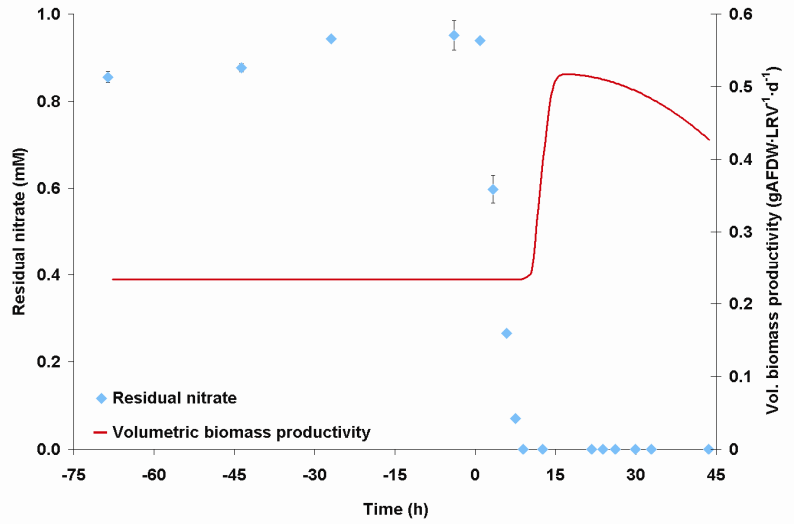

Figure 4.4 *Effect of nitrogen depletion on the volumetric biomass productivity of D. salina cells cultivated in a turbidostat. Error bars indicate standard deviations of duplicate measurements. The volumetric biomass productivity was calculated from the biomass concentration measurements and the online-measured changes in dilution rate.*

In the GCMS chromatograms of the hydrolyzed and methylated methanol-chloroform extracts of *D. salina* cells, 9 major peaks were detectable. Based on their mass spectra and retention times, they were identified as the fatty acid species C16:0 to C16:4 and C18:0 to C18:3. The total intracellular fatty acid concentration, calculated from the sum of these 9 fatty acid species, did not change significantly upon nitrogen depletion (Figure 4.6).

However, the individual fatty acids were differentially affected by nitrogen starvation (Figures 4.7 and 4.8). The contents of C16:1, C16:3, C16:4 and C18:3 decreased upon nitrogen depletion, while those of C16:0, C16:2, C18:0, C18:1 and C18:2 increased. These changes in fatty acid composition resulted in a reduced degree of unsaturation of the total fatty acid pool. The degree of unsaturation, which is expressed as the average number of double bonds per fatty acid molecule, dropped from 2.2 under nitrogen replete conditions to 1.8 at 35 hours after nitrogen depletion (at t = 43h).

Discussion

High light intensity and nitrogen deficiency are two of the most potent inducing factors of β-carotene accumulation in *D. salina* (Lamers et al. 2010). Previously, we examined the effect of light intensity and the possible role of fatty acid metabolism in carotenoid production by *D. salina* (Lamers et al. 2010). High-light-induced carotenoid overproduction appeared to be associated with oil globule formation and a decrease in the degree of fatty acid unsaturation. With regard to fatty acids, β-carotene accumulation in high-light-stressed *D. salina* cells correlated with the production of the specific fatty acid species C16:0 and C18:1, rather than with total fatty acid accumulation (Lamers et al. 2010).

In the following sections, the specific effects of nitrogen deficiency on the growth characteristics, β-carotene accumulation and fatty acid metabolism of *D. salina* are discussed, while similarities and differences between high-light stress and nitrogen starvation are highlighted.

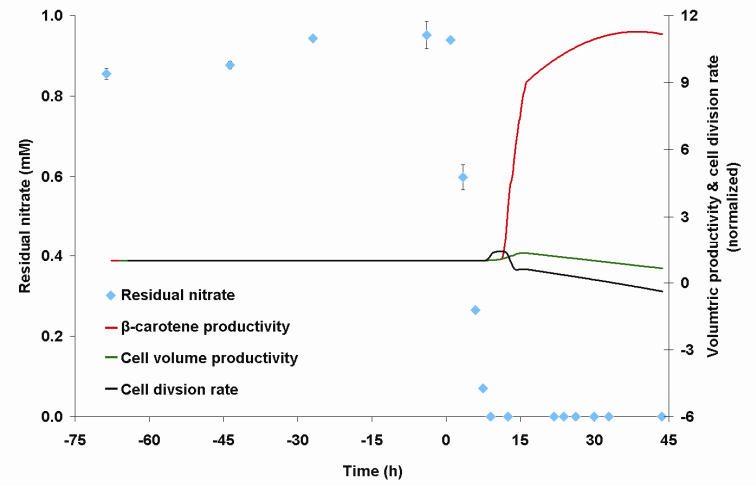

Figure 4.5 *Effect of nitrogen depletion on the normalized cell division rate and the normalized volumetric β-carotene and cell volume productivities of D. salina cells cultivated in a turbidostat. Error bars indicate standard deviations of duplicate measurements. The volumetric productivities and cell division rate were calculated from the algal hold-up, cell number and β-carotene measurements and the online-measured changes in dilution rate.*

Cell growth and biomass yield on light

In order to maintain a constant turbidity upon nitrogen depletion, the dilution rate of the turbidostat first increased and then decreased (Figure 4.1). This temporary increase was caused at least partly by a transient increase in the apparent cell division rate, which is defined as the true cell division rate minus the cell lysis rate (Figure 4.2). Since it seems unlikely that nitrogen depletion induces a reduction of cell lysis, we propose that an acceleration of cell division contributed to this transient increase in dilution rate.

This hypothesis is supported by the fact that a similar nitrogen-starvation-induced transient increase in cell division rate has been observed in the fission yeast *Schizosaccharomyces pombe* (Fantes 1984). This phenomenon has been explained by nutritional control over the cell size at which cells become committed to cell division: following transfer of *S. pombe* cells into medium without nitrogen, the size requirement for cell division shifts towards smaller cells, resulting in a transient acceleration of the division rate (Fantes and Nurse 1977). In our turbidostat experiments with *D. salina* we observed a simultaneous acceleration of cell division and a small, though significant ($p < 0.01$, $n = 30$), transient decrease of the average cell size upon nitrogen depletion (Figure 4.2), which might suggest the involvement of a similar nutritional control mechanism in this algal species.

Besides cell division, cell growth through swelling of the cells, which followed the transient decrease in cell size (Figure 4.2), also contributed to the transient increase in the dilution rate of the turbidostat. Swelling of *D. salina* cells is a typical response to various extreme conditions such as high light intensity and/or low temperature (Cowan et al. 1995) and a similar, initially linear, increase in *D. salina* cell size has recently been observed upon stress by a seven-fold increase in the incident light intensity in a comparable turbidostat experiment (Lamers et al. 2010). In the latter case, however, the light-induced cell size increase rate was 3.6 times higher than observed for nitrogen starvation in this study, suggesting that the type of environmental stress influences the initial rate of stress-induced swelling of *D. salina* cells.

The swelling of the cells may give some extra insight in the mechanisms involved in the observed enhancement of cell-specific density upon nitrogen depletion (Figure 4.3). Since the observed swelling does not support a water out-flux, the increase in cell-specific density must have been the result of enhanced production of metabolites which were densely packed inside the cells. Only a small fraction (6%) of this accumulated organic matter was accounted for by the cumulative production of all intracellular metabolites that were determined in this study (*i.e.* carotenoids and fatty acids, see Figures 4.3 and 4.6). However, other major biochemical constituents of *D. salina* biomass, such as carbohydrates (mainly glycerol and starch) and proteins, have not been determined in this study.

Research on *D. salina* and other algal species indicates that the intracellular concentration of protein may decrease upon nitrogen depletion, while carbohydrates may increase (Fabregas et al. 1989; Kilham et al. 1997; Uriarte et al. 1993; Vorst et al. 1994). Such changes resulted in an increase in the cell-specific density of *D. primolecta* cells (Uriarte et al. 1993), whereas an opposite trend was found in the green alga *Ankistrodesmus falcatus* (Kilham et al. 1997). For *D. salina* it remains to be tested experimentally, however, whether carbohydrate accumulation could account for the observed enhancement of cell-specific density upon nitrogen depletion.

The present study indicates that nitrogen depletion affects cell division, cell size and cell-specific density which results in a temporary doubling of volumetric biomass productivity and hence a doubling of the biomass yield per absorbed light unit (*i.e.* from 0.45 to 1.0 gAFDW·mol photons^{-1}). In the previously described turbidostat experiment with high-light stressed *D. salina* cells (Lamers et al. 2010), the volumetric biomass productivity increased as well (6.1-fold) upon induction, but the biomass yield on absorbed light energy actually decreased since the incident light intensity was increased 7-fold. Such a decrease in biomass yield on absorbed light is generally expected for high-light stress in algal cells, since a substantial part of the oversaturating light will be quenched non-photochemically by the cells (Telfer 2005).

Similarly, for nitrogen starvation of marine phytoplankton it is expected that the biomass yield on absorbed light energy decreases rather than increases (Berges and Falkowski 1998; La Roche et al. 1993). Although we actually observed an elevated volumetric biomass productivity, and thus also an elevated biomass yield on absorbed light energy, a decline in volumetric biomass productivity started at t = 16.5h, *i.e.* approximately 8.5 hours after complete nitrogen depletion (Figure 4.4). When this decline is extrapolated, the biomass yield on absorbed light energy is expected to ultimately fall below that of nitrogen sufficient *D. salina* cells. Nevertheless, it seems worthwhile to study the potential of nitrogen-limitation strategies for permanent improvement of the biomass yield on absorbed light in large scale microalgal production systems.

<u>β-*carotene and fatty acid metabolism*</u>

In our turbidostat experiments, an increase in β-carotene production was observed only after complete depletion of residual nitrogen (at t = 8h). The switch from moderate to massive increase in β-carotene productivity (at t = 12h)

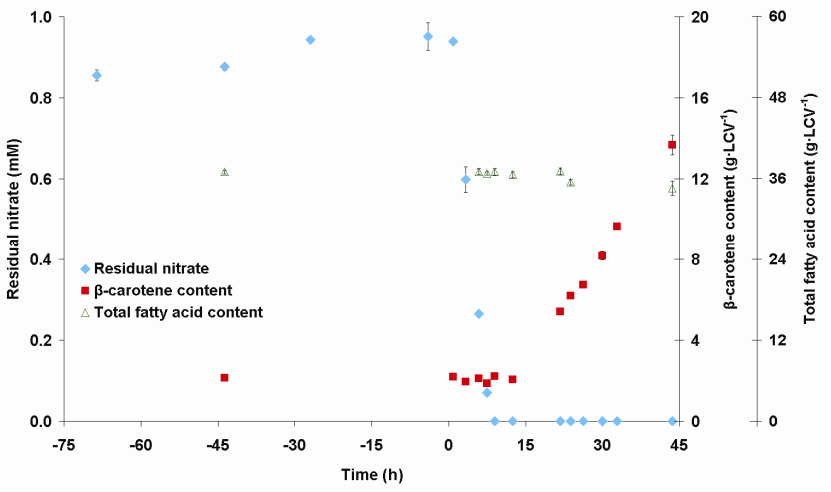

Figure 4.6 *Effect of nitrogen depletion on the intracellular levels of* β*-carotene and total fatty acids of D. salina cells cultivated in a turbidostat. Error bars indicate standard deviations of duplicate measurements.*

coincided with the onset of reduction in cell division rate (Figure 4.5), which suggests a correlation between these two events. Such correlation was also observed in a turbidostat experiment during which the algae were induced by a seven-fold increase in incident light intensity (Lamers et al. 2010), suggesting that the mechanisms underlying β-carotene overproduction might be similar for both high-light stress and nitrogen depletion.

The maximum β-carotene productivity observed in this study (18.5 mg·LRV^{-1}·d^{-1}) was approximately half of that found for the aforementioned high-light-induced turbidostat cultures (37 mg·LRV^{-1}·d^{-1}; Lamers et al. 2010) indicating that high light is a more potent inducer of β-carotene overproduction than nitrogen depletion. However, β-carotene production through nitrogen depletion appears more efficient in terms of energy use, since 7 times more light energy was applied in the high-light experiment. Nevertheless, both inducing factors resulted in maximum β-carotene productivities that were a factor 12 to 25 larger than the average productivity reported for small commercial open pond systems (1.5 mg·LRV^{-1}·d^{-1}; Ben-Amotz 1995). These gaps between maximum and average

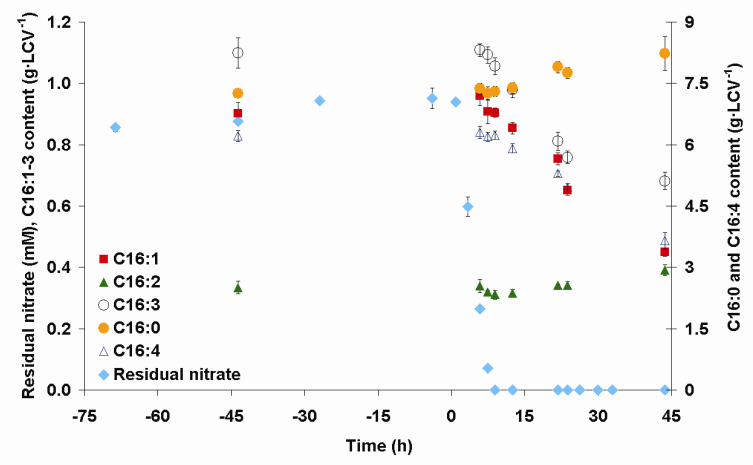

Figure 4.7 *Effect of nitrogen depletion on the intracellular levels of C16 fatty acids of D. salina cells cultivated in a turbidostat. Error bars indicate standard deviations of duplicate measurements.*

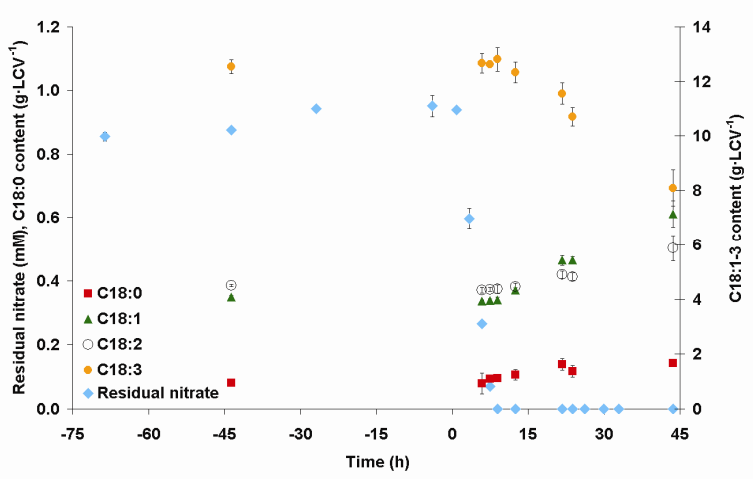

Figure 4.8 *Effect of nitrogen depletion on the intracellular levels of C18 fatty acids of D. salina cells cultivated in a turbidostat. Error bars indicate standard deviations of duplicate measurements.*

productivity suggest that there is a large potential for improvement of commercial β-carotene production.

