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Research review paper

Accumulation of energy reserves in algae: From cell cycles to biotechnological applications


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ABSTRACT

Starch and lipids are key components of algal cells and responsible for buffering variable supplies of energy and carbon that are vital for cell growth and reproduction, particularly DNA replication, nuclear and cellular division. The basic characteristics of energy reserves, their ultrastructure and localization inside the cell, regulation of their synthesis in relation to cell cycle phases, and their control by external factors, including light intensity, temperature, and carbon dioxide are described. Over the last two decades, research in this field has been boosted by possible biotechnological applications of algae for the production of biofuels from energy conserving compounds (bioethanol from starch and biodiesel from lipids). Recent findings on mechanisms that lead to an accumulation of exceptionally high levels of starch and lipids in algae will be summarized in this review. Macroelement (N, S, P) limitation, or depletion in mineral medium, as the most widely used approaches for enhancing both starch and lipid accumulation, are reviewed in detail. Potential biotechnological strategies for the economically viable overproduction of lipid and starch, such as a two-step procedure exploiting the effects of nutrient limitation and depletion, as well as the means and rationale for selecting appropriate strains, are discussed.

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1. Introduction

The role of starch energy reserves in the regulation and progression of algal cell cycle has been the subject of basic research since the 1970s (Ballin et al., 1988; Duynstee and Schmidt, 1967; Hirokawa et al., 1982; Klein, 1987; Semenenko and Zvereva, 1972; Šetlík et al., 1988; Zachleder et al., 1988). However, up until about 1990, there was only moderate interest until a recent boost in both basic and applied research in energy reserves. This increased interest was motivated by the view that algae could be a high-yielding source of energy-rich components for bioethanol production from starch (John et al., 2011) and biodiesel production from lipids (Chisti, 2007; Chisti and Yan, 2011; Pfišl et al., 2014; Singh et al., 2011a; Singh et al., 2011b).

To study how starch and lipid reserves are formed and regulated, it is crucial to understand why and where they are produced, how their accumulation can be affected by external growth conditions or genetic manipulation, and what processes or events are involved in their utilization (degradation) within the algal life and cell cycle. For the sake of simplicity, the term starch will be used throughout the manuscript because most of the research was done on green algae producing starch; however, rules similar to these described here for starch will also be generally valid for other polyglucans produced by other algal species (glycogen in cyanobacteria and red algae, chrysolaminarin in diatoms, paramylon in euglenophytes, and floridean starch in red algae).

Both starch and lipid metabolism start with a common initial pool of molecules consisting of three carbons, such as 3-phosphoglycerate (3PG) and glyceraldehyde 3-phosphate (GAP) (de Jaeger et al., 2014) (Fig. 1). From 28 molecules of 3PG, one molecule of C18 TAG or 14 molecules of glucose in a starch matrix can be formed (de Jaeger et al., 2014). Most algae can produce both starch (or other polyglucans) and lipids as energy reserves, with ratios that differ depending on growth conditions. In the most common situation, starch is the primary energy compound but how its production switches to triacylglycerol production (carbon partitioning) under specific conditions is not completely understood. Existing knowledge on the relationship between metabolic pathways influencing starch and reserve lipid accumulation by common C3 precursors is illustrated in Fig. 1. Starch functions as a primary storage of energy, being formed in light, providing energy for key metabolic processes independently of external energy sources (for details, see Section 2) (de Winter et al., 2013). Reserve lipids (TAG), however, usually serve as a secondary energy source and an electron sink when their production is more economical for the cell than production of starch (usually under conditions of nutrient limitation) (Hu et al., 2008).

The production and accumulation of energy rich molecules, like starch and lipids, are an indispensable buffer against varying external growth conditions and allows the photosynthetic cell to survive periods of light-energy absence at night, as well as during periods of varying light intensity. Both compounds serve as a source of energy and carbon and thus not only ensure cell survival but also supply energy for processes related to cell multiplication, such as DNA replication, nuclear division, cytokinesis, and daughter cell formation and liberation.

From a historical point of view, research on microalgae and the application of microalgae as sources of renewable energy covers a span of approximately 140 years. The first report on growing microalgae (diatoms) as a source of lipids for food or fuel, and their large-scale production, was published in 1942 in an effort to substitute for limited

sources of fossil fuels during the Second World War (Harder and von Witsch, 1942a; Harder and von Witsch, 1942b). For a more detailed historical tour, we refer readers to a recent excellent overview of the topic *Algae for Biofuels and Energy* (Borowitzka and Moheimani, 2013). From that time up to now, much effort was dedicated to improving algal biomass growth on a large scale, leading to testing, optimization and production of different bioreactors and cultivation regimes for different algal species. However, we feel that the effort was driven by trial and error in many cases and was not backed up by basic research, an approach that may have inhibited further development and improvement. We strongly believe that understanding algal physiology, beyond the simple characteristics of growth, such as biomass increase or photosynthetic efficiency, is essential for improving industrial productivity. The life- and cell-cycle stages of algae are of paramount importance for their behavior, and understanding the background of behavior is crucial for efficient manipulation of biomass and, particularly, energy reserve productivity. This present review will attempt to bridge the gap between basic research and its industrial application. The review concentrates on basic research of energy reserve regulation in the algal cell cycle and discusses the possible applications of such knowledge for scaled-up industrial production of high-energy content reserves.

This paper reviews the current state of knowledge of mechanisms that control the synthesis of starch and lipids as well as the relationship between synthesis and events of the cell cycle. The accumulation of energy reserves under optimal conditions will be compared with increased accumulation of starch and lipids after the inhibition of cell cycle progression.

The effect of light, temperature, carbon dioxide, and nutrient stress on the accumulation of energy reserves will be described, and the procedures for obtaining algal cells enriched in starch or lipids will be discussed. The main attention will be focused on the most effective methods and approaches leading to the overproduction of energy reserves, mainly limitation of single macro-elements like nitrogen, sulfur, or phosphorus.

The reviewed species belong mostly to the taxonomic orders Chlorococcales (*Chlorella*, *Scenedesmus*) and Volvocales (*Chlamydomonas*, *Dunaliella*) of the phylum Chlorophyta green algae, but other biochemically important taxonomic groups, such as red algae (Rhodophyta), diatoms (Heterokontophyta, Bacillariophyceae) and genus *Isochrysis* (Chromophyta, Haptophyceae) will be also reviewed (for detailed information on taxonomic categorization of reviewed algal species, see van den Hoek et al., 1995).