Parallel increases in β-carotene and total fatty acid levels have been reported for both high-light stressed *D. salina* cells (Rabbani et al. 1998) and nitrogen depleted *D. salina* cells (Mendoza et al. 1999). In order to compare fatty acid biosynthesis with that of β-carotene, we followed their intracellular concentrations every 2-5 hours after changing towards nitrogen-free culture medium (Figure 4.6).

While the total intracellular fatty acid concentration did not change significantly during the entire experiment, the concentration of β-carotene increased markedly once nitrogen was depleted completely (Figure 4.6). Thus, in contrast to previous reports (Mendoza et al. 1999; Rabbani et al. 1998), our results on nitrogen-starved *D. salina* cells in a turbidostat indicate that β-carotene accumulation did not coincide with total fatty acid accumulation, as was also found in our previous turbidostat experiments with high-light stress (Lamers et al. 2010).

However, β-carotene levels did correlate positively ($p < 0.01$) with the level of oleic acid (C18:1), which supports previous findings for high-light treated *D. salina* cells (Lamers et al. 2010) and nitrogen depleted *D. salina* cells cultivated under various light intensities (Mendoza et al. 1999). It has been reported that triacylglycerols are present in high quantities in the β-carotene-containing lipid globules (Rabbani et al. 1998) that are formed under carotenogenic conditions (Ben-Amotz et al. 1982). The strong positive correlation between oleic acid and β-carotene levels suggests that oleic acid may be a key component of globule-localized triacylglycerols.

Similarly to high-light treatment of *D. salina* cells (Lamers et al. 2010) and nitrogen starvation of several green algae (Pohl and Wagner 1972; Stephenson et al. 2010), nitrogen depletion of *D. salina* cells led to a reduced degree of unsaturation of the total fatty acid pool. This reduction in the degree of unsaturation, which correlated negatively ($p < 0.01$) with the intracellular β-carotene concentration for both high-light stress (Lamers et al. 2010) and nitrogen starvation, resulted from a decrease in polyunsaturated fatty acids and a simultaneous increase in the more saturated fatty acids, including oleic acid (Figures 4.7 and 4.8).

Polyunsaturated fatty acids are particularly sensitive to oxidation and the observed specific loss of unsaturated fatty acids upon nitrogen depletion thus suggests oxidative damage. Since β-carotene accumulation requires *de novo* synthesis of fatty acids for the formation of lipid globules (Rabbani et al. 1998), the increased levels of the more saturated fatty acids might reflect *de novo* synthesis of triacylglycerol molecules within the lipid globules.

Conclusions

We found that nitrogen depletion, as opposed to high-light treatment, of *D. salina* cells led to an increase in biomass yield on absorbed light, which was mediated by an increase in cell-specific density as well as enhancement of cell size and a transient acceleration of cell division rate. Furthermore, β-carotene

accumulated rapidly upon nitrogen depletion reaching a maximum productivity which was approximately half of that found for high-light treated cells in similar turbidostat experiments. Nonetheless, the β-carotene productivity significantly exceeded reported average productivities of commercial open pond systems, thus pointing out the potential for improvement of such systems.

Finally, we found that upon nitrogen starvation, just as upon high-light stress, β-carotene accumulation correlated negatively with the degree of unsaturation of the total fatty acid pool and did not correlate with total fatty acid accumulation, but rather with the biosynthesis of oleic acid.

Acknowledgements

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Nomenclature

AFDW: ash-free dry weight
LCV: liter cell volume
LRV: liter reactor volume
PAR: photosynthetic active radiation (400-700 nm)

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Chapter 5

Metabolic profiling as a tool in studying stress-induced carotenogenesis in *Dunaliella salina*

The contents of this chapter have been submitted for publication as:
Lamers PP, De Vos RCH, Janssen M, Bino RJ, Wijffels RH. Metabolic profiling as a tool in studying stress-induced carotenogenesis in *Dunaliella salina.*

Abstract

Both high-light stress and nitrogen deficiency lead to β-carotene accumulation in *Dunaliella salina*. To investigate what cellular mechanisms are related to this response, we cultivated *D. salina* cells under either high light or nitrogen deprivation. The use of turbidostat-operated photobioreactors ensured a constant and well-defined light regime throughout the entire experiment.

Samples were taken both before and during stress induction, and were analyzed for polar metabolites using an untargeted GC-TOF-MS-based profiling approach. This technique, including unbiased peak picking and clustering of signals into metabolite mass spectra, resulted in the detection of 87 unique compounds. The polar metabolite profiles obtained were correlated to the stress-induced changes in lipid-soluble isoprenoids, including β-carotene, and composition of fatty acids.

The alterations in polar and apolar metabolites indicated essentially similar overall responses of *D. salina* towards both types of stress, with the main variance being caused by only 3 out of the 44 metabolites that were detected for both treatments. The accumulation of various energy-storage-related metabolites under either inducing condition suggests that excess energy storage is a uniform approach to imbalances in absorbed and required light energy.

This study shows the added value of combining highly controlled algae growth experiments with metabolomics approaches, and thus emphasizes the importance of similar follow-up studies aimed at a better understanding of microalgal stress responses and at developing optimal strategies for the production of microalgal products such as carotenoids.

Introduction

The microalga *Dunaliella salina* is used to produce β-carotene-rich biomass (Ben-Amotz and Avron 1983a; Borowitzka et al. 1984). The success of the commercial production depends largely on *D. salina*'s potential to grow in hypersaline environments, which reduces the risk of contamination, and on its high productivity of β-carotene. This orange pigment is used as antioxidant and as colorant in food, feed, cosmetics and nutraceuticals. *D. salina* can accumulate β-carotene up to approximately 10% of its dry weight when subjected to stress conditions such as high light intensity, high salinity, nitrogen deficiency or temperatures suboptimal for growth (Ben-Amotz 1996; Ben-Amotz and Avron 1983b; Ben-Amotz et al. 1982; Borowitzka et al. 1990; Chapter 4; Kleinegris et al. 2009; Krol et al. 1997; Lamers et al. 2010; Shaish et al. 1993).

β-Carotene overproduction in *D. salina* cells requires both transcriptional and translational activity, as was found using the inhibitors actinomycin D, chloramphenicol and cycloheximide under inducing conditions (Lers et al. 1990). Many genes and enzymes have been linked to the regulation of this β-carotene production, but a clear understanding is still lacking for *D. salina* (Coesel et al. 2008; Rabbani et al. 1998; Ramos et al. 2007; Ramos et al. 2009; Sanchez-Estudillo et al. 2006). There are indications that β-carotene overproduction is driven by the formation of lipid globules in the cells (Rabbani et al. 1998). These globules might serve as a metabolic sink, transporting β-carotene away from its biosynthetic machinery and consequently avoiding end-product inhibition of the carotenoid biosynthetic enzymes (Rabbani et al. 1998).

Previously, we studied the stress-induced changes in carotenoid and fatty acid metabolism of *D. salina* cells upon switching to either excess light or nitrogen depletion (Chapter 4; Lamers et al. 2010). These experiments did not confirm a direct correlation between β-carotene synthesis and total fatty acid content. However, a positive correlation was observed between β-carotene overproduction and the biosynthesis of oleic acid (C18:1), while β-carotene accumulation correlated negatively with the degree of fatty acid unsaturation (Chapter 4; Lamers et al. 2010). These findings suggest a stress-induced

breakdown of predominantly polyunsaturated fatty acids, which are relatively abundant in thylakoid membranes (Cho and Thompson 1987; Fried et al. 1982), and a concomitant *de novo* synthesis of lipid-globule-localized triacylglycerol molecules, which contain predominantly (monoun)saturated fatty acids such as oleic acid (Chapter 4; Klyachko-Gurvich et al. 1997; Lamers et al. 2010; Mendoza et al. 1999).

In our previous studies, we focused on the apolar fractions of *D. salina* biomass, which includes the valuable compound β-carotene. Here, we integrate these findings with the stress-induced changes in polar metabolites with the goal of phenotyping the changes in *D. salina*'s metabolite profile upon stress-induced carotenogenesis. Such polar metabolite profiling approaches mainly provide information on primary metabolites involved in central carbon metabolism, and have been successfully applied in plant research, e.g. to characterize metabolic networks of plants in response to environmental stress (Cook et al. 2004; Ducruix et al. 2006), to elucidate gene function and regulatory mechanisms in plant metabolic networks (Kaplan et al. 2004), and to phenotype specific mutants and wild species of crop plants like tomato (e.g. Bino et al. 2005; Schauer et al. 2005).

With regard to microalgae, metabolite profiling has been described earlier for *Scenedesmus vacuolatus*, for which it was found that the inhibitor prometryn impaired energy metabolism and affected various other parts of the algal metabolism (Kluender et al. 2009). Furthermore, *Chlamydomonas reinhardtii* has been the subject of two studies aimed at optimizing metabolite profiling methods (Kempa et al. 2009; Lee and Fiehn 2008). In addition, a third study aimed to determine the effects of various nutrient limitations on *C. reinhardtii* polar metabolites, showing that phosphorus depletion leads to a markedly different phenotype compared to nitrogen, sulfur or iron depletion (Bolling and Fiehn 2005).

In this paper, we compared the polar metabolite profiles in *D. salina* cells in response to stress induced by high light and nitrogen starvation. We subsequently correlated the time-dependent changes in the primary metabolites

to the previously observed changes in the carotenoid and fatty acid biosynthetic pathways, in order to get insight into metabolic pathways that may play a role in carotenogenesis.

Materials and methods

Algae, cultivation and sampling

Dunaliella salina (CCAP 19/18) cells were cultivated in turbidostats as described previously (Chapter 4; Lamers et al. 2010). In turbidostats, the turbidity of the culture, and thus the light perception of the algal cells, is maintained at a fixed and defined level by a constant and automatic adjustment of the dilution rate of the culture broth (Myers and Clark 1944). The turbidostats were used because light intensity is one of the most important factors that influence β-carotene accumulation (Lamers et al. 2010).

After a steady state was reached, samples were taken from the reactor during a period of 3 days. Upon induction with either high-light or nitrogen deficiency the sampling rate was increased to once per every 1.5 to 5 hours. For metabolite profiling, samples were quenched by transferring 3.5 mL samples, in duplicate, to pre-cooled glass tubes followed by a short centrifugation step (4500 g, 2°C, 3 min). Cell pellets were quickly frozen and stored at -80°C.

Metabolite extraction

Ice-cold aqueous methanol (MeOH : MQ = 3 : 1), containing 2.5 $\mu g \cdot mL^{-1}$ ribitol as internal standard, was added to each frozen cell pellet (250 µL per 1.75 mg AFDW), after which the tubes were vortexed (15 sec), put on ice and briefly vortexed again prior to a 20 minute sonication treatment. The metabolites were then extracted by transferring the tubes into a waterbath (70°C) with agitation for 30 minutes. The tubes were subsequently cooled on ice, vortexed, sonicated for 20 minutes and then centrifuged for 5 minutes at 3000g (4°C). A volume of 200 µL was transferred to a clean tube and 200 µL of MQ-water and 143 µL of $CHCl_3$ were added, after which the samples were vortexed. The samples were

centrifuged (21000g, 5 min) and 100µL of the upper (polar) phase was transferred into a GCMS vial with glass insert. The samples were dried overnight at 30°C in a speedvac and capped the next day under argon.

On-line derivatization and GC-TOF-MS analysis

The GCMS vials were placed in a CTC Combi Pal autosampler/pipetting robot (CTC analytics AG). The robot was programmed to carry out on-line derivatization with methoxyamine/N-Methyltrimethylsilyltrifluoroacetamide (MSTFA). Derivatization was done in an agitator (600 RPM, 40°C) attached to the robot. To each sample 12.5 µL of pyridine, containing 20 $mg \cdot mL^{-1}$ methoxyamine hydrochloride and 25 $\mu g \cdot mL^{-1}$ octadecane (to correct for injection variation), was added by the pipetting robot. After 30 minutes of agitation, 17.5 µL MSTFA was added and after another 60 minutes agitation, 2 µL of the mixture was taken and injected into a GC coupled to a time-of-flight (TOF) MS (Pegasus III, LECO) operating as described previously (Fu et al. 2009). For samples of the high-light treatment a split flow of 5 $mL \cdot min^{-1}$ and a column flow of 2 $mL \cdot min^{-1}$ was used, whereas samples of the nitrogen-starvation experiment were injected in splitless mode.

Untargeted processing of GC-TOF-MS data

Since the turbidostat experiments and sample analyses were performed separately per type of stress, data from each experiment were separately processed in an untargeted manner. Raw data were firstly processed by LECO ChromaTOF software for baseline correction, using a baseline offset through the middle of the noise (0.5), and for smoothing, by averaging 5 data points. The resulting netCDF files from 34 (HL) or 16 (NS) samples were subsequently subjected to unbiased peak picking and mass signal alignment using MetAlign software (Lommen 2009). This was followed by removal of the aligned mass signals that were present in less than 3 samples, or did not have a single measurement that exceeded 3 times the noise value. The dataset was then normalized for the ribitol and octadecane internal standards, after which the

ribitol and octadecane masses were removed. Mass signals of poor reproducibility, *i.e.* mass signals with a coefficient of variation higher than 60% for both the technical replicates and duplicate samples, were deleted as well.
The remaining mass signals were then processed using a multivariate mass spectra reconstruction (MMSR) approach (Tikunov et al. 2005), which groups all mass signals derived from the same metabolite, based on their corresponding retention times and relative intensity ratios across all samples. The resulting reconstructed metabolite mass fragmentation patterns, so-called mass clusters, consisting of less than 8 mass signals were subsequently discarded. Finally, the most abundant mass signal within each mass cluster was selected as a representative of the relative level of the corresponding metabolite and was used in our further data analyses.

Metabolite identification

For the identification of metabolites, the reconstructed mass spectra were matched to the Golm Metabolome Database (Kopka et al. 2005) and NIST 08 Mass Spectral Library (www.nist.gov) as described previously (Tikunov et al. 2005). In addition, positive identification was achieved by matching the retention times and mass spectra with those of authentic standards derivatized on-line and analyzed by GC-TOF-MS as described above.

Data fusion, data normalization and multivariate analysis

The representative mass signals of duplicate samples were averaged and normalized to intracellular concentration, after which the data matrices (mass signals x samples) of both stress treatments were concatenated along the metabolite dimension. Only representative mass signals that were detected in both treatments were included. The resulting dataset was then fused with the previously obtained data resulting from the analyses of lipid-soluble pigments and fatty acid methyl esters (Table 5.1; Chapter 4; Lamers et al. 2010). Following fusion, the data matrix was normalized using $\log_2$ transformation and subsequently mean centered per metabolite. Unsupervised multivariate analysis

was performed using the principal-component-analysis (PCA) application of the GeneMaths version 1.5 software package (www.applied-maths.com). Metabolite-metabolite correlations were evaluated using Pearson's correlation analysis.

Table 5.1 *Fatty acids and isoprenoids analyzed previously (Chapter 4; Lamers et al. 2010).*

Metabolite	PCA-variable[a]	Fold change upon HL[b]	Fold change upon NS[b]
C16:0	1	**0.66**	**1.09**
C16:4	2	**0.19**	**0.69**
C18:1	3	0.91	**1.57**
C18:2	4	**0.70**	**1.21**
C18:3	5	**0.34**	**0.75**
Chlorophyll *a*	6	**0.19**	**0.61**
Chlorophyll *b*	7	**0.12**	**0.66**
All-trans β-carotene	8	**5.01**	**3.70**
9-cis β-carotene	9	**7.29**	**5.52**
Lutein	10	**0.47**	1.14
Lycopene	11	**3.32**	**1.38**
Phytoene	12	**13.07**	**27.50**
Zeaxanthin	13	**2.96**	**1.38**

[a] *Numbers correspond with those in Figure 5.3B.*

[b] *Fold change in intracellular concentration upon induction. Bold numbers indicate significant changes as determined by a Student's t-test ($p < 0.05$) using 12 NS-samples or 15 HL-samples taken in steady state and after 14-50 hours of induction.*

Results

Untargeted data processing and metabolite identification

GC-TOF-MS analysis of MSTFA-derivatized polar extracts was used to profile the primary metabolite composition of *D. salina* cells in response to either nitrogen

starvation (NS) or high-light stress (HL). Examples of baseline corrected chromatograms of non-stressed (control), HL and NS cell extracts are given in Figure 5.1. Following peak picking and alignment with MetAlign, 4372 and 9640 mass signals were obtained for the samples of the HL- and NS-experiments, respectively. The higher number of mass signals in the NS-samples was due to the higher amount of biomass applied to the GC-TOF-MS.

The data were filtered for noise, normalized using the internal standard and filtered for poor reproducibility (see materials and methods section), which reduced the number of mass signals by approximately 60%. A median coefficient of variation of 19% over all replicate samples (based on representative mass signals) was found.

The reproducible mass signals were clustered into 69 (HL) and 138 (NS) compounds, of which only those represented by at least 8 mass signals (HL: 41 and NS: 77 compounds) were used in our further data analysis. The compounds of the HL-treatment were then matched with those of the NS-treatment based on their retention time and on their filtered and raw mass spectral data. In total, 87 unique compounds were discerned, of which 31 were detected in both treatments, 46 were only observed in the NS-experiment and 10 only in the HL-experiment (Figure 5.2).

By automatically matching the reconstructed mass spectra with the Golm Metabolome Database (Kopka et al. 2005) and the NIST 08 Mass Spectral Library (www.nist.gov), and subsequent manual evaluation of the resulting annotations in the raw GC-TOF-MS data, 38% of these mass clusters (*i.e.* 33 metabolites) could be matched to these libraries (solely based on mass fragmentation patterns; Table 5.2). Subsequent injection of reference standards yielded 22 positively identified metabolites (Table 5.2).

Overall metabolite profiles during stress

We recently reported on the effects of high-light stress and nitrogen stress on the carotenoid and fatty acid metabolism of *D. salina* cells that were cultivated in turbidostats (Chapter 4; Lamers et al. 2010). The metabolites included in the

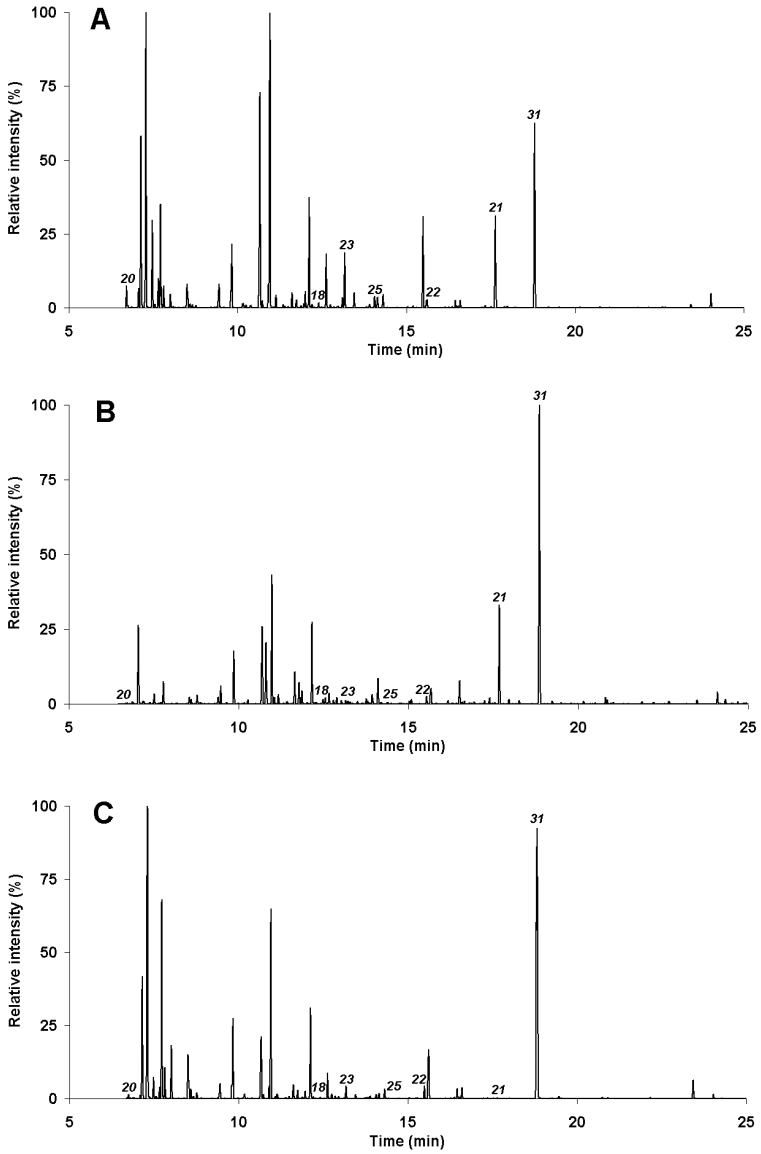

Figure 5.1 *Total ion count chromatograms of polar extracts of D. salina cells exposed to various cultivation conditions. A: Standard growth conditions, B: High-light stress (31 hours), C: Nitrogen starvation (34 hours). Metabolites with the largest fold changes for both types of stress are indicated (numbers in italics correspond with those of Table 5.2).*

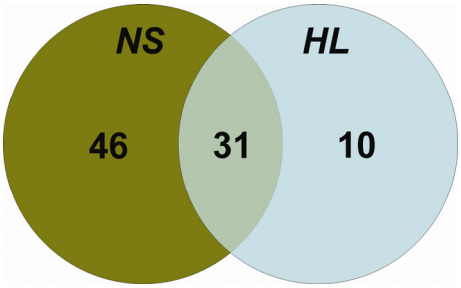

Figure 5.2 *Venn diagram showing the correspondence of polar metabolite mass clusters observed for nitrogen starvation (NS) and high-light treatment (HL) of D. salina cells.*

present study are shown in Table 5.1, together with their stress-induced fold changes in intracellular concentration. The intracellular fatty acid concentrations were differentially affected by stress, as was discussed previously (Chapter 4; Lamers et al. 2010). Furthermore, the contents of both chlorophyll *a* and chlorophyll b decreased upon induction by either type of stress, whereas all determined carotenoid levels, except for lutein, increased significantly.

Principal component analysis (PCA) using information of the 31 polar metabolites in common (Table 5.2) plus the data of the 13 apolar metabolites from our previous analyses, including β-carotene and fatty acids (Table 5.1), is shown in Figure 5.3. All samples taken before the stress inductions were clustered into a separate and closely related group (Figure 5.3A), indicating high similarity of the metabolite profiles and confirming the steady state condition of the cell cultures.

Upon stress induction, however, a clear time-dependent change in the metabolite profiles was observed. The metabolite profiles of the NS-treated and the HL-treated algae resulted in a stress-dependent separation of algal samples in the PCA plot. The difference between NS and HL metabolite profiles was mainly determined by differential changes in glycine, *myo*-inositol, guanine (Figure 5.3B). The effects of high-light treatment and nitrogen starvation on the relative abundance (in terms of intracellular concentration) of these metabolites were different (Figure 5.4): *myo*-inositol and guanine reacted oppositely to the

Table 5.2 *Effect of high-light stress or nitrogen starvation on the intracellular concentrations of identified metabolites.*

Metabolite	PCA-variable[a]	Retention time (sec)	ID method[b]	Fold change upon HL[c]	Fold change upon NS[c]
Adenine		912.1	MF (909)	ND	1.00
Adenosine-5-monophosphate	14	1441.8	MF (922)	**0.81**	**0.71**
Asparagine		720.6	MF (959)	ND	**0.09**
β-Alanine		488.2	MF (932)	ND	1.81
Citrate		779.2	Standard	**1.79**	ND
Galactosylglycerol	15	995.8	MF (937)	1.19	**2.18**
Glucose		771.4	Standard	**7.34**	ND
Glucose-6-phosphate		1040.4	Standard	ND	**1.92**
Glutamate	16	657.4	Standard	1.23	**1.58**
Glutamine		786.4	Standard	ND	**0.09**
Glycerate-3-phosphate	17	808	MF (958)	**0.52**	0.69
Glycerol		349.4	Standard	ND	3.72
Glycerol-2-phosphate	18	732.4	Standard	**0.32**	1.26
Glycerol-3-phosphate	19	757.6	MF (963)	**0.44**	1.00
Glycine	20	402.6	Standard	0.92	**0.30**
Guanine	21	1058.4	MF (958)	**2.37**	**0.05**
L-DOPA	22	936.8	Standard	**1.87**	**7.31**
Lysine	23	790.6	Standard	**0.26**	0.67
Malate	24	567	Standard	**1.49**	0.98
myo-Inositol	25	848.8	Standard	**0.25**	0.78
N-acetylglutamate		646.5	Standard	0.79	ND
Phenylalanine		689.5	Standard	ND	**2.68**
Phosphate	26	428.2	MF (966)	0.93	**1.78**
Proline		424.1	Standard	ND	0.73
Putrescine	27	639.6	Standard	0.8	**0.38**
Pyroglutamate	28	668.3	Standard	1.21	0.74
Serine		448.5	Standard	ND	**0.52**
Spermidine	29	929.9	MF (956)	1.09	**0.43**
Succinate	30	468.7	Standard	**2.19**	1.15
Sucrose	31	1128.2	Standard	**2.39**	**5.20**
Threitol	32	515.3	Standard	**1.16**	**2.45**
Threonate	33	589.9	MF (965)	1.41	1.23
Threonine		459.4	Standard	ND	1.18

[a] *Numbers correspond with those in Figures 5.1 and 5.3B.*

[b] *Identification by authentic standards (Standard) or based only on mass fragmentation patterns (MF). Numbers between parentheses give the similarity (out of 1000) with the library match.*

[c] *Fold change in intracellular concentration upon induction. Bold numbers indicate significant changes as determined by a Student's t-test ($p < 0.05$) using 12 NS-samples or 15 HL-samples taken in steady state and after 14-50 hours of induction. ND: not detected.*

two different types of stress, while glycine showed a much more pronounced transient increase upon HL treatment (8-fold transient increase for HL vs. 1.5-fold for NS).

The effect of both treatments on the intracellular metabolite concentrations was also evaluated by the Student's t-test (significance threshold of 0.05) on samples taken in steady state and taken between 14–50 hours after stress was induced. Using this method, it was found that out of the total of 44 polar (31) and apolar (13) metabolites that were detected in both experiments, 22 exhibited a similar change upon induction (*i.e.* either an increase, decrease or no significant change in intracellular concentration), and the remaining metabolites were found to be differentially regulated by the two treatments.

Effect of NS- or HL-stress on nitrogen-containing metabolites

To assess the influence of nitrogen starvation on the intracellular concentrations of nitrogenous metabolites, we determined the stress-induced changes of all 19 nitrogen-containing compounds (Tables 5.1 and 5.2). Induction by nitrogen starvation caused a significant reduction in the intracellular concentration of 10 of these nitrogenous metabolites, with the most notable decreases for the amino acids asparagine and glutamine, and for the nucleobase guanine (Tables 5.1 and 5.2). In contrast, glutamate (1.6-fold), phenylalanine (2.7-fold) and L-DOPA (7.3-fold) showed a significant increase upon nitrogen starvation. In the high-light stress experiment, we detected 12 nitrogen-containing metabolites, of which 2 increased (guanine and L-DOPA), and 4 decreased significantly in their intracellular concentrations (Tables 5.1 and 5.2).

Metabolic changes related to β-carotene accumulation

To get insight into metabolic pathways that may play a role in carotenogenesis, we compared the time dependent changes in intracellular β-carotene content with those of the other apolar and polar metabolites. High-light-induced β-carotene accumulation correlated positively (Pearson's correlation, $p < 0.01$, $n = 17$) with 14 of the 54 polar and apolar metabolites detected in this

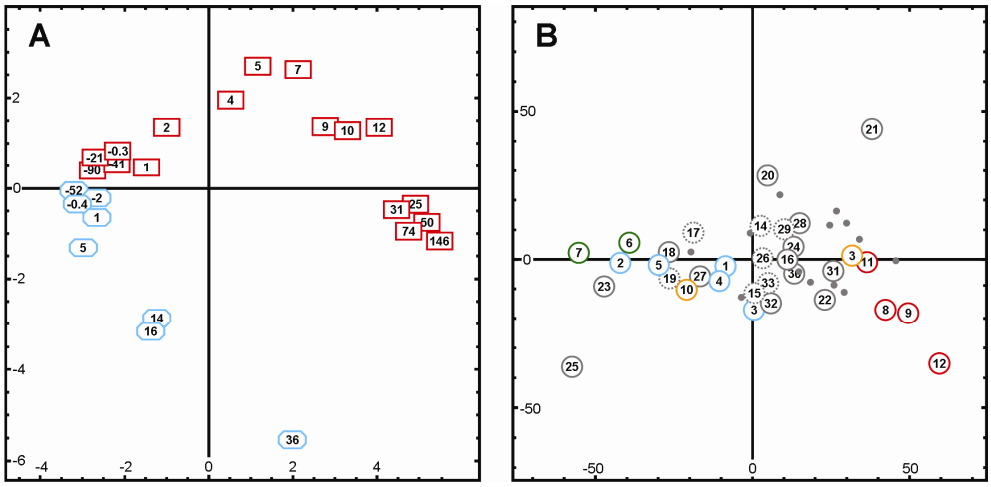

Figure 5.3 *Principal component analysis of algal samples (A) taken before and during either high-light stress (red rectangles) or nitrogen starvation (blue octagons). The variables used were the intracellular concentrations of both aqueous methanol-soluble metabolites and apolar metabolites, of which the distribution is shown in (B). The sample numbers in (A) represent the time (h) upon induction. The numbers in (B) signify the metabolites, which can be found in Tables 5.1 and 5.2. Color coding of (B): Chlorophylls (green), fatty acids (blue), carotenes (red), xanthophylls (orange), polar metabolites identified using an authentic standard (grey closed circles), polar metabolites identified using mass spectrum (grey dotted circles), yet-unidentified metabolites (grey small dots). PC1: 51% (horizontal), PC2: 25% (vertical).*

experiment (Table 5.3). For nitrogen starvation, 11 out of 90 metabolites were found to correlate positively with β-carotene accumulation (n = 8).