2. Storage reserves characteristics

Polysaccharides used as energy and carbon reserves, are typically homopolysaccharides composed of glucose units (polyglucans). They are species specific and not affected by changes in growth conditions. Starch is the most common type of polyglucan produced by most green algae (as well as higher plants) as a primary store of carbon and energy, and comprises two different α -polyglucans, amylose and amylopectin, in different species-dependent ratios. Amylose and amylopectin differ in the degree of branching. Amylose is the linear form of starch whereas amylopectin is the branched one. Other algae produce different types of polyglucans, for example, chrysolaminarin

on the conditions, some strains of *Chlorella* or *Parachlorella* can overproduce starch (Brányiková et al., 2011), whereas other strains of the same species overproduce lipids instead (Li et al., 2013). Moreover, mutants can be isolated from what were originally “starch” alga, such as *Chlamydomonas reinhardtii*, but are unable to produce starch, producing lipids as a reserve instead (Goodenough et al., 2014; Li et al., 2010a; Li et al., 2010b; Saut et al., 2011; Wang et al., 2009; Work et al., 2010; Zabawinski et al., 2001). Usually, strains that overproduce starch in response to stress can synthesize lipids in a small amount but do not overproduce them. In the case of overproduction of lipids, starch is often synthesized at the beginning of treatment, whereas lipids start to accumulate much later. Even in cells enriched in lipids, starch remains in small but significant amounts in cells, particularly as grains around pyrenoids. Stress treatments, for example, nutrient limitation or depletion, are the most widely used approaches to overproduce starch and lipids. Such treatments efficiently block further cell cycle progression, not only preventing consumption of the majority of energy reserves but simultaneously stress the cells. Under such conditions, lipids are often produced as an energetically more compact storage pool, yet they also serve as an electron sink preventing the over-reduction of photosynthetic electron transport chain (Breuer et al., 2014). Starch, even when overproduced or accumulated, will be of the same structure as under standard conditions. In contrast, the composition of lipid reserves not only differs substantially in different species or strains but also can vary depending on growth conditions (temperature or other environmental factors, type of stress, for example). Based on fatty acid composition, lipid-enriched algal biomass can be utilized for the production of biodiesel, as food and feed additives, or in pharmaceutical applications.

3. Dynamics of energy reserves during the cell cycle

3.1. The course of cell cycle events

The cell cycle of chlorococcal algae, particularly those dividing by multiple fission, was described in detail (Přibyl et al., 2013; Šetlík and Zachleder, 1984; Šetlík et al., 1972; Zachleder and Šetlík, 1990) and recently reviewed (Bišová and Zachleder, 2014). In summary, the multiple-fission algal cell cycle is composed of several consecutive steps consisting of growth, DNA replication, nuclear division and cellular division, with the preparative phases preceding each step. The cells dividing into two daughter cells (binary fission) pass through only one growth and reproductive sequence (e.g., DNA replication, nuclear division and cellular division) within one cell cycle. After completing a certain amount of synthetic work enabling accumulation of energy and material reserves, the cell attains the “commitment point” which “empowers” it to undergo and terminate DNA replication, nuclear and cellular division without an external energy supply; i.e., the cell can divide in the dark (Šetlík et al., 1972). In cases where the cells divide by multiple fission into more than two daughter cells (in general 2^n , where n is an integer), several (n) reproductive sequences, mutually overlapping each other, are performed within one cell cycle to terminate in multiple cellular divisions.

3.2. Utilization of energy reserves in cell cycle events

In autotrophically growing algae, light energy for photosynthesis is essential for completion of the growth steps during which energy reserves (polyglucans, lipids) accumulate. Consequently, DNA replication, nuclear and cellular division use the reserves to meet their carbon and energy requirements. Interestingly, they do so even when photosynthesis is active (Vítová et al., 2011b), and individual steps of the reproductive sequence proceed, leading to the completion of cell cycles without any external carbon or energy supply, for example, at low light intensity or in the dark (Bišová and Zachleder, 2014; Šetlík and Zachleder, 1984; Šetlík et al., 1972; Vítová and Zachleder, 2005;

Zachleder and Šetlík, 1988; Zachleder and Šetlík, 1990; Zachleder and van den Ende, 1992).

3.3. Synthesis of energy reserves during the cell cycle

The time course of starch accumulation during the cell cycle was described in several synchronized cultures of green algae, for example, *Chlorella pyrenoidosa* (Duynstee and Schmidt, 1967), *Chlorella ellipsoidea* (Hirokawa et al., 1982), *Chlorella vulgaris* (Brányiková et al., 2011), *Scenedesmus quadricauda* (Ballin et al., 1988; Šetlík et al., 1988; Zachleder et al., 1988), and *C. reinhardtii* (Klein, 1987; Vítová et al., 2011b). In all cases, the rules for starch accumulation are almost the same. The dark-born daughter cells at the beginning of the cell cycle had very low starch content. Starch started to accumulate rapidly upon illumination until the content was sufficient to cover the energy and carbon requirement for DNA replication, nuclear and cellular division; starch is apparently one of the prerequisites to attain a commitment point. Starch content decreased dramatically during nuclear and cellular division, even in light grown cells, to accommodate the energy consumption (Vítová et al., 2011b). Moreover, starch is also used to cover all energy requirements in the dark.

Degradation of starch during nuclear and cellular divisions was observed microscopically (Murakami et al., 1963) and by chemical analysis (Cole et al., 1998; Curnutt and Schmidt, 1964; Herrmann and Schmidt, 1965; Johnson and Schmidt, 1966; Schmidt, 1966; Vítová et al., 2011b). During starch degradation, increased activity of starch degrading enzymes occurred (Levi and Gibbs, 1984; Wanka et al., 1970). Due to reconstruction of the chloroplast during its division, photosynthetic rate was markedly decreased, as observed in *C. pyrenoidosa* (Sorokin, 1957) and *S. quadricauda* (Berková et al., 1972). Concomitant with cessation of photosynthesis, the endogenous respiratory rate accelerated during the onset of nuclear division (Curnutt and Schmidt, 1964). In phototrophically grown algae, starch accumulates during the cell cycle prior to the period of nuclear and cellular division and is utilized as a carbon and energy reserve to ensure that all division processes are independent of varying supplies of carbon and energy. In photoautotrophic prokaryotic cyanobacteria, organic carbon is stored in the form of glycogen instead of starch, but similar to eukaryotic algae, it is produced in the light via photosynthesis. In darkness, organic carbon is degraded in at least two species: *Crocospheera watsonii* (Dron et al., 2012; Dron et al., 2013) and *Cyanotheca* sp. (Červený et al., 2013). In both species, glycogen degradation in the dark by respiration leads to decreased oxygen levels, which is a prerequisite for nitrogen fixation, a unique feature of cyanobacteria. The cells are able to fix carbon dioxide, form glycogen, and release oxygen in the light, whereas in darkness the oxygen and glycogen are consumed and nitrogen is fixed instead. The mechanisms governing such fascinating changes in cell physiology are not clear but are a matter of interest for both basic research and industrial applications because nitrogenases, enzymes responsible for nitrogen fixation, can also fix hydrogen. In such cells, glycogen is not only used to support processes of DNA replication and cell division but also exploited by enormously energy-demanding nitrogen fixation mechanisms.