A total number of 6 metabolites correlated positively with β-carotene overproduction independent of the type of stress (Figure 5.5). These 6 metabolites included threitol, L-DOPA, lycopene, phytoene and 2 unknown metabolites, named DSL1222 and DSL2160 (retention times and mass spectra are given in Figure 5.6).

Metabolites that only correlated positively to high-light-induced β-carotene accumulation included glutamate, succinate, guanine, sucrose and 4 unidentified metabolites (Table 5.3). Galactosylglycerol, unknown metabolite DSL3149 and the fatty acid species C16:0, C18:1 and C18:2, exhibited a nitrogen-starvation-

specific positive correlation with β-carotene overproduction. Additionally, various metabolites correlated negatively with β-carotene accumulation, with glycerate-3-phosphate, chlorophyll *a*, chlorophyll *b* and the unsaturated fatty acids C16:4 and C18:3 showing negative correlations during both treatments (Table 5.3).

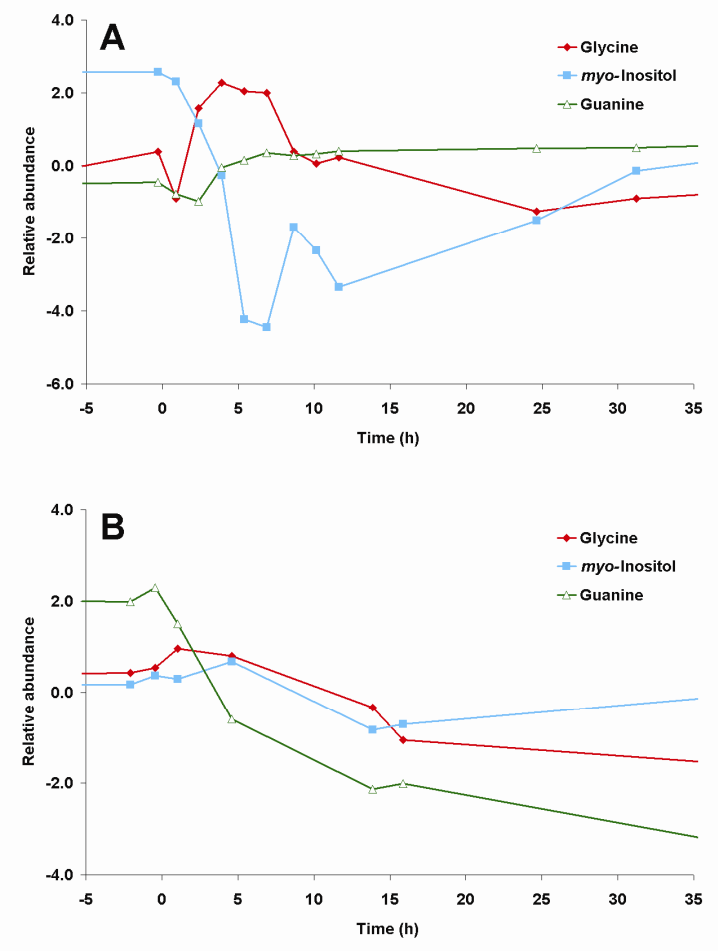

Figure 5.4 *Effect of high-light stress (A) and nitrogen starvation (B) on the relative abundances (expressed as intracellular concentration) of glycine, myo-inositol and guanine. Time point 0 hours indicates the shift to high light intensity (A) or the complete depletion of residual nitrogen (B).*

Table 5.3 *Metabolites correlating with* β*-carotene.*

HL + [a]	**HL -** [a]	**NS +** [a]	**NS -** [a]
Succinate	Glycerol-2-phosphate	Threitol	Serine
Threitol	Glycerol-3-phosphate	L-DOPA	Malate
Glutamate	Glycerate-3-phosphate	Galactosylglycerol	Putrescine
L-DOPA	Chlorophyll *a*	Phytoene	Lysine
Guanine	Chlorophyll *b*	Lycopene	Glycerate-3-phosphate
Sucrose	Lutein	C16:0	Spermidine
Lycopene	C16:4	C18:1	Chlorophyll *a*
Phytoene	C18:3	C18:2	Chlorophyll *b*
5 unidentified	2 unidentified	3 unidentified	C16:4
			C18:3
			Adenosine-5-monophosphate
			2 unidentified

[a] *HL: High-light treatment. NS: Nitrogen starvation. Plus and minus symbols indicate positive and negative correlations with the level of* β*-carotene, respectively (Pearson's correlation, $p < 0.01$, $n = 17$ for HL, $n = 8$ for NS).*

Discussion

Using controlled turbidostat reactors, we subjected *D. salina* cells to either high-light stress or nitrogen starvation. Subsequent untargeted data analysis from GC-TOF-MS profiling resulted in 87 reconstructed mass spectra, containing at least 8 mass signals. The obtained amounts of mass signals and reconstructed mass spectra are about 4-10 times lower as compared to other untargeted metabolite profiling studies in microalgae (Bolling and Fiehn 2005; Kluender et al. 2009; Lee and Fiehn 2008).

The relative low amount of metabolites detected in the present study was mainly caused by the low biomass concentrations in the samples and the small sample volumes that were collected during the experiments, to ensure proper turbidostat operation. Nevertheless, the described method ensures accurate and reproducible analysis of the most abundant metabolites in *D. salina* samples.

Principal component analysis based on the detected polar and apolar metabolites of *D. salina* cells indicated that the majority of compounds showed similar changes upon both types of stresses. In fact, the variance between both types of stress was mainly caused by only 3 compounds (7 % of the total metabolites detected for both treatments, including unknowns): guanine, glycine and *myo*-inositol (Figure 5.3).

The intracellular concentrations of guanine and *myo*-inositol both responded oppositely to the two different types of stress: guanine decreased upon nitrogen starvation but increased upon high-light stress, probably reflecting the differential effect of nitrogen availability. High-light stress also induced an immediate and transient decline in the cellular *myo*-inositol content, whereas nitrogen stress induced a small transient increase upon depletion (Figure 5.4). The rapid light-induced decrease in *myo*-inositol suggests rapid utilization of this compound, possibly for the mediation of the high-light stress. Studies in *Arabidopsis* have shown that *myo*-inositol, a well-known mediator of stress responses in plants (Loewus and Murthy 2000), acts as the initial substrate for the biosynthesis of the antioxidant ascorbic acid (Lorence et al. 2004), which is known to accumulate in *D. salina* under high-light stress, but also during nitrogen limitation (Abd El-Baky et al. 2004). However, we were not able to substantiate this suggested role of *myo*-inositol in *D. salina*, as we did not detect ascorbic acid in our samples, despite the fact that the GC-TOF-MS platform usually enables the detection of this antioxidant in plant samples (Ebert et al. 2010; Han and Yuan 2009).

Glycine was among the metabolites explaining the most variance between the time-dependent effects of HL and NS stress (Figure 5.3), although for both types of stress a similar transient accumulation upon induction was observed (Figure 5.4). Glycine is one of the products of photorespiration (Wingler et al. 2000). Photorespiration can protect plants from damage by photooxidation (Niyogi 1999) and the observed transient accumulation of glycine might therefore reflect a temporary enhancement of photorespiration, to cope with the photooxidative stress induced by the energy imbalance.

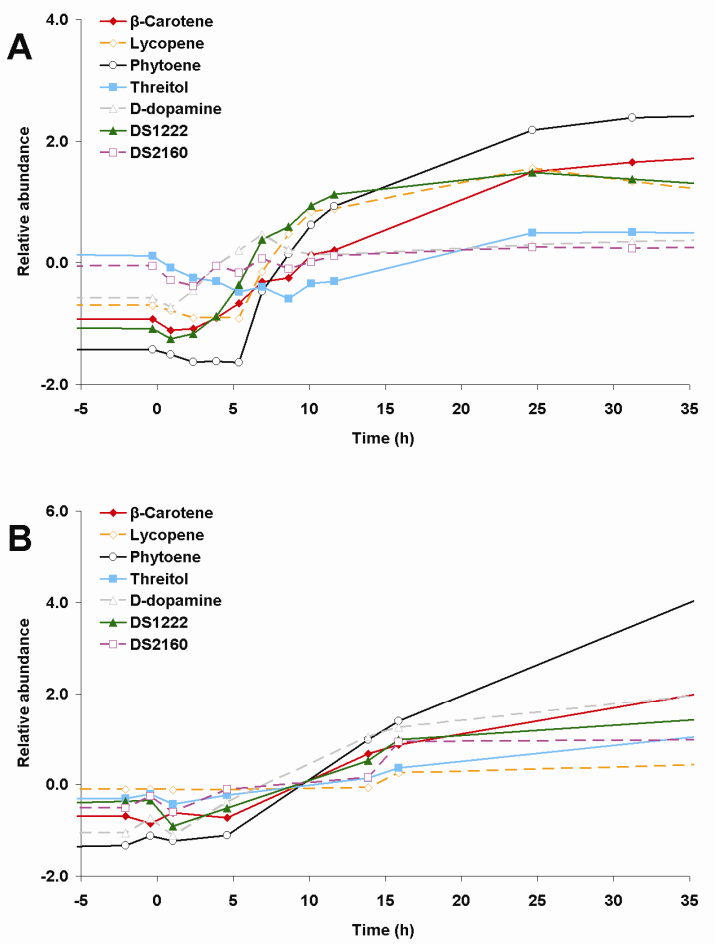

Figure 5.5 *Effect of high-light stress (A) and nitrogen starvation (B) on the relative abundances (expressed as intracellular concentration) of* β*-carotene and 6 metabolites correlating positively with* β*-carotene (Pearson's correlation, $p < 0.01$, $n = 17$ for HL, $n = 8$ for NS). Time point 0 hours indicates the shift to high light intensity (A) or the complete depletion of residual nitrogen (B).*

Glycine accumulation can, however, also be explained by photooxidative stress-mediated inhibition of the glycine decarboxylase enzyme complex, which is responsible for the conversion of glycine to serine during operation of the photorespiratory cycle, as observed for pea plants (Taylor et al. 2002; Vauclare

et al. 1996). While photooxidative stress is likely to occur under both NS and HL treatment of *D. salina* cells (Lamers et al. 2008), we did not detect serine during the HL treatment and no transient decrease in the serine level was observed for the NS treatment. Thus, it remains unclear whether such inhibition of glycine-serine conversion may be the cause of the observed transient increase in the glycine level.

Finally, the accumulation and subsequent decrease of glycine might also be coupled to the synthesis of glutathione, which is also involved in stress protection (Buwalda et al. 1990; Noctor et al. 1999). Unfortunately, information on changes in this metabolite is lacking as well, clearly indicating the need for additional and more comprehensive metabolomics experiments to elucidate the temporal stress-type-specific accumulation of glycine in *D. salina* cells.

Nitrogen deficiency caused a reduction in the cellular contents of most nitrogen-containing metabolites, including almost all amino acids (Table 5.1, 5.2). However, the intracellular concentrations of 3 nitrogen-containing metabolites significantly increased upon induction by nitrogen depletion: glutamate, phenylalanine and L-DOPA.

Such increases in the level of nitrogen-containing metabolites during nitrogen depletion of photosynthetic organisms are not unusual. Tryptophan and 2-amino-adipate levels were found to increase in *C. reinhardtii* cells subjected to nitrogen starvation (Bolling and Fiehn 2005). Likewise, lysine, leucine and γ-aminobutyric acid increased when tomato plants were grown under low-light and nitrogen-deficient conditions (Urbanczyk-Wochniak and Fernie 2005).

However, whereas glutamate was termed a key regulator of amino acid biosynthesis in tobacco (Masclaux-Daubresse et al. 2002) and was found to decrease shortly after or along with other amino acids upon nitrate-starvation of tomato plants (Urbanczyk-Wochniak and Fernie 2005), we observed a nitrogen-starvation-induced increase in glutamate, while most other amino acids decreased (Table 5.2). Nevertheless, the observed increase in glutamate and concomitant decrease in glutamine upon nitrogen starvation are in support of

previous findings that low glutamine:glutamate ratios are indicative of nitrogen deficiency in microalgae (Flynn et al. 1989).

Phenylalanine and L-DOPA are compounds that both serve as precursors of dopamine and other catecholamines in plants. To our knowledge, this is the first report on the presence of L-DOPA in microalgae, although dopamine production as a deterrent to grazers has been reported for the green alga *Ulvaria obscura* (Van Alstyne et al. 2006).

In plants, the synthesis of catecholamines is regulated by various stress conditions and the accumulation of catecholamines in potato plants, resulting from overexpression of tyrosine decarboxylase (Swiedrych et al. 2004) or human dopamine receptor (Skirycz et al. 2005), was found to positively correlate with the levels of glucose, sucrose and fructose, suggesting a regulatory role for catecholamines in plant carbohydrate metabolism (Kulma and Szopa 2007).

In our experiments with high-light-stressed and nitrogen-starved *D. salina* cells, we found simultaneous and significant increases in L-DOPA and sucrose, as well as an increase in glucose for HL-stress and an increase in phenylalanine levels during nitrogen starvation (Table 5.2). Moreover, the observed changes during high-light treatment were positively correlated (Pearson's correlation, $p < 0.01$, $n = 17$), just as the observed increases in phenylalanine and sucrose upon nitrogen depletion (Pearson's correlation, $p < 0.01$, $n = 8$). Therefore, the observed stress-induced increases in phenylalanine and L-DOPA levels possibly reflects changes related to energy storage metabolism.