The level of energy reserves is not the sole requirement for cell division. Nutrient limitation by cultivation in diluted mineral medium in *Parachlorella kessleri* leads to the accumulation of high levels of lipids, while the cells also maintained a certain content of starch. The cells stopped growth and did not divide once the medium was completely depleted, even though they clearly had sufficient energy reserves to perform reproduction. Only after transfer into a complete mineral medium were they able to grow into large mother cells and subsequently divide more or less synchronously while expending all energy and carbon reserves (both starch and lipids) (Fernandes et al., 2013). The same mechanism is at work in other algae such as *C. reinhardtii* and *C. vulgaris*. Growth blockage due to nutrient limitation, is readily overcome by fragmentation of lipid bodies and a decrease in lipid and starch content once

the cells are re-supplemented with complete medium (Siaut et al., 2011). The process can occur even in the dark (Přibyl et al., 2013) suggesting that the cells were limited by nutrients rather than by the absence of energy reserves, and once the nutrient limitation is lifted, the high levels of storage compounds that accumulated during nutrient starvation can serve as an exclusive source of energy and carbon for recovery of growth and reproductive processes.

Most starch analyses during algal cell cycles record only the net content of starch, the sum of starch that was synthesized minus that which was utilized. It is apparent that net accumulation data underestimate the value of actual starch synthesized during the cell cycle. This was clearly demonstrated in *C. reinhardtii*, where starch content increased at the beginning and at the end of the light period while it decreased in the middle of the light period and in the dark. Labeled bicarbonate was, however, incorporated into starch even at times when the total intracellular level of starch was decreasing, which indicated that the rate of synthesis and degradation can be different but both processes can run simultaneously (Klein, 1987).

The aim of any biotechnological application is to produce biomass with a maximal content of starch or lipid. As a matter of course, growth conditions, particularly light intensity, should be kept at optimal values because starch content increases with light intensity (Brányiková et al., 2011; Šetlík et al., 1972; Zachleder and Brányiková, 2014). It is relatively simple to manage optimal conditions in laboratory settings. Under natural conditions in scaled-up industrial photobioreactors, it is more complicated; the desire to attain optimal conditions has been the main bottleneck for the inexpensive production of starch- or lipid-enriched algal biomass. To economically produce starch or lipids from algae, the following conditions should be optimized: intensive sunlight for most of the year, sufficient sources of water, free or inexpensive sources of carbon dioxide and fertilizers. From this point of view, there is only a very limited number of locations that fulfill all of these requirements, and most of the sites used provide only some of the conditions. Sufficient light intensity can be found in subtropical areas, such as Southern Europe or North Africa and similar regions, where the sunlight together with optimal temperature is available for most of the year (Doucha and Lívanský, 2006; Rodolfi et al., 2009). Inexpensive or free-of-charge CO₂ can be supplied by fuel gas from different industrial sources, such as incinerators (Douskova et al., 2009), biogas plants (Dousková et al., 2010), burning

natural gas (Doucha et al., 2005), or lime or cement works. Similarly, fertilizers can be obtained from waste water. However, to avoid increasing the price of such “wastes” by transporting them, the bioreactor needs to be placed close to the waste production sites. Using nutrient limitation, approaches for energy rich storage accumulation could be an easier or more efficient approach for energy rich storage accumulation (see Subsection 6.2 Nutrient starvation).

It should also be kept in mind that even under optimal light conditions, during the night, the cultures usually degrade all or most of the reserves accumulated during the day; this is the main problem. Consequently, even under optimal growth conditions, the only way to attain maximal energy reserves is to minimize those cellular activities that utilize reserves as a source of carbon or energy, particularly during the night in outdoor bioreactors. This stems from the fact that during the cell cycle, nuclear and cellular division processes are the greatest consumers of energy reserves and these processes must be blocked without disturbing the synthesis of starch and other energy reserves. The following sections will deal with this problem in detail.

4. External factors

4.1. Light intensity

There has been unequivocal agreement in the literature since the 1970s that the synthesis and accumulation of starch increases with increasing light intensity (Brányiková et al., 2011; Hirokawa et al., 1982; Šetlík et al., 1972; Sukenik, 1991; Yao et al., 2012). Under high light intensity ($\geq 180 \mu\text{mol photons s}^{-1} \text{m}^{-2}$) with 2.5% CO₂ aeration, the total amount of carbohydrates (e.g., both starch and structural carbohydrates) can reach up to about 73–80% of dry weight (DW) (Ho et al., 2011). Sufficient light intensity is also crucial for overproduction of lipids (Khotimchenko and Yakovleva, 2005). In marine red alga *Tichocarpus crinitus* at low light intensities, only membrane lipids and unsaturated fatty acids were increased, whereas at high light intensities, storage lipids increased more than 1.5 fold, together with an increase in the concentration of saturated fatty acids (Khotimchenko and Yakovleva, 2005). Oleaginous alga *Nannochloropsis* sp. produced the highest amount of lipids (47% of DW) at the highest temperature used ($700 \mu\text{mol photons s}^{-1} \text{m}^{-2}$) (Pal et al., 2011). Eight algal strains

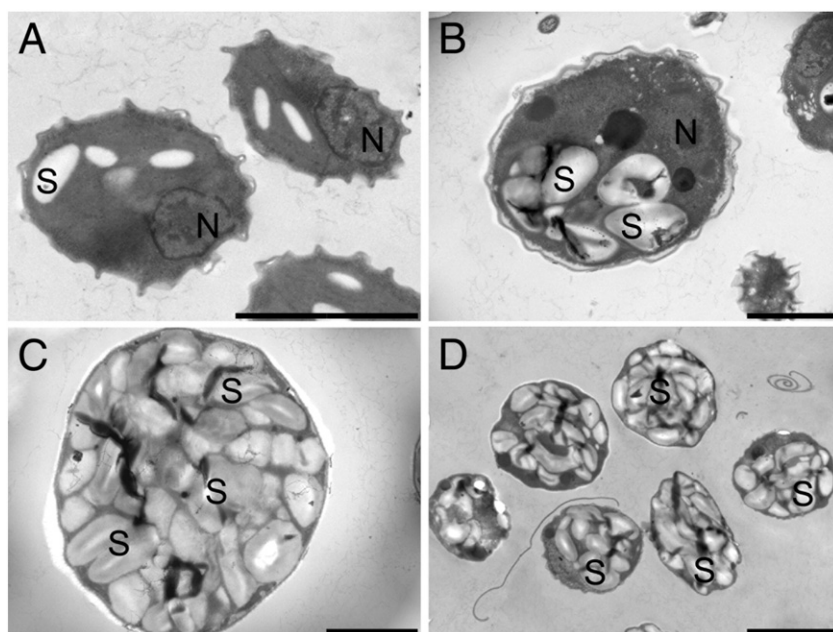


Fig. 2. Electron microscopic photographs of daughter (A) and mother (B) cells of *Chlorella vulgaris* grown in complete mineral medium, in the presence of cycloheximide (1 mg L^{-1}) (C), and in sulfur limiting medium (D). N, nucleus; S, starch granules. Bar panels A–C: $2 \mu\text{m}$; bar panel D: $5 \mu\text{m}$ (Brányiková et al., 2011).

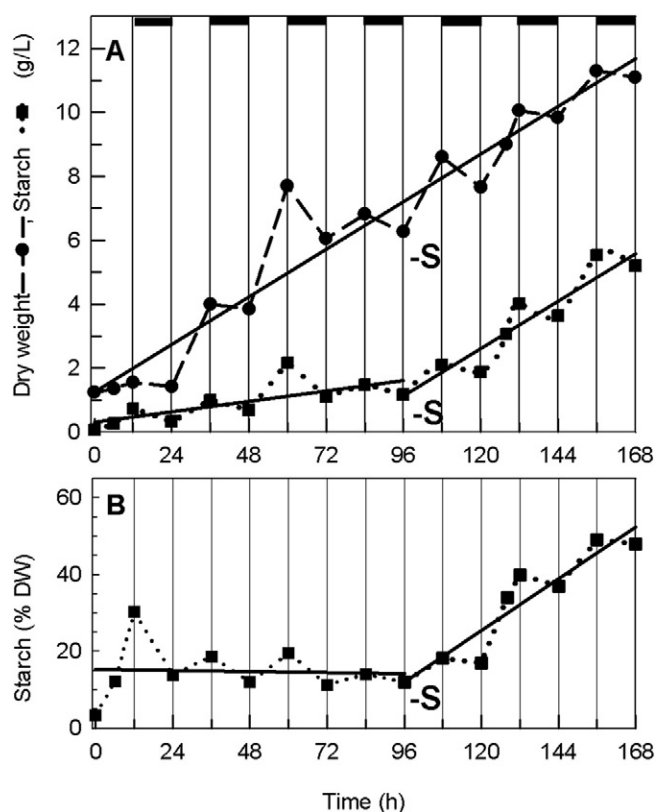


Fig. 3. Changes in biomass concentration (DW in g L^{-1}), starch content (g L^{-1}) (A), and relative starch content (% of DW) (B) in cultures of *Chlorella vulgaris*. The cultures were grown in an outdoor scaled-up thin-layer photobioreactor in complete mineral medium for 96 h and then in sulfur limited medium (-S). Dark periods (nights) are marked by black stripes and separated by vertical solid lines. Linear regression curves of the 1st order (solid lines) were fitted to the experimental data separately for the periods of culture grown in complete medium and sulfur-limited cultivation (Brányiková et al., 2011).

(*Chlorella viscosa*, *C. vulgaris*, *Chlorella sorokiniana*, *C. emersonii*, *Parachlorella beijeirinkii*, *P. kessleri* CCALA255, NIES-2159, and NIES-2152), increased starch and lipid productivity under high light intensity ($600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) (Takeshita et al., 2014). For *Scenedesmus obliquus*, the maximal yield of reserve lipids was observed at pH 7, 27.5°C and a high incident light intensity of $200 \mu\text{mol photons s}^{-1} \text{m}^{-2}$. Suboptimal pH and temperatures reduced both lipid content and yield. A further increase in light intensity resulted in the same TAG content but lower yields (Breuer et al., 2013). The positive effect of increasing light intensity on the accumulation of starch and lipids functions only up to a point, usually equal to saturation of photosynthesis under the given conditions in a particular algal species. At very high light intensities above saturation level, photoinhibition occurs, leading to the cessation of starch synthesis (Ho et al., 2011; Yao et al., 2012) and lower TAG yields (Breuer et al., 2013). Preventing photoinhibition by the accumulation of lipid bodies associated with carotenoids is described by Solovchenko (2012); see also Subsection 6.3.2 (Function of lipid bodies). At very low light intensities below the compensation point or in darkness, both starch and lipid syntheses are stopped and reserves are rapidly degraded.

The effect of light intensity is also strongly dependent on the phase of the cell cycle. It was shown in *C. ellipsoidea* (Hirokawa et al., 1982), *C. vulgaris* (Brányiková et al., 2011), and the marine green alga *Tetraselmis subcordiformis* (Yao et al., 2012) that at the end of the cell cycle, starch content decreased rapidly, independent of the light. In *C. vulgaris*, a maximum starch content of 46% of DW was attained just prior to the first cell division and decreased during cell division to 13% of DW, even in cells grown in the light (Brányiková et al., 2011). Besides light intensity, the illumination regime also plays a significant role in regulating starch and lipid accumulation, as previously mentioned. In *C. vulgaris*, the daily net

starch content was degraded during the dark period, and starch levels oscillated during light and dark periods between 10% and 20% of DW (Brányiková et al., 2011). The degradation of starch during the dark can be prevented by using starvation treatments targeting nutrients such as sulfur, that inhibit division processes (Brányiková et al., 2011).

Unfortunately, the light intensity data often cited in the literature mostly represents incident light intensities at the surface of bioreactors, whereas the actual, often variable, absorbed mean light intensity (the difference between incident and transmitted light intensities) is not considered. The two values are close at a low cell concentration, where only slight shadowing by cells occurs. In contrast, they differ significantly, particularly in batch cultures, with increasing cell density. Therefore, the mean light intensity decreases markedly despite the fact that incident light intensity remains constant. For example, in a given batch culture of *C. vulgaris* illuminated by a constant incident light intensity of $780 \mu\text{mol photons s}^{-1} \text{m}^{-2}$, the mean light intensity of $410 \mu\text{mol photons s}^{-1} \text{m}^{-2}$ decreased to $170 \mu\text{mol photons s}^{-1} \text{m}^{-2}$ at the end of the second cell cycle. This was concomitant with a gradual decrease in the content of accumulated starch caused by the decreasing mean light intensity as culture density increased (Brányiková et al., 2011).