Next to sucrose and glucose, the polyol compound threitol also accumulated upon induction (Table 5.2). Polyols are known to be involved in osmoregulation and cryoprotection in microalgae (Arnold et al. 2003; Gustavs et al. 2009). However, as the temperature and culture salinity were constant during our turbidostat experiments, a key role of threitol in osmoregulation and cryoprotection seems unlikely. Alternatively, the accumulation of threitol may reflect the storage of carbohydrate reserves. As discussed above, the accumulation of L-DOPA, phenylalanine, sucrose and glucose levels also suggest a metabolic switch towards energy storage.

Other indications that both HL and NS stress affected the energy metabolism of *D. salina* cells are the changes in adenosine-5-monophosphate (AMP), glucose-6-phosphate and the TCA-cycle intermediates citrate, malate and succinate (Table 5.2), which are all indicative of a cellular energy surplus (Hardie 2003). We did not determine the amount of triacylglycerol (TAG), another important energy storage compound, but did observe NS-induced increases in the levels of fatty acids that are typically associated with TAG (*i.e.* C16:0, C18:1 and C18:2; Table 5.1). Although these fatty acids did not increase during high-light stress, TAG accumulation was confirmed through electron-microscopic observation of a

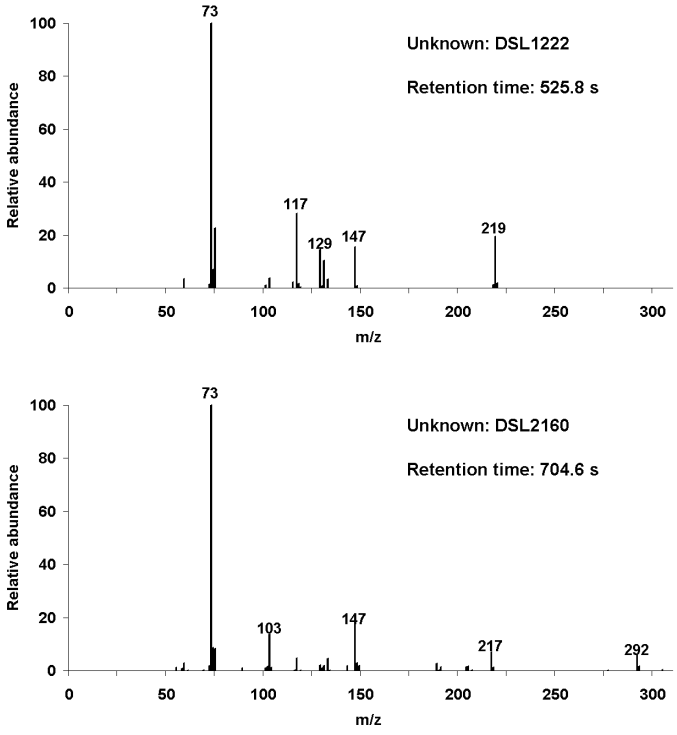

Figure 5.6 *Mass fragmentation pattern of 2 unidentified metabolites that correlate positively with* β*-carotene for both HL and NS stress. Only mass signals that exceeded 3 times the noise were included. The correctness of mass alignment was manually evaluated.*

massive increase in the amount of TAG-containing lipid globules (Lamers et al. 2010).

Therefore, we propose that the switch towards energy storage represents a uniform approach of *D. salina* to imbalances between the light energy absorbed by the cell and the energy required for normal growth. More specifically, the high-light stress will cause an overshoot of energy supply, whereas nitrogen starvation will reduce the energy demand for cell growth. To reduce the amount of photooxidative damage that is normally caused by this excess of energy (Noguchi 2002), *D. salina* overproduces energy storage molecules, which can act as electron sinks and thus increase the demand for energy, as well as β-carotene, which can act both as an electron sink and as a direct quencher of excess light by releasing the absorbed energy as heat (Ben-Amotz et al. 1989).

This suggestion of energy-balance-mediated regulation is supported by findings that the level of β-carotene was positively correlated to the overall amount of irradiation perceived by *D. salina* cells during the cell-division cycle (Ben-Amotz and Avron 1983b). Nevertheless, validation of this hypothesis of stress-induced switching towards energy storage requires further studies, e.g. using other stress factors, such as high salinity and temperature shock, and by studying cells restoring from a temporary stress.

Conclusions

The combination of stress-experiments in controllable photobioreactors and metabolite profiling techniques is a novel and promising approach for the study of microalgal stress-metabolism. Because the supply rate of light energy is one of the most important factors influencing microalgal physiology, well-defined light regimes are essential for gaining detailed insight into the responses to changes in culture conditions other than light. In addition, metabolic profiling enables unbiased phenotyping of such responses, providing a framework for the definition of new hypotheses regarding the mechanisms involved in stress-metabolism.

Indeed, by employing such an approach we were able to link energy and carotenoid metabolism during both high-light stress and nitrogen starvation. We therefore suggest that β-carotene accumulation and an increased production of energy storage molecules reflect a uniform and concerted effort of *D. salina* cells to cope with stress-induced damaging imbalances between the absorbed and required energy.

Acknowledgements

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Nomenclature

AFDW: ash-free dry weight
GCMS: gas chromatography and mass spectrometry
GC-TOF-MS: gas chromatography with time-of-flight mass spectrometry
LCV: liter cell volume
LRV: liter reactor volume
TIC: total ion count

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Chapter 6

General discussion: Paving the way for optimal productivity of microalgal metabolites

A part of the contents of this chapter have been submitted for publication as:
Lamers PP, Janssen M, De Vos RCH, Bino RJ, Wijffels RH. Regulation of carotenoid accumulation in *Dunaliella salina*: a role for the plastoquinone pool redox state?

Introduction

The demand for products derived from renewable resources is steadily growing. Not only does this concern the currently much discussed automotive fuels, but also other industrial products such as coatings, food and fine chemicals like β-carotene. Being one of the most promising renewable feedstocks for such commodities, microalgae have gained a great deal of attention in recent years. Just like all other photosynthetic organisms, microalgae convert solar energy into chemical energy using only sunlight, water, carbon dioxide and a few other nutrients. Photosynthetic products therefore offer a sustainable alternative to their (petro)chemical counterparts as long as nutrients, water, carbon dioxide and sunlight are available.

Microalgae (and cyanobacteria) can be grown under (semi)-controlled conditions preventing any limitations apart from light limitation. In comparison with other photosynthetic crops, much higher areal productivities (Chisti 2007; Chisti 2008; Nielsen et al. 1996) can be reached with microalgae and they do not have to compete for arable land. This potential has inspired many researchers and entrepreneurs, leading to various commercial microalgal production systems. Nevertheless, the relatively high production cost of microalgal biomass, estimated to be ~4 € per kilogram dry matter for large-scale systems using current state-of-the-art technology (Wijffels et al. 2010), has limited commercial production to high-value compounds such as carotenoids and omega-3 fatty acids.

The production of low-value bulk chemicals and biofuels from microalgae is suggested to become cost-effective within the next 10-15 years through a 10-fold reduction in biomass production costs (Wijffels and Barbosa 2010). Such improved economics should follow from breakthroughs in all areas of microalgal biotechnology, which include molecular biology, physiology, photobioreactor design and downstream processing.

Although optimizing the production processes for microalgal derived products requires a multidisciplinary approach integrating all these subjects, the following sections will mainly focus on the current state and road ahead of the fields of

microalgal physiology and photobioreactor design and operation. In view of our previous studies (this thesis), β-carotene accumulation by *Dunaliella salina* will be used as a reference point for discussing several important aspects in these fields of research.

Microalgal physiology

Microalgal physiology deals with questions on how microalgae function in their environment and what physical and biochemical factors and processes are involved. With respect to cultivation of microalgae for the production of their metabolites, these questions mostly regard the relationship between process conditions, such as light regime, temperature, pH and nutrient availability on one side and the productivity and metabolite content of biomass on the other side. Studies aimed at resolving these questions have provided and will provide a detailed understanding of the responses of microalgae to (changes in) process conditions, which will ultimately aid the realization of optimal production strategies for microalgal metabolites.

Stress metabolism

Many of the microalgal metabolites that are presently in demand, such as carotenoids and triacylglycerols, are overproduced when microalgae are exposed to certain stress conditions. Typical stress parameters include high light intensities, suboptimal growth temperatures, high salinity, extreme pH and nutrient deprivation.

Apart from high light intensities, all these conditions cause reduced growth rates. This has led to the suggestion that stress metabolism, is regulated by an imbalance in absorbed light energy and the energy required for growth (Lamers et al. 2008). According to this theory, secondary metabolites are accumulated as electron sinks that quench the excess light energy, while accumulated pigments, such as β-carotene in *D. salina*, additionally serve as screening agents, quenching light directly and dissipating the energy as heat (Ben-Amotz et al. 1989).

Understanding how microalgae detect such an imbalance in the energy supply:consumption ratio is important, not only from a fundamental point of view, but also because such knowledge could, for example, enable the chemical or physical induction of stress-metabolism under otherwise non-inducing conditions. Especially, energy-inefficient induction processes, such as the high-light-intensity induction of β-carotene accumulation in *D. salina*, would benefit from such advances.

Stress sensory mechanism

The potential signaling mechanisms involved in β-carotene accumulation by *D. salina* have recently been reviewed and both singlet oxygen and a plastoquinone-redox-state signaling-mechanism were named as likely candidates (Lamers et al. 2008). Singlet oxygen's main origin is the reaction of oxygen with chlorophyll molecules of photosystem II that have become over-excited as a result of more light being absorbed than what is required for growth (Noguchi 2002). As such, singlet oxygen may readily serve as a global stress sensor and initiator of secondary metabolism in other microalgae as well.

The same holds for the plastoquinone redox-state sensing mechanism as this, too, is capable of sensing imbalances between energy supply and consumption (Durnford and Falkowski 1997; Wilson et al. 2003). The plastoquinone pool is part of the photosynthetic electron transport chain (Figure 6.1) and its possible role in stress signaling is based on the fact that when PSII is excited by light, it transfers electrons via a cascade of redox reactions to plastoquinone, which is thereby reduced into plastoquinol (Figure 6.1).

The subsequent oxidation back into plastoquinone is widely assumed to be the rate-limiting step in the entire photosynthetic electron-transport chain (Cramer and Crofts 1982; Junge 1977). When the light intensity increases or the growth rate decreases (and hence the need for reduction equivalents in the form of NADPH), the rate of electron production by PSII might exceed the rate of oxidation of plastoquinol. As a consequence, the plastquinone pool will become highly reduced. It was shown that, in *D. tertiolecta*, reduction of the

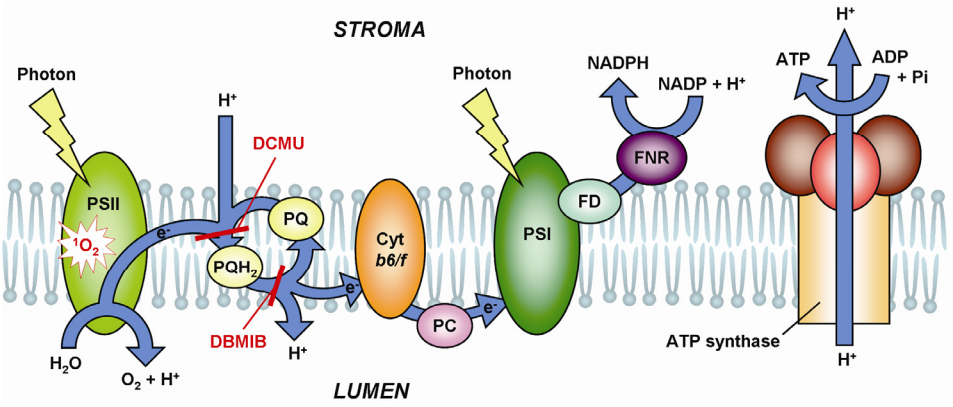

Figure 6.1 *Schematic overview of the photosynthetic electron transport system at the thylakoid membrane. Abbreviations: $^1O_2$ – singlet oxygen; Cyt b6/f – cytochrome b6/f complex; DBMIB – 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FD – ferredoxin; FNR – ferredoxin-NADP reductase; PC – plastocyanin; PQ – plastoquinone; PQH_2 – plastoquinol; PSI – photosystem I; PSII – photosystem II.*

plastoquinone pool caused a decreased expression of a nuclear *cab* gene encoding a thylakoid associated light-harvesting complex II protein (Escoubas et al. 1995).

We therefore performed preliminary experiments to evaluate whether this redox hypothesis could be matched to β-carotene accumulation in *D. salina* as well. This was done by using the photosynthetic electron-transport-chain inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB). These inhibitors block either the reduction or the oxidation of the plastoquinone pool (Figure 6.1) and, therefore, provide ways to alter the plastoquinone redox state independently of the environmental conditions.

D. salina cells were cultivated for 44 hours in wells plates in the presence and absence of these inhibitors under both inductive (high light or nitrogen starvation) and non-inductive low-light conditions (Table 6.1). The inhibitor

concentration was adjusted to inhibit algal growth by ~15-50%, ensuring that the electron transport was not completely blocked. DBMIB was not added to the high-light condition, because the inhibitor was not stable enough under these conditions. We found that DCMU partially inhibited β-carotene accumulation under otherwise inducing conditions, whereas DBMIB did not significantly influence the β-carotene accumulation (Table 6.1).

D. salina cells were subsequently cultivated in small flat-panel photobioreactors equipped to determine the quantum yield of PSII *in-situ* (Nedbal et al. 2008). Cultivation under low light, high light and low light combined with nitrogen starvation indicated that the adverse growth conditions affected linear electron transport negatively. Cultivation in the presence of DCMU again prevented the normal stress-induced accumulation of β-carotene, while we observed only small decreases of linear electron flow in comparison with the cultivations without DCMU. Addition of DBMIB decreased linear electron flow to a similar extend as DCMU, but, in contrast to DCMU, no significant changes in β-carotene accumulation were observed.

The results in the flat panel reactors indicate that DCMU and DBMIB indeed inhibited the photosynthetic electron transport chain in *D. salina in vivo*. Furthermore, DCMU inhibited β-carotene production under carotenogenic conditions, which suggests that the redox state of the plastoquinone pool is indeed involved in the regulation of β-carotene accumulation. The inhibitory effect of DCMU on β-carotene could not have resulted from a DCMU-induced reduction in energy availability, since DBMIB induced similar reductions in growth rate and linear electron flow without affecting β-carotene accumulation (Table 6.1). It cannot be excluded, however, that DCMU interacts with one or more specific enzymatic steps in the biosynthetic pathway of β-carotene.

Moreover, for complete validation of the redox hypothesis, a positive effect of DBMIB on the β-carotene levels under non-inducing growth conditions should have been observed. We did not record such accumulation, although this could have been the result of the instability of the inhibitor in light. This characteristic forced us to apply a very low light intensity (~50 µmol photons·m^{-2}·s^{-1}), which,

Table 6.1 *Effect of inhibitors of photosynthesis on growth and carotenoid production by D. salina cells cultivated under various conditions.*[a]

	LL[b]		**HL**[c]		**NS (LL)**[d]	
	growth	β-*carotene*	*growth*	β-*carotene*	*growth*	β-*carotene*
No inhibitors	100 %	1.7	100 %	6.9	100 %	10.6
+ DCMU	86 %	1.9	65 %	4.6	48 %	5.2
+ DBMIB	69 %	1.9	-	-	66 %	10.0

[a] *Percentages indicate growth relative to cultures without inhibitors at the same stress condition.* β-*carotene is expressed as gram per liter cell volume.*
[b] *Low light condition.*
[c] *High light condition.*
[d] *Nitrogen starvation + low light condition.*

together with the DBMIB-induced reduction in linear electron transport, perhaps prevented β-carotene accumulation through energy shortage.

Nevertheless, these preliminary results indicate that the plastoquinone redox-state hypothesis for regulation of β-carotene accumulation in *D. salina* is plausible, although additional validation studies that use light-stable DBMIB equivalents, such as DNP-INT (2-iodo-2',4,4'-trinitro-3-methyl-6-isopropyl-diphenylether), are needed.

Another important conclusion from these studies is that singlet oxygen, despite its ability to induce carotenogenesis (Shaish et al. 1993), most probably is not a sensor of stress in *D. salina*. This conclusion is based on the fact that β-carotene accumulation was inhibited in the presence of DCMU, while this inhibitor is actually known to stimulate release of singlet oxygen (Fufezan et al. 2002).

Finally, regulatory mechanisms involving the plastoquinone redox state are not limited to *Dunaliella* species (Escoubas et al. 1995; Maxwell et al. 1995), but have also been implicated in the regulation of carotenoid biosynthetic genes in the alga *Haematococcus pluvialis* (Steinbrenner and Linden 2003) and photoacclimation in plants (Frigerio et al. 2007; Yang et al. 2001). Based on its putative function as a sensor of the energy supply:consumption ratio, the

plastoquinone redox state is one of the candidates for the global regulation of secondary metabolism in microalgae.

Combined stress effects in turbidostats

Light intensity and growth-reducing stress conditions both affect the energy supply:consumption ratio and, as such, together act upon the accumulation of microalgal secondary metabolites. The definition of process conditions optimally suited for microalgal metabolite production, therefore, requires a detailed understanding of the combined effects of all environmental conditions on both the metabolite accumulation/secretion rate and cellular growth rate. This asks for studies employing sophisticated cultivation techniques with defined and controlled light conditions.

One way to meet this criterion is the use of turbidostat cultures (Figure 6.2) in which the turbidity of the culture, and thus the light perception of the algal cells,

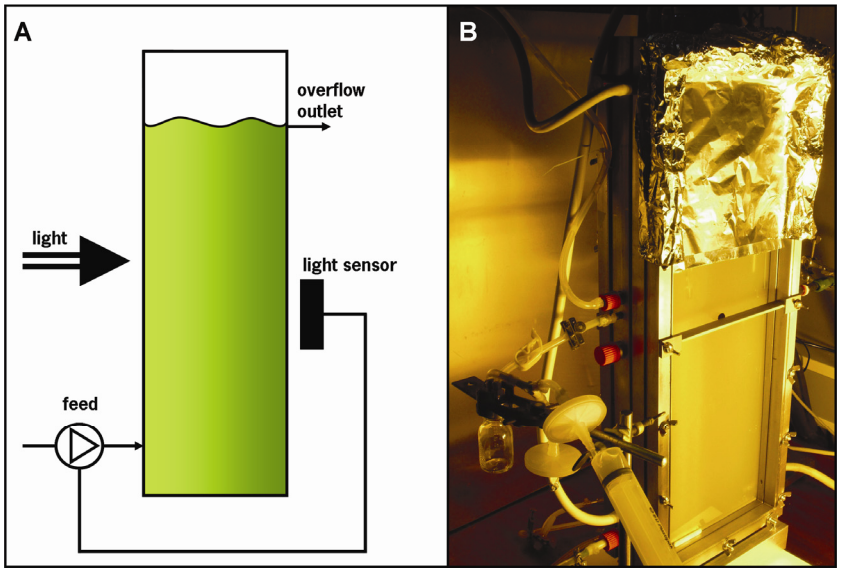

Figure 6.2 *Flat-panel turbidostat in which the turbidity of the culture is maintained at a fixed set point through automated dilution of the culture. A: Schematic overview. B: Practical setup at our lab.*

is maintained at a fixed and defined level by a constant and automatic adjustment of the dilution rate of the culture broth (Myers and Clark 1944). We applied such turbidostat cultures successfully to study the effects of high-light stress and nitrogen starvation on the accumulation of β-carotene in the alga *D. salina* (Chapter 4 and 5; Lamers et al. 2010). Whereas these studies targeted the dynamics of *D. salina*'s response to single changes in environmental conditions, turbidostats may also be applied to study the combined effects of, for instance, light intensity and nutrient availability on both the accumulation of secondary metabolites and on the cellular growth rate.

We hypothesize that for any given light intensity that is continuously perceived by algal cells (up to a certain maximum at which cells will die), a cell-specific nutrient supply rate should exist at which metabolite productivity is optimal. Indirect evidence for the existence of such optima follows from experiments with *Nannochloris* sp. and *Nannochloropsis* sp., which showed that different nitrogen-replenishment strategies resulted in differential volumetric lipid productivities (Rodolfi et al. 2009; Takagi et al. 2000).

Nevertheless, proper testing of the hypothesis requires the use of low-density turbidostat cultures fed with various limiting amounts of a nutrient at various light intensities. Such experiments will give detailed insight in the relation between the volumetric nutrient supply rate, the volumetric light energy supply rate and the volumetric productivities of biomass and secondary metabolites. Ultimately, these findings may be translated into a model and combined with a suitable model describing the light profile inside photobioreactors (Bosma et al. 2007; Pottier et al. 2005), which should enable adequate control of continuous commercial-scale photobioreactors used for the production of microalgal secondary metabolites.

Metabolite profiling

Apart from efforts directed at understanding specific individual steps of algal metabolism, large-scale untargeted approaches such as metabolomics should be employed as well. These comprehensive profiling techniques might provide

global clues and novel insights into the algal response to stress stimuli and into the cellular regulation of their response. Metabolic profiling will be particularly helpful in understanding the metabolite alterations taking place inside the cell upon induction of secondary metabolism.

Indeed, recent untargeted analysis of the aqueous methanol-soluble extracts of *D. salina* cells subjected to either high light stress or nitrogen starvation, showed that 11 metabolites correlated either positively or negatively with β-carotene accumulation, independent of stress treatment (Chapter 5). As such, these compounds may be key to β-carotene biosynthesis. This is not the case for chlorophyll *a*, chlorophyll *b* and the fatty acid species C16:4 and C18:3, however, as their negative correlations with β-carotene likely reflected the simultaneous stress-induced accumulation of β-carotene and degradation of photosystems and thylakoid membranes (Lamers et al. 2010). The 6 positively correlating metabolites included L-DOPA, threitol and 2 unidentified metabolites, which may be linked to changes in energy metabolism.

The remaining 2 positively correlating metabolites were lycopene and phytoene, both intermediates towards β-carotene biosynthesis (Figure 6.3). This suggests that both high-light stress and nitrogen starvation induced an upregulation of the entire β-carotene biosynthetic pathway, rather than only the final step in β-carotene biosynthesis, because precursor levels are expected to decrease upon upregulation of downstream steps in the biosynthetic pathway.

For similar reasons, the oil-globule-driven induction of β-carotene accumulation, as proposed previously (Rabbani et al. 1998), seems improbable. This theory describes a mechanism for β-carotene accumulation that does not require upregulation of structural genes encoding enzymes of the carotenoid biosynthetic pathway but relies on activity-enhancement of these enzymes through removal of their end-product (*i.e.* β-carotene) by newly formed oil globules (Rabbani et al. 1998). If this would be correct, however, the precursor levels would be expected to have decreased since it was found that β-carotene essentially accounts for the total carotenoid content of the lipid globules (Ben-Amotz et al. 1982). The observed increases in phytoene and lycopene levels in

this study, therefore, suggest otherwise.

Our hypothesis that the entire β-carotene biosynthetic pathway is upregulated is supported by various studies reporting stress-activated expression of several genes involved in carotenoid biosynthesis, *i.e.* phytoene synthase, phytoene desaturase, lycopene β-cyclase and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (Coesel et al. 2008; Ramos et al. 2007; Ramos et al. 2009). In contrast, however, it has been reported for *D. salina* that nitrogen deprivation resulted in a decreased expression of 1-deoxyxylulose-5-phosphate synthase, while the expression of phytoene synthase transcripts remained constant (Sanchez-Estudillo et al. 2006). Also, no upregulation of phytoene synthase and no changes in phytoene desaturase and its transcript levels were observed under carotenogenic conditions (Rabbani et al. 1998).

Although our findings point towards regulation of the entire biosynthetic pathway of β-carotene, these conflicting results on transcriptional and translational regulation illustrate the need for more detailed studies on its regulation. Such studies should also be aimed at elucidating the rate limiting step in β-carotene accumulation. In that respect, phytoene synthase has been named as a likely candidate because it catalyses the first committed step of the carotenoid pathway and is regarded as an important regulatory step in many plant species (Sandmann et al. 2006). However, we found relatively large increases in the intracellular concentrations of phytoene, the product of phytoene synthase (Figure 6.3), compared to the other carotenoids (Chapter 5). This suggests that phytoene desaturase, the next biosynthetic step, is rate limiting in β-carotene accumulation.

In conclusion, the combination of stress-experiments in highly controllable photobioreactors and broad-scale metabolite profiling techniques, as discussed here, is a novel and promising approach for the study of microalgal stress-metabolism. Indeed, by employing such an approach we were able to evaluate various hypotheses concerning the regulation of β-carotene accumulation in *D. salina*, and also to uncover a possible link between energy and carotenoid

metabolism in stressed *D. salina* cells. This shows the potential of such methods and justifies similar follow-up studies aimed at developing optimal strategies for the production of microalgal products such as carotenoids and biofuels.

Photobioreactor design and operation

Current status

The past few decades have witnessed the development of many different microalgal cultivation systems (Carvalho et al. 2006), ranging from large-diameter bubble columns to floating bag systems and from short light-path flat panel reactors to raceway ponds (Figure 6.4). This diversity in photobioreactor design stems mainly from the large variety in microalgal characteristics, environmental conditions, desired products and designer's expertise.

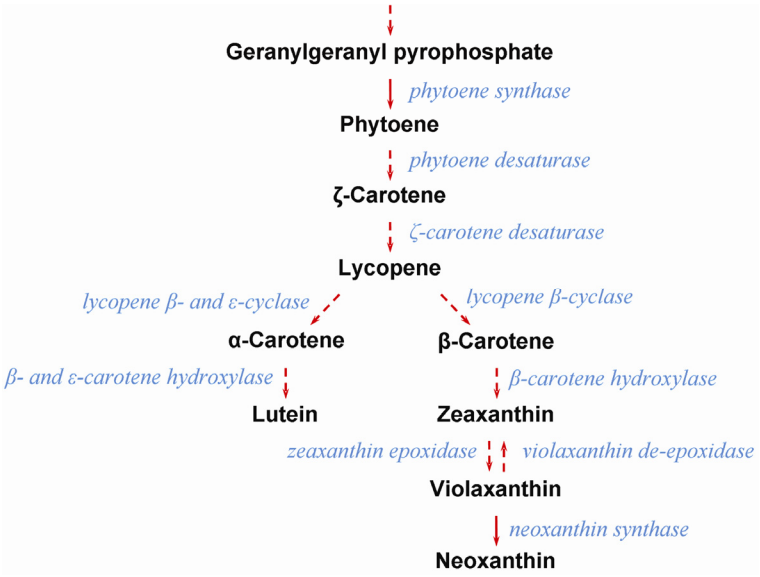

Figure 6.3 *Schematic representation of the carotenoid pathway found in green algae. Metabolites written in bold black characters; enzymes written in blue italics. Dotted arrows indicate non-shown intermediate steps.*

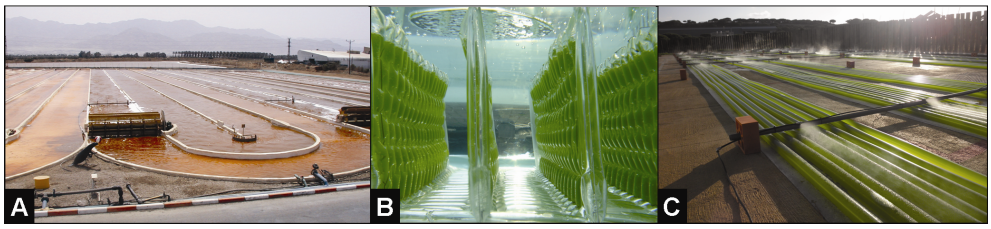

Figure 6.4 *Diversity in photobioreactor design. A: Open pond photobioreactor of Nature Beta Technologies, Israel (courtesy of A. Ben-Amotz). B: Flat panel photobioreactor of Proviron, Belgium (courtesy of M. Michiels). C: Horizontal tubular photobioreactor of University of Huelva, Wageningen University and Neste Oil, Spain (courtesy of R. Wijffels).*

Nevertheless, requirements which should be met for every successful photobioreactor have been defined (Posten 2009). First of all, the ideal photobioreactor should enable biomass production at high photosynthetic efficiency. Photosynthetic efficiency is defined as the amount of solar energy that is converted into biochemical energy and has a theoretical maximum of 9% (Wijffels 2008).

Such conversion of light energy is optimal when algae experience light intensities below the strain- and acclimation-specific saturation level of approximately 100-200 µmol photons·m^{-2}·s^{-1}, above which part of the excess energy is dissipated as fluorescence and heat (Mussgnug et al. 2007). Since for most locations on Earth the average light intensity falling on a horizontal surface during midday is several-fold higher than this saturation intensity, dilution of the incident light is necessary to ensure high photosynthetic efficiency.

Such dilution can be achieved by designing vertically arranged (e.g. flat panel) photobioreactors, which enable spreading of the incident light over a much larger illuminated area (Schenk et al. 2008). In view of the rather low incident light intensity, the optical path of such a reactor should be short to prevent the light intensity that is attenuated inside the reactor from dropping below the critical threshold level where light energy is just sufficient to match photosynthesis and respiration rates. Having a zone inside the reactor with light intensities lower than this so-called compensation point will result in a

suboptimal productivity of the system (Takache et al. 2010). Therefore, designing photobioreactors with high surface-to-volume ratios is a necessity, which implicitly points to the importance of low cost reactor materials.