4.2. Temperature

Similar to the effect of light intensity, the rates of biomass growth as well as lipid and starch accumulation increased to a certain extent with increasing temperature. The optimal temperature at which the highest biomass concentration and the highest biomass productivity are attained, differs among species. The alga *Nannochloropsis oculata* (Eustigmatophyceae) and *C. vulgaris* (Chlorophyceae) produced maximum lipid yields at 25°C . Decreasing the temperature caused a decrease in lipid content (Converti et al., 2009). The optimal temperature for lipid production in *Scenedesmus* sp. (Chlorophyta) was 20°C (Li et al., 2011). In *S. obliquus*, the final lipid store content varied between 18% and 40% of DW at temperatures between 20°C and 27.5°C . In general, stored starch and lipids are synthesized at all temperatures in the suboptimal range, but the final lipid or starch content is highest at the optimal temperatures favored for biomass growth (Breuer et al., 2013; Gigova et al., 2012; Rolea et al., 2013).

However, there are some apparent exceptions to this rule. Increasing temperature to a sub-lethal value (38°C) caused an increase in lipid content (up to 34% DW) in *N. oculata* and *C. vulgaris* (Converti et al., 2009). It should be kept in mind that there are multiple parameters affecting growth, and the cells will respond primarily to the most limiting or even stressful parameter. Sub-lethal temperatures and macroelement starvation not only affect cell growth (Liu et al., 2012) but also block division (see Section 6 Approaches to increase accumulation of energy reserves). Therefore, the cell response is no longer responding simply to light intensity or temperature. Under stress conditions, the production of energy reserves operate as an energy sink (safety valve) against over-excitation when the energy cannot be used for protein whose synthesis is limited by a lack of nitrogen, phosphorus, or sulfur (Solovchenko, 2012).

4.3. Carbon dioxide

In phototrophically grown algae, carbon dioxide ensures the supply of photosynthetic carbon for cell growth. Optimal growth of cultures requires sufficient dissolved CO_2 , which should be orders of magnitude higher than in air. The increased CO_2 requirements are commonly ensured using an aeration gas mixture with a high content of CO_2 (usually about 2% (v/v) CO_2 is applied). Aeration with pure air caused a decrease in growth of *P. kessleri* (Li et al., 2013). Interestingly, even when growth rate was decreased by other factors, such as under conditions of nutrient limitation by depleting mineral medium, rates of lipid accumulation were decreased by CO_2 limitation (Li et al., 2013) suggesting that the amount of carbon dioxide in air is not sufficient even under

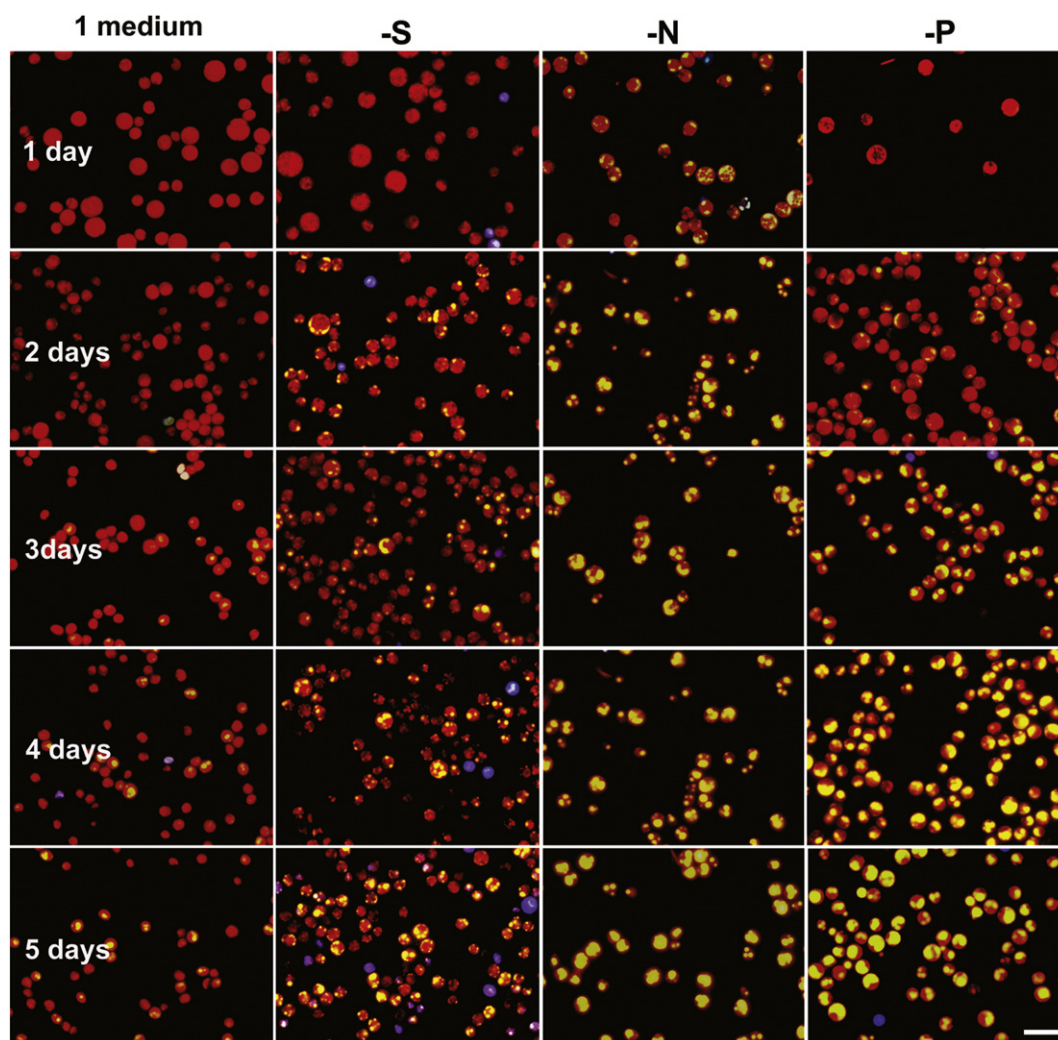


Fig. 4. Time course of lipid accumulation during batch cultivation of *Parachlorella kessleri*. Lipid bodies were stained using Nile Red (yellow); autofluorescence of chloroplasts is seen in red. Cultures were grown in complete mineral medium (1 medium), or in either nitrogen (–N), phosphorus (–P) or sulfur (–S) free media. The cultivation time for all variants is indicated in the left column. The same initial biomass concentration (0.3 g L^{-1}) was used for all cultures and no detectable lipid bodies were seen in cells at the beginning (0 h; not shown in figure). Scale bar = $10 \mu\text{m}$ (Li et al., 2013).

such conditions. The concentration of CO_2 in the aeration mixture did not affect the final content of accumulated lipid reserves only when growth ceased completely (Li et al., 2013). Similar results were also found for other organisms and energy reserves. *C. reinhardtii* grew rapidly with 5% CO_2 under nitrogen limitation and quickly accumulated starch, which was followed by accumulation of TAG. The low CO_2 cultures exhibited carbon limitation and slow metabolic growth with minimal TAG or starch accumulation (Gardner et al., 2013).