In spite of these general guidelines, the desired product will be of great influence on photobioreactor design. For instance, the production of secondary metabolites, such as energy storage molecules or other overflow metabolites, generally requires adverse growth conditions like high light intensities and nutrient deprivation. Whereas nutrient deprivation can rather easily be applied in the above sketched systems, a high light intensity is not possible.
Therefore, two-stage production processes are being applied for the production of, for instance, β-carotene by *D. salina* (Ben-Amotz 1995). First, algal biomass is produced in a dedicated growth stage open pond reactor, after which the culture is diluted with nitrogen-deficient medium and transferred to several large pond systems. This shift to a higher light intensity and nitrogen depletion induces massive accumulation of β-carotene.
Despite being cheap in investment costs, these open pond systems are characterized by low biomass densities, subsequent significant harvesting costs and low metabolite productivities relative to the biological capacity of the organism (Chapter 4; Lamers et al. 2010).

Future directions
Thus, the presently used two-stage production processes are characterized by low volumetric productivities of secondary metabolites. Usually, scientific literature only refers to the algal metabolite content that is achieved and rarely to the time required to reach this concentration. It is important to take the rate of accumulation into account and for that reason the productivity (kg of product per reactor volume or reactor surface area per day) is the parameter we actually need to optimize the process for.
It has been suggested that β-carotene productivity in *D. salina* cultures, and possibly also the productivity of other secondary metabolites such as

triacylglycerols in all types of microalgal cultures, is regulated by the imbalance of absorbed light energy and the energy requirement for growth in terms of cell division (Ben-Amotz and Avron 1983; Lamers et al. 2008). Indeed, we calculated that continuous production of *D. salina* biomass, containing moderate levels of β-carotene, under high-light conditions in a turbidostat (Kleinegris 2010) was approximately 2 to 20 times more productive than two-stage open pond systems, when the difference in light source and reactor down-time were taken into account (Ben-Amotz 1995; Borowitzka 1999).

Furthermore, we also observed a transient increase in the biomass yield on light energy when *D. salina* cells were subjected to nitrogen starvation (Chapter 4) and we therefore propose to evaluate new photobioreactor designs and operation modes based on such new insights in the optimal conditions for secondary metabolite production. This means that such cultivation systems should be tailored to enable tight control of the light intensity and nutrient availability at the same time.

Control of light in large-scale outdoor systems is not easy, however. For instance, the adaptive mechanical adjustment of both photobioreactor orientation (Zijffers et al. 2008) and optical path length, with the possible exception of submerged plastic sheet reactors (Figure 6.4B), will be too costly for large-scale production. In contrast, continuously operated turbidostat systems are well-suited for the economical control of both light and nutrient regimes. In turbidostats, it is possible to control parameters, such as nutrient concentration or light intensity:biomass concentration ratios, to such an extent that the process is always steered towards a maximization of metabolite productivity.

For successful implementation of large-scale turbidostat cultivation, the influence of these process parameters on the metabolite productivity (and also on the biomass productivity) needs to be determined using lab-scale experiments. The gained knowledge should then be evaluated in a large-scale turbidostat-controlled reactor system. This is the approach we are presently further developing in several new PhD projects and already apply in the system shown in Figure 6.4C.

Conclusion

Photobioreactor design and operation define the conditions to which microalgae are exposed and the physiological response of the cells in turn affects these conditions as well as the metabolite productivity. This shows that the topics of photobioreactor design, photobioreactor operation and physiology are intricately connected and a detailed understanding of this relatedness, gained through experiments with sophisticated turbidostat photobioreactors, inhibitor studies and metabolomics profiling techniques, will pave the road for the realization of optimally designed microalgal metabolite production processes.

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Summary

Innovative production processes based on renewable resources are required to stop the exhaustion of our natural resources. Microalgae are one of the most promising feedstocks for such sustainable processes, since they can produce valuable biochemicals at high productivity using sunlight, water, carbon dioxide and a few other nutrients, without the need for arable land. This high productivity is attained in (semi)controlled photobioreactors designed to prevent any limitation but light.

Currently, the high costs for investment and operation of such systems only allow for economic production of high-value molecules such as carotenoids and omega-3 fatty acids. Though economically sound, these production processes typically require large amounts of energy and thus cannot be termed sustainable. Nevertheless, they do provide the essential know-how to develop sustainable and cost-effective production of microalgal low-value bulk chemicals and biofuels. To this end, breakthroughs are needed in all areas of microalgal biotechnology, including cell physiology.

One of the most important objectives of physiological studies is the improved understanding of how microalgal metabolism is affected by cultivation conditions. Design of optimal production process conditions can be enabled if the metabolism is fully understood and controlled.

A good example of the interaction of cultivation conditions on microalgal metabolism is the stress-induced accumulation of β-carotene in the unicellular green microalga *Dunaliella salina*. β-Carotene is a lipid-soluble orange pigment and an antioxidant, that is used in cosmetics and as a colorant for feed and food. This metabolite is accumulated by *D. salina* up to 10% of the cellular dry weight when the alga is exposed to extreme environmental conditions. However, the knowledge about the regulatory mechanisms that underlie β-carotene accumulation in *D. salina* is limited.

Therefore, the aim of this thesis was to gain more insight in how *D. salina* senses and responds to changes in process conditions with the ultimate goal of defining optimal strategies for β-carotene production, and possibly also for other stress-

inducible metabolites in other microalgae.

In **Chapter 2**, the cell-factory potential of *D. salina* was reviewed and the general ideas concerning mechanisms related to β-carotene accumulation were discussed. *D. salina* accumulates β-carotene when exposed to a high light intensity or to conditions that lead to a reduced growth rate, such as high salinity, nutrient deprivation or extreme temperatures. In addition, the levels of β-carotene in *D. salina* correlate positively with the overall amount of irradiation perceived during the cell division cycle. This positive correlation suggests the involvement of a sensing mechanism that is responsive to the imbalance between energy input, *i.e.* light harvesting in the chloroplasts, and energy demand, *i.e.* requirement for cell growth.

Singlet oxygen, an excited oxygen species that can be produced upon excess of electron transfer in the chloroplast, seems a likely candidate, as it can induce β-carotene accumulation and is known to be released under the conditions that favor β-carotene accumulation. Another potential sensing mechanism involves the redox state of the plastoquinone pool of the photosynthetic electron transport chain. Since oxidation of the reduced form of plastoquinone is generally assumed to be the rate-limiting step in this electron transport chain, an imbalance between the supply and demand of photosynthetic products may be reflected in the redox-state of the plastoquinone pool. Upon excess of electron production by the photosynthetic machinery, the plastoquinone redox state will shift towards a more reduced form.

However, the way these stress-induced changes in singlet oxygen or plastoquinone redox state are transduced and finally stimulate β-carotene accumulation is still largely unknown. Transcriptional and translational activity have been found essential for β-carotene accumulation, although a clear understanding of the involvement of structural genes that encode enzymes of the carotenoid biosynthetic pathway is still lacking.

Finally, β-carotene accumulation may also be driven by the formation of β-carotene-sequestering lipid globules. In this case, β-carotene accumulation does

not require upregulation of genes encoding enzymes of the carotenoid biosynthetic pathway, but relies on activity-enhancement of these enzymes through removal of their end-product (*i.e.* β-carotene) by newly formed oil globules.

In **Chapters 3** and **4**, the effects of either a sudden light increase or nitrogen starvation on the carotenoid metabolism of *D. salina* were described. Since high-light is one of the most potent inducers of β-carotene accumulation, the experiments were done in flat panel photobioreactors that were run in turbidostat mode to ensure a constant light regime throughout the entire duration of the experiments.

A 7-fold increase in light intensity proved more powerful in inducing β-carotene accumulation than complete nitrogen starvation, as was demonstrated by a 2 times higher maximum productivity (37 mg per liter reactor volume per day as compared to 18.5 mg per liter reactor volume per day). However, nitrogen depletion appears more efficient with regard to energy usage, since 7 times more light energy was used in the high-light experiment.

Interestingly, in our experimental turbidostat systems the maximal productivities found for both stresses were about an order of magnitude larger than the average productivity reported for a commercial β-carotene production facility, which indicates a vast potential for improvement in the latter.

Apart from β-carotene accumulation, the growth characteristics of the *D. salina* cells were also influenced by the stress treatments. Induction by either type of stress resulted in cell swelling and an increase in the cell-specific density. The increased amount of β-carotene could only partially explain the increase in cell-specific density, which suggests that other metabolites accumulated as well.

The initial cell division rate was differentially affected, with high-light causing a temporary cell cycle arrest and nitrogen starvation leading to a transient increase in the cell division rate. Nevertheless, the volumetric biomass productivity increased temporarily for both nitrogen starvation (2-fold) and high-light stress (6-fold).

These results implied that the 7-fold increase in incident light caused a decrease in the biomass yield on absorbed light energy, whereas nitrogen depletion led to a transient increase in the yield caused by the accumulation of non-nitrogenous biomass. This finding points towards the potential of nitrogen-limitation strategies for permanent improvement of lipid or carbohydrate yield on light in large scale microalgal production systems.

Since *D. salina* accumulates β-carotene in lipid globules and it has been suggested that fatty acid biosynthesis determines the amount of accumulating β-carotene, we determined the fatty acid content and composition during both stress treatments and correlated the exact time course of fatty acid levels to that of β-carotene production. The intracellular concentration of the total fatty acid pool did not change significantly during nitrogen starvation and decreased following treatment with high-light.

These results indicated that β-carotene and total fatty acid accumulation were unrelated, at least in our turbidostat experiments. Nevertheless, carotenoid overproduction was associated with oil globule formation and a decrease in the degree of fatty acid unsaturation. The accumulation of β-carotene appeared to correlate positively with oleic acid production, suggesting that oleic acid may be a key component of the lipid globule-localized triacylglycerols and thereby in β-carotene accumulation.

In **Chapter 5**, the cellular mechanisms that could be related with stress-induced β-carotene accumulation in *D. salina* were investigated. Samples were taken from the aforementioned high-light and nitrogen-starvation experiments, both before and during stress induction. Subsequent untargeted GC-TOF-MS-based analysis of derivatized polar extracts, mainly containing primary metabolites, in combination with unbiased peak picking and clustering of signals into metabolite mass spectra, yielded 87 unique polar metabolites of which 31 were in common between both stress experiments.

By combining these polar metabolite profiles with the previously determined levels of carotenoids, chlorophylls and fatty acids, it was found that *D. salina*

cells exhibit essentially similar overall responses towards both types of stress, with the principal stress-specific variation being caused by only 3 out of the 44 polar and apolar metabolites.
Furthermore, we observed accumulation of various metabolites that are usually linked with energy storage, both during high-light stress and during nitrogen depletion. Because both stress treatments are known to cause an imbalance in absorbed and required light energy, it was suggested that β-carotene accumulation and an increased production of energy storage molecules reflect a uniform and concerted effort of *D. salina* cells to cope with such damaging imbalances.

In **Chapter 6**, data of preliminary experiments towards elucidating the mechanisms potentially involved in the sensing of imbalances in the ratio between energy supply and energy consumption in *D. salina* are presented. In these experiments we used specific inhibitors of the reduction (DCMU) and the oxidation (DBMIB) of the plastoquinone pool in the photosynthetic electron transport chain, under both inducing and non-inducing conditions. It was found that DCMU inhibited both growth and β-carotene accumulation under otherwise carotenogenic conditions, whereas DBMIB inhibited only the growth.
These results pointed towards a possible role of the plastoquinone redox state in the regulation of β-carotene accumulation in *D. salina*. In addition, these findings suggested that singlet oxygen did not serve as a signaling agent for stress-induced β-carotene accumulation, since this accumulation was inhibited in the presence of DCMU, despite this inhibitor being a well-known stimulator of singlet oxygen release.
Next to performing these photosynthesis inhibitor experiments, we also showed the importance of metabolic profiling techniques for a better understanding of microalgal metabolism. We discussed the relations between β-carotene accumulation and the observed temporal changes in polar and apolar metabolites. This enabled evaluation of several hypotheses concerning the mechanisms involved in β-carotene accumulation, with upregulation of the

entire β-carotene biosynthetic pathway proving a likely element of such a mechanism.
Furthermore, because light intensity and growth-reducing stress conditions act together upon the accumulation of secondary metabolites in microalgae, proper physiological studies, as well as optimal commercial production strategies, require cultivation systems that enable tight control of both light and nutrient supply. We therefore advocated the use of turbidostats, both for research on and for commercial production of stress-induced accumulation of microalgal metabolites.

In conclusion, this thesis illustrates the potential of combining sophisticated cultivation techniques with broad-scale metabolite profiling approaches for the detailed study of microalgal metabolism. Hence, similar studies should be applied for gaining the understanding that is needed for the development of optimal and sustainable production processes that will ultimately put a stop to the exhaustion of our natural resources.

Samenvatting

Om de uitputting van onze natuurlijke grondstoffen een halt toe te roepen, zijn innovatieve productieprocessen, die gebaseerd zijn op hernieuwbare grondstoffen, noodzakelijk. Microalgen zijn één van de meest veelbelovende bronnen voor zulke duurzame processen omdat ze, gebruikmakend van zonlicht, water, koolstofdioxide en enkele andere nutriënten, waardevolle biochemicaliën kunnen produceren met een hoge productiviteit zonder dat hier landbouwgrond voor nodig is. Deze hoge productiviteit is mogelijk dankzij (semi)gecontroleerde fotobioreactoren die ontworpen zijn om elke groeilimitatie, anders dan licht, te voorkomen.

De hoge investerings- en operationele kosten van dergelijke reactoren laten momenteel slechts ruimte voor de productie van hoogwaardige moleculen als carotenoïden en omega-3 vetzuren. Hoewel dit soort productieprocessen economisch haalbaar zijn, verbruiken ze doorgaans veel energie en kunnen ze derhalve niet als duurzaam aangemerkt worden. Desalniettemin leveren deze processen de essentiële kennis die nodig is voor de ontwikkeling van duurzame en kosteneffectieve productie van laagwaardige bulkchemicaliën en biobrandstoffen uit microalgen. Om dit te bewerkstelligen zijn doorbraken op alle gebieden van de biotechnologie van microalgen noodzakelijk, zo ook het gebied van celfysiologie.

Eén van de meest belangrijke doelstellingen van fysiologische studies is beter begrijpen hoe het metabolisme van microalgen beïnvloed wordt door kweekomstandigheden. Een volledig begrip en controle van het metabolisme zullen de ontwikkeling van optimale procesomstandigheden mogelijk maken.