The starch content of the marine alga, *T. subcordiformis*, was low (17.76%) at low CO_2 supply but increased after supplying higher concentrations of CO_2 (Zheng et al., 2011). The higher concentration of CO_2 also affected the rate of starch hydrolysis, which caused changes in the amylose to amylopectin ratio at higher CO_2 concentrations (Tanadul et al., 2014).

For scale-up production of algal biomass, an increased supply of CO_2 represents an increase in biomass cost. The only way to maintain production costs for algal biomass at an elevated CO_2 concentration is to use cheap or free sources of CO_2 , such as flue gases from biogas plants, incinerators of domestic or other organic waste, lime works, cement works, or power stations (Brennan and Owende, 2010). The production of algal biomass was successfully tested using flue gas generated by burning natural gas (Doucha et al., 2005) derived from the incineration of municipal waste (Douskova et al., 2009); a cogeneration unit provided flue gas by

the burning of biogas (Kaštánek et al., 2010). It is advantageous that microalgae can utilize CO_2 at high concentrations because its content in flue gases is usually very high. Flue gases can contain about 13% CO_2 (Doušková et al., 2010), but *Chlorella* could even grow on biogas from anaerobic digestion, that is, a gas mixture containing methane and about 40% of CO_2 and no oxygen (Doušková et al., 2010). In addition to producing cheaper biomass, utilizing waste CO_2 is an environmentally effective method for the bioremediation of CO_2 (Douskova et al., 2009; Doušková et al., 2010; Kaštánek et al., 2010); for review, see (Brennan and Owende, 2010). The apparent tolerance of algae to high CO_2 is also affected by limited dissolution of CO_2 gas in the medium. Thus, even at a higher CO_2 concentration, only a limited amount of CO_2 is dissolved in medium and available for algal growth (details see below); the rest of the CO_2 could not be utilized and remained in the outgoing gas mixture. This is nicely shown by the effect of different CO_2 concentrations (from 5% to 50% (v/v)) on the growth of the green alga, *Chlorococcum littorale*, where the highest lipid content under nitrogen starvation was found at a concentration of 5% CO_2 (Ota et al., 2009).

In most papers, only concentrations of CO_2 in external sources (aeration mixtures, biogas, or flue gas mixtures) are given, whereas the content of dissolved CO_2 is not known, which is understandable because determining the concentration of dissolved CO_2 acceptable for the growth of algal cells is complicated. Firstly, at a constant partial pressure of carbon

dioxide in the atmosphere, solubility of CO₂ varies markedly depending on the temperature of the algal suspension. Secondly, CO₂ in water can be present in three forms carbonic acid (H₂CO₃) formed by dissolving of CO₂ and the products of its dissociation, bicarbonate HCO₃⁻ and carbonate CO₃⁻ anions. The ratio of the three forms is pH dependent. From these three forms, the bicarbonate anion (HCO₃⁻) is the most important for growth of algae, and whose maximum dissociation is in the pH range from 7 to 9. Thirdly, in the presence of Ca and Mg ions in the nutrient medium, insoluble carbonates can be formed, particularly at a higher pH, and this decreases the level of anions available for growth (Reid et al., 1987).

Because it is so complex to detect dissolved forms of CO₂, researchers chose a more practical empirical approach to determine the concentration of external CO₂ in air mixtures, and use it to prevent growth limitation at a given light and temperature. For example, cultures of *P. kessleri* growing in complete mineral medium required higher CO₂ concentrations because aeration with pure air (0.03% of CO₂) caused both decreased biomass and storage lipid accumulation (Li et al., 2013). The same culture, grown slowly in 10-fold diluted mineral medium, showed no difference if aerated by pure air or air supplemented with 2% of CO₂ (Li et al., 2013), which suggests that simple growth is not the only parameter that should be monitored during optimization.

5. Selection of algal species for overproduction of energy reserves in algal suspension

The selection of appropriate algal species or strains is the first and most important step when aiming for production of starch- or lipid-enriched algal biomass. A high growth rate of algal cultures, particularly for open air bioreactors, is an important selection criterion to prevent contamination by faster growing species. Algal species such as *Chlorella*, *Parachlorella*, and *Chlamydomonas* usually fulfill this condition and have the ability to produce large amounts of starch. Maximum starch production, including highest productivity, is usually tightly coupled with optimal growth conditions (appropriate light intensity, temperature, and CO₂ supply) (see Section 4 External factors). As mentioned above, starch production, which is of interest in any scaled-up industrial process, is measured as net production, that is, the total production of starch minus starch consumed in darkness and that is spent by enormous consuming activities such as nuclear and cellular divisions. To increase net starch production as much as possible, processes and activities consuming energy and carbon from starch must be minimized or stopped completely. Various treatments leading to the overproduction of starch reserves in the form of chloroplastic starch grains (net starch synthesis) are described in Section 6 Approaches to increase accumulation of energy reserves.

The oleaginous algae Eustigmatophyceae (*Nannochloropsis* and *Trachydiscus*) produce lipids exclusively as an energy reserve and therefore must be omitted as starch producers. This also applies, to some extent, to diatoms, in which production of polyglucans is scarce (Xia et al., 2014). Most green algae produce both starch and oil but in different amounts and different ratios (Breuer et al., 2012; Fernandes et al., 2013; Mizuno et al., 2013; Takeshita et al., 2014; Vigeolas et al., 2012). Strains can differ even within a single species. In some strains (e.g., *P. kessleri* and some strains of *Chlorella*), oil production is inducible (Fernandes et al., 2013; Li et al., 2013; Přibyl et al., 2013), whereas in others (e.g., some strains of *Chlorella*, *Tetraselmis*, and *Chlamydomonas*), starch production is inducible. This phenomenon is complex because both types of species or strains, under standard environmental conditions in complete nutrient medium, produce both types of reserves. Starch grains produced in chloroplasts are the primary source of energy, whereas small amounts of lipid occur as lipid bodies in both chloroplasts and the cytoplasm. In all species, starch intensively accumulates shortly after the beginning of nitrogen starvation or other stress treatments, whereas storage lipid accumulation starts within a few hour to days later (Breuer et al.,

2012; Fernandes et al., 2013; Ho et al., 2011; Lacour et al., 2012; Mizuno et al., 2013; Siaut et al., 2011; Takeshita et al., 2014). The particular dynamics of starch and lipid accumulation, and ratios of compounds, depends both on the species and the type of induction. In nitrogen deficient *C. reinhardtii*, starch initially increased, but then declined while lipids started to accumulate later than starch, and continued to high levels (Siaut et al., 2011). In contrast, in oleaginous species like *S. obliquus*, the rate of lipid productions as well as the final lipid content under similar treatment was much higher (Breuer et al., 2012). In *P. kessleri*, where depletion of medium was used as a stress treatment, starch accumulated rapidly within the first day, and then slowly decreased to a relatively low level. Accumulation of lipids started 1 or 2 days later but was slower, and the final lipid content was much higher than that of starch (Fernandes et al., 2013). This trend was nicely evidenced by comparison of lipid and starch accumulation in different strains and species of algae under nitrogen (Breuer et al., 2012) or sulfur (Takeshita et al., 2014) starvation. Individual species and strains differed in their maximum content of accumulated starch. After the peak of starch accumulation (Breuer et al., 2012; Takeshita et al., 2014), starch storage tended to decrease in most strains, with one exception, *Dunaliella tertiolecta*. Lipid accumulation started later than starch but increased rapidly during the period when starch was continuously degraded (Breuer et al., 2012; Takeshita et al., 2014). All the above mentioned studies provide evidence that the duration of starvation is decisive in determining which type and amount of energy storage will be synthesized, suggesting that the duration of starvation could be an important parameter to manipulate the accumulation of the two energy-rich components, lipids and carbohydrates. For industrial applications, it would be ideal to achieve higher productivity and a higher relative content of reserves. However, optimizing both parameters is difficult because nitrogen limitation is required to trigger the accumulation of both starch and lipids (Ho et al., 2011).

Considering the types of reserves and their practical utilization for the production of bioethanol (from starch) or biodiesel (from lipids), green algae *P. kessleri* would be a promising microalgal strain to produce lipids for making biodiesel (Li et al., 2013), and *C. vulgaris* (strain CCALA 924) would be appropriate as a feedstock for the production of bioethanol (Brányiková et al., 2011). Due to the high accumulation of starch, as well as lipids, under nitrogen starvation of *S. obliquus*, this strain would be suitable both for the production of bioethanol and biodiesel (Breuer et al., 2013; Breuer et al., 2014; Ho et al., 2010; Ho et al., 2011; Vigeolas et al., 2012). Production can be further increased by raising starchless mutants that produce less starch and have improved lipid productivity because they direct most of their energy towards lipids while growth is not affected (Breuer et al., 2014; de Jaeger et al., 2014). This approach was originally used in *C. reinhardtii* (Li et al., 2010a; Li et al., 2010b; Wang et al., 2009; Work et al., 2010), where it led to at least a 10-fold increase in storage lipids (Li et al., 2010a; Li et al., 2010b; Wang et al., 2009; Work et al., 2010). It was suggested that overproduction of neutral lipid by preventing starch synthesis is more effective than genetic engineering of the lipid biosynthetic pathway, which has only achieved limited success (Li et al., 2010a). Importantly, productivity can be further increased by nitrogen starvation in mixotrophic conditions (in the presence of 0.2% acetate) (Wang et al., 2009) and can be successfully used in other species, *C. sorokiniana*, for example (Vigeolas et al., 2012).

In the starchless mutant *S. obliquus*, both reserve lipids and starch accumulated markedly (45% and 38% DW, respectively). Under nitrogen starvation, lipid content reached 49.4% of DW without any decrease in production (de Jaeger et al., 2014).

In addition to the green algae (*Chlorophyta*), representatives of taxonomically different algal species were also found to be candidates for industrial utilization as carbohydrate or lipid producers. The alga *Isochrysis* sp. (*Chromophyta*, *Haptophyceae*) overproduced both carbohydrate and lipids under nitrogen starvation but in a different way. Carbohydrates increased and remained stable during starvation, while

neutral lipids also accumulated but their content varied (Lacour et al., 2012). The lipid accumulation in the marine red alga *T. crinitus* (Rhodophyta) increased markedly in algae grown at a high light intensity (Khotimchenko and Yakovleva, 2005).

6. Approaches to increase accumulation of energy reserves

6.1. Applying specific inhibitors

6.1.1. Inhibition of nuclear DNA replication

Specific inhibition of nuclear DNA synthesis by fluorodeoxyuridine (FdUrd) was used to study the regulation of cell cycle events and starch reserve synthesis in the chlorococcal alga *S. quadricauda* (Zachleder, 1994; Zachleder, 1995). FdUrd inhibits the enzyme thymidylate synthase, which catalyzes the reductive methylation of 2'-deoxyuridylate to form deoxythymidylate (Bachmann et al., 1983; Cisneros et al., 1993; Follmann, 1983).

Inhibition of nuclear DNA (nuc-DNA) synthesis prevented nuclear division and cytokinesis (Zachleder et al., 1996), and in a synchronized culture, the daughter cells remained uninuclear with their initial nuc-DNA content and did not divide but continued to grow to a giant size and replicate chloroplast DNA for an interval corresponding to two or three unperturbed cell cycles and continuously accumulated starch.

6.1.2. Inhibition of cytoplasmic proteosynthesis

The antibiotic cycloheximide, a specific inhibitor of cytoplasmic proteosynthesis, prevented cultures of *S. quadricauda* (Zachleder et al., 2002) and *C. vulgaris* (Brányiková et al., 2011) from undergoing nuclear division and cell division. Starch, however, was intensively synthesized and in the case of *C. vulgaris*, reached about 60% of DW (Fig. 2) (Brányiková et al., 2011), implying that starch synthesis in the chloroplast is independent of cytoplasmic protein synthesis and nuclear DNA replication.

On the one hand, application of specific inhibitors is advantageous for basic research because the mechanisms by which they affect cellular processes are known. On the other hand, their application in the industrial production of starch-enriched biomass is unacceptable because of their toxicity.

Therefore, for the prospective industrial scale-up of starch production, economically viable treatments that do not cause environmental contamination, but do enhance starch or lipid enriched biomass, must be chosen. Such treatments not only include the already widely used nutrient starvation discussed below but also may involve protocols with optimized combinations of light, carbon dioxide and temperature, or sub-lethal temperature as discussed above. Regardless of treatment or approaches, cellular processes and events consuming energy reserves must be minimized, while chloroplast processes such as photosynthesis and starch or lipid synthesis must be preserved. DNA replication, nuclear division, and cytokinesis belong to the main processes or events consuming most of the energy reserves (even under conditions favoring concomitant production of energy by photosynthesis). Indeed all treatments that in some way inhibit these processes led to the accumulation of reserves (mostly in the form of starch). Examples include the effects of FdUrd, cycloheximide, sub-lethal temperature or high light intensities described in sections above, the effects of benzopyrene (Abarzua et al., 1985), and high concentration of selenium compounds (Geoffroy et al., 2007; Vítová et al., 2011a).