Een goed voorbeeld van de invloed van procesomstandigheden op het metabolisme van microalgen is de stressgeïnduceerde ophoping van β-caroteen in de eencellige groene microalg *Dunaliella salina*. β-caroteen is een vetoplosbare oranje kleurstof en antioxidant, die gebruikt wordt in cosmetica en als kleurstof voor humane en dierlijke voeding. Dit metaboliet wordt tot zoveel als 10% van het cellulaire drooggewicht opgehoopt in *D. salina* wanneer deze wordt blootgesteld aan extreme omstandigheden. Echter, de kennis van de

reguleringsmechanismen die betrokken zijn bij β-caroteenophoping in *D. salina* is beperkt.
Het doel van dit proefschrift was daarom het verkrijgen van meer inzicht in hoe *D. salina* veranderingen in procesomstandigheden signaleert en erop reageert. Uiteindelijk zou deze kennis het mogelijk moeten maken om optimale strategieën te definiëren voor de productie van β-caroteen en mogelijk ook voor andere stress-induceerbare metabolieten in andere microalgen.

In **Hoofdstuk 2** is de potentie van *D. salina* als celfabriek behandeld en zijn de algemene ideeën met betrekking tot β-caroteenophoping bediscussieerd. *D. salina* hoopt β-caroteen op wanneer deze wordt blootgesteld aan een hoge lichtintensiteit of aan omstandigheden die leiden tot een verlaagde groeisnelheid, zoals een hoog zoutgehalte, afwezigheid van nutriënten of extreme temperaturen. Bovendien is het β-caroteengehalte in *D. salina* positief gecorreleerd met de hoeveelheid licht die gedurende een celdelingscyclus geabsorbeerd wordt. Deze positieve correlatie suggereert het bestaan van een signaleringsmechanisme voor de onbalans tussen energie-invoer, d.w.z. lichtabsorptie in de chloroplasten, en vraag naar energie, d.w.z. verbruik voor celgroei.
Singletzuurstof, de aangeslagen toestand van moleculaire zuurstof die vrij kan komen bij een overmaat van elektronentransport in de chloroplast, zou een rol kunnen spelen in dit signaleringsmechanisme omdat het β-caroteenophoping kan induceren, en omdat het bekend is dat singletzuurstof vrijkomt onder de omstandigheden die kunnen leiden tot β-caroteenophoping.
Een ander mogelijk signaleringsmechanisme betreft de redoxstaat van de plastoquinonvoorraad in de fotosynthetische elektronentransportketen. Omdat over het algemeen aangenomen wordt dat de oxidatie van plastoquinol naar plastoquinon de snelheidsbeperkende stap is in deze elektronentransportketen, zou een onbalans tussen de aanvoer en vraag naar fotosynthetische producten zich kunnen weerspiegelen in de redoxstaat van de plastoquinonvoorraad. Bij een overmaat van elektronenproductie door het fotosynthetisch apparaat zal de

redoxstaat van plastoquinonvoorraad naar de gereduceerde staat verschuiven.
Echter, de manier waarop deze stressgeïnduceerde veranderingen in singletzuurstof of de plastoquinon redoxstaat doorgegeven worden en uiteindelijk β-caroteen ophoping stimuleren is grotendeels onbekend. Het is aangetoond dat transcriptionele en translationele activiteit essentieel zijn voor de ophoping van β-caroteen, maar het ontbreekt nog aan een duidelijk begrip van de eventuele betrokkenheid van structurele genen die coderen voor enzymen uit de biosynthetische route voor carotenoïden.
Tot slot zou de ophoping van β-caroteen ook gedreven kunnen worden door de vorming van β-caroteensequestrerende vetdruppels. In dit geval behoeft de ophoping van β-caroteen geen opregulering van genen die coderen voor enzymen uit de carotenoïdensynthese route, maar wordt de ophoping gestimuleerd door activiteitstoename van diezelfde enzymen als gevolg van de verwijdering van hun eindproducten (d.w.z. β-caroteen) door de nieuw gevormde oliedruppels.

In **Hoofdstuk 3** en **4** zijn de effecten van ofwel een plotselinge verhoging van de lichtintensiteit, ofwel een stikstofverhongering op het carotenoïdenmetabolisme van *D. salina* beschreven. Omdat hoog licht één van de meest bepalende factoren voor β-caroteenophoping is, zijn de experimenten uitgevoerd in vlakke plaat fotobioreactoren die als turbidostaat bedreven werden om zo een constant lichtregime gedurende de experimenten te waarborgen.
De ophoping van β-caroteen werd beter geïnduceerd door een 7-voudige verhoging van de lichtintensiteit dan door een complete stikstofverhongering. Dit bleek uit de tweemaal hogere maximum productiviteit die bij de lichtstress werd gevonden (37 versus 18.5 mg per liter reactorvolume per dag). Met betrekking tot energieverbruik lijkt stikstofverhongering echter een efficiëntere methode voor β-caroteenproductie, omdat 7 maal meer lichtenergie gebruikt was voor deze verdubbeling van de maximale productiviteit.
In onze experimentele turbidostaatsystemen zijn voor beiden typen stress maximale β-caroteenproductiviteiten gevonden die ongeveer een ordegrootte

hoger zijn dan de gemiddelde productiviteiten die voor bestaande commerciële systemen gerapporteerd zijn. Dit duidt op een aanzienlijke potentie voor verbetering van deze commerciële systemen.

Naast de ophoping van β-caroteen werd de groei van de *D. salina* cellen ook beïnvloed door de stressbehandelingen. Beiden typen stress resulteerden in een vergroting van het celvolume en de celspecifieke dichtheid. De toename in celspecifieke dichtheid kon slechts gedeeltelijk verklaard worden door de toename in de hoeveelheid β-caroteen, hetgeen suggereert dat er ook andere metabolieten opgehoopt moeten zijn.

Afhankelijk van het type stress werd de initiële celdelingssnelheid verschillend beïnvloed. Hoog licht veroorzaakte een tijdelijke stop van de celdeling, terwijl stikstofverhongering een tijdelijke toename van de celdelingssnelheid teweeg bracht. Desalniettemin nam de volumetrische biomassa productiviteit tijdelijk toe voor zowel stikstofstress (verdubbeling) als lichtstress (verzesvoudiging).

Deze resultaten betekenen dat de 7-voudige toename in invallend licht leidde tot een afname in de biomassaopbrengst per hoeveelheid geabsorbeerd licht, terwijl stikstofverhongering juist leidde tot een tijdelijke verhoging van deze opbrengst door ophoping van stikstofloze biomassacomponenten te stimuleren. Deze bevindingen wijzen op het potentieel van stikstoflimitatie-strategieën voor de permanente verbetering van vet- of koolhydraatopbrengsten in grootschalige productiesystemen.

Omdat *D. salina* β-caroteen ophoopt in vetdruppels, en omdat gesuggereerd is dat de aanmaak van vetzuren de hoeveelheid opgehoopt β-caroteen bepaalt, hebben wij de vetzuurinhoud en -samenstelling tijdens beide typen stress bepaald en het verloop van de vetzuur- en β-caroteenspiegels met elkaar vergeleken. De intracellulaire concentratie van de totale vetzuurvoorraad veranderde niet significant tijdens stikstofverhongering en nam af bij hoog licht stress.

Deze resultaten wezen erop dat β-caroteenophoping en de ophoping van alle vetzuren samen niet gerelateerd waren, in elk geval in onze turbidostaatexperimenten. Desalniettemin bleek β-caroteenophoping

geassocieerd te zijn met de vorming van vetdruppels en een toename in de verzadigingsgraad van de vetzuren. Daarnaast bleek de ophoping van β-caroteen ook positief te correleren met de productie van oliezuur (C18:1), hetgeen suggereert dat oliezuur een belangrijke component van de triglyceriden in de β-caroteenbevattende vetdruppels zou kunnen zijn.

In **Hoofdstuk 5** zijn de cellulaire mechanismen, die gerelateerd zouden kunnen zijn aan stressgeïnduceerde β-caroteenophoping in *D. salina*, bestudeerd. Hiervoor zijn monsters genomen van de eerdergenoemde stikstofverhongerings- en lichtstress-experimenten, zowel vóór als tijdens de stressinductie. Daarop volgde een ongerichte GC-TOF-MS-gebaseerde analyse van gederivatiseerde polaire extracten, die voornamelijk primaire metabolieten bevatten. Dit leverde, in combinatie met onbevooroordeelde piekkeuze en clustering van signalen in massaspectra van metabolieten, 87 unieke polaire metabolieten op, waarvan er 31 bij beide typen stress gedetecteerd werden.
Door het verloop van deze polaire metabolieten met de eerder bepaalde carotenoïden-, chlorofyl- en vetzuurspiegels te combineren, bleek dat *D. salina* cellen in essentie een vergelijkbare respons vertonen op beide typen stress. De belangrijkste verschillen werden veroorzaakt door slechts 3 van de 44 polaire en apolaire metabolieten.
Verder bleken verscheidene metabolieten, die gewoonlijk geassocieerd worden met energie-opslag, op te hopen onder zowel stikstofverhongering als hoog lichtstress. Omdat het bekend is dat beide typen stress een onbalans veroorzaken tussen de hoeveelheid geabsorbeerde en benodigde lichtenergie, opperden wij dat een verhoogde productie van β-caroteen en energiereserves een uniforme en gezamenlijke inspanning weerspiegelt, die erop gericht is om *D. salina* cellen een dergelijke schadelijke onbalans te laten overleven.

In **Hoofdstuk 6** zijn data gepresenteerd van preliminaire experimenten die erop gericht waren om de mechanismen te ontrafelen die mogelijk betrokken zijn bij het vaststellen van de onbalans tussen energie aanvoer en energieconsumptie in

D. salina. Bij deze experimenten hebben we gebruik gemaakt van specifieke remmers van de reductie (DCMU) en oxidatie (DBMIB) van de plastoquinonvoorraad in de fotosynthetische elektronentransportketen. Deze remmers zijn onder zowel inducerende als niet-inducerende omstandigheden toegevoegd en het bleek dat DCMU zowel de groei als de ophoping van β-caroteen verlaagde onder omstandigheden die anderszins tot β-caroteenophoping zouden leiden. Onder vergelijkbare omstandigheden leidde toevoeging van DBMIB alleen tot een verlaging van de groei.

Deze resultaten duiden op een mogelijke rol van de plastoquinon redoxstaat in de regulatie van β-caroteenophoping in *D. salina*. Daarnaast suggereren deze bevindingen dat singletzuurstof niet als signaleringsmolecuul dient voor de stressgeïnduceerde ophoping van β-caroteen. Dit omdat β-caroteenophoping verlaagd was in de aanwezigheid van DCMU dat juist een bekende stimulator is van singletzuurstofproductie.

Naast de beschrijving van deze experimenten, illustreert dit hoofdstuk ook het belang van metabole profileringstechnieken voor de begripsvergroting van het metabolisme van microalgen. De relaties tussen β-caroteenophoping en de geobserveerde tijdelijke veranderingen in polaire en apolaire metabolieten zijn bediscussieerd. Hierbij zijn verschillende hypotheses met betrekking tot de mechanismen, die mogelijk betrokken zijn bij β-caroteenophoping, geëvalueerd. Het bleek dat opregulering van de volledige biosynthetische route voor β-caroteen zeer waarschijnlijk onderdeel uitmaakt van de stressrespons in *D. salina*.

Verder is vastgesteld dat het gezamenlijk effect dat lichtintensiteit en groeiremmende kweekomstandigheden op de ophoping van secondaire metabolieten in microalgen hebben, vereist dat zowel correcte fysiologische studies als optimale commerciële productiestrategieën gebruik maken van kweeksystemen waarin een nauwgezette regeling van de licht- en nutriënttoevoer mogelijk is. Wij bepleitten daarom het gebruik van turbidostaten voor onderzoek aan en commerciële productie van stressgeïnduceerde metabolietophoping in microalgen.

Tot besluit illustreert dit proefschrift het potentieel van de combinatie van verfijnde kweektechnieken en breedschalige metabolietprofilering voor gedetailleerde studies van het metabolisme in microalgen. Vergelijkbare studies zijn daarom aanbevolen om zo de kennis te verwerven die nodig is voor de ontwikkeling van optimale en duurzame productieprocessen die uiteindelijk de uitputting van onze natuurlijke grondstoffen zullen voorkomen.

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Packo

Curriculum Vitae

Packo Lamers was born in Bennekom, the Netherlands, on April 20th, 1980. He went to the Christelijk Lyceum in Veenendaal where he obtained his gymnasium diploma in 1998. After that, he studied Bioprocess Technology at Wageningen University, specializing in cellular and molecular biology. He did both a minor and major thesis on the metabolism of hybridoma cells at Diosynth BV in Oss. These projects, which were performed under the auspices of Process Engineering of Wageningen University, resulted in a peer-reviewed scientific publication and involved the construction of a metabolic flux model and a study on the metabolic response to different carbon sources. Another publication resulted from his second major thesis, during which he molecularly characterized a metalloregulator of the archaea *Sulfolubus solfataricus* at the Bacterial Genetics Group of Wageningen University. Packo then went to the Triple-J group of Stellenbosch University, South Africa, for an internship on the validation of a kinetic model of glucose and pyruvate metabolism in *Lactococcus lactis*. After graduating in 2005, he started his PhD-project at Bioprocess Engineering of Wageningen University, of which this thesis is the tangible result. Packo currently holds a postdoctoral position at Bioprocess Engineering and is involved in projects that are centered around microalgal lipid metabolism, energy metabolism and secondary metabolite production.

Publications

Lamers PP, Janssen M, De Vos RCH, Bino RJ, Wijffels RH. Regulation of carotenoid accumulation in *Dunaliella salina*: a role for the plastoquinone pool redox state? Submitted for publication.

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Training activities

Courses

Good manufacturing practice	2002
VLAG PhD introduction week	2005
Metabolomics	2005
Biosystems engineering	2005
Systems biology course	2006
PhD scientific writing	2006
PhD competence assessment	2006
Project and time management	2006
Career perspectives	2009

Conferences

Netherlands biotechnology congress, Ede, The Netherlands	2006/07/08/09
BSDL symposium, Delft, The Netherlands	2006/07
International marine biotechnology conference, Eilat, Israel	2007
International algae congress, Amsterdam, The Netherlands	2008
Wetsus congress, Leeuwarden, The Netherlands	2008
Algae biomass summit, Phoenix, USA	2010

Optionals

Preparation of PhD research proposal	2005
Brainstorm week Process Engineering	2005
Process Engineering PhD study tour to Denmark and Sweden	2006
Process Engineering PhD study tour to Japan	2008
Brainstorm day Bioprocess Engineering	2008/09

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