6.2. Nutrient starvation

Macroelement (nitrogen, sulfur, or phosphorus) limitation is the most widely explored and so far the most successful strategy for enhancing starch accumulation (Ball et al., 1990; Brányiková et al., 2011; Dragone et al., 2011) as well as for accumulating lipid reserves (Breuer et al., 2013; Li et al., 2013; Wu et al., 2013) and it is a viable

and environmentally friendly option for the control of the cell cycle as a crucial condition for this enhancement.

6.2.1. Nitrogen

The intracellular partitioning of newly fixed carbon was studied using enzymatic and isotopic techniques in synchronized cells of *C. reinhardtii*. Starch and growth metabolism, that is, the use of carbon in biosynthesis, were found to be the major sinks for photosynthetically fixed carbon in the alga. Sucrose does not accumulate in significant quantities. The amount of carbon partitioned into either starch or growth varies during the 12-hour-light/12-hour-dark cell cycle. As we discussed above, starch is accumulated at the beginning and at the end of the light period, whereas a net breakdown is observed in the middle of the light period and in the dark. In contrast, non-synchronized cells accumulated starch all the time in the light, which suggested that carbon partitioning was controlled by the cell cycle. Accumulation of starch in the absence of nitrogen was observed in two consecutive cell cycles in synchronized cultures of *S. quadricauda* (Ballin et al., 1988), where the absence of nitrate (KNO₃) prevented rRNA synthesis from the very beginning of the cell cycle and caused a decline to half of the initial content. Protein content slightly increased and then stopped. No DNA replication, and consequently, no nuclear or cellular division occurred. In contrast, starch accumulation proceeded at a rate similar to that in the control culture during the first half of the cycle but later slowed, attaining about half the amount per cell, characteristic of mother cells in the unlimited culture; no further synthesis occurred during prolonged incubation. Nevertheless, the relative starch content was very high (the ratio of starch to protein was more than four times higher than in normal cells) (Ballin et al., 1988). This report is, to our knowledge, the first one comprehensively studying the relationship between starch synthesis in nutrient limitation and cell cycle progression. Nutrient (sulfur) limitation was studied in synchronized algae *Chlorella elipsoidea* more than 50 years ago, but synthesis of energy reserves was not studied (Hase et al., 1958; Hase et al., 1959; Hase et al., 1960a; Hase et al., 1961). Starch synthesis during the cell cycle was also studied in synchronized *Chlamydomonas* (Duynstee and Schmidt, 1967; Klein, 1987) but not under nitrogen-limited conditions. In the first report, over-accumulation of energy reserves, both polysaccharides and lipids, under nitrogen starvation were described in two different species of the genus *Chlorella*. One of them, *Chlorella* sp. K, overproduced reserves in the form of polysaccharides (they called it the "carbohydrates strain"), while the second one, *C. pyrenoidosa*, produced energy reserves in the form of lipids (the "lipid strain") (Semenenko et al., 1967; Semenenko et al., 1969; Zukova et al., 1969). Increased accumulation of starch (from 20% to 55% of DW) was also observed in a batch culture of *C. vulgaris* grown photosynthetically under nitrogen limitation (Behrens et al., 1989). Later, the overproduction of starch under nitrogen starvation (10- to 50-fold increase in cellular starch content) was observed in *C. reinhardtii* (Ball, 1998; Ball et al., 1990). All these studies on the effect of nitrogen starvation on the accumulation of starch reserves were, at the time of publication, solely basic research without any vision with regard to biotechnological application. Today, however, this potential can be exploited.

The recent revival of research in directed accumulation of algal starch has been due to the assumption that algae could synthesize starch for the production of bioethanol for biofuels (for review, see John et al., 2011)). The experimental approaches described for *S. quadricauda* more than 25 years ago (Ballin et al., 1988; Šetlík et al., 1988; Zachleder et al., 1988) were recently applied to obtain starch-enriched cultures of the industrially promising species *C. vulgaris* (Brányiková et al., 2011) (Figs. 2 and 3). Some experimental approaches from this work were then successfully applied to the study of starch overproduction in the marine alga *T. subcordiformis* (Yao et al., 2012). The authors proved that under nitrogen and sulfur starvation conditions, concomitantly with starch accumulation, changes in the ratio of amylose and amylopectin occurred. Nitrogen starvation was also applied in *S. obliquus* to trigger the accumulation of both lipid and carbohydrate (Ho et al., 2011).

Nitrogen starvation for increased starch accumulation is not, however, used as frequently as the directed overproduction of lipids. The main reason is that the absence of nitrogen causes severe stress, leading to a nearly immediate cessation of biomass synthesis and, after a relatively short time, cell death (Ballin et al., 1988; Brányiková et al., 2011). Cell lysis due to nitrogen limitation (Eriksen et al., 2007) can lead to a decline in starch to half of its initial value (Brányiková et al., 2011), suggesting that nitrogen starvation is not the optimal approach for the production of starch-enriched algal biomass for industrial applications. Moreover, the time after which total (g L^{-1}) as well as relative (% of DW) starch content began to decrease is short, particularly if the time needed to yield such a culture in a large-scale industrial photobioreactor is considered (Brányiková et al., 2011) (Fig. 3). Some authors tried to solve the problem by decreasing the concentration of nitrogen in mineral medium instead of a total absence of the element (Yao et al., 2012). By this approach, they succeeded in increasing the maximum biomass and starch concentrations, but maximum starch productivity ($\text{g L}^{-1} \text{ day}^{-1}$) and relative content of starch (% of DW) decreased. Therefore, for potential scaled-up applications, it would be important to find a compromise between maximum starch content (g L^{-1}) and maximum starch relative content (% of DW) (Yao et al., 2012); for example, it would be crucial to find the optimal harvesting time so that the biomass yield is achieved before a decline in starch content due to cell lysis. However, we believe that nitrogen starvation is by no means the optimal approach to induce starch overproduction, and sulfur starvation should be used in this case (see below).

While nitrogen starvation was not found to be the best way to achieve increased starch synthesis, it did effectively increase the accumulation of storage lipids. The first report on the effect of nitrogen starvation on *C. pyrenoidosa* was published as early as 1949 (Spoehr and Milner, 1949) and showed that lipids increased from 5% DW to 85% of DW in nitrogen starved cells. A 75% decrease in nitrogen concentration in the medium, with respect to the optimal values for growth, increased the lipid fractions of *N. oculata* from 7.90% to 15.31% and of *C. vulgaris* from 5.90% to 16.41%, respectively (Converti et al., 2009). Nitrogen deficiency is now the most efficient and frequently used approach to increase lipid content in various, often taxonomically different, algae, such as in Chlorophyta: *P. kessleri* (Li et al., 2013), *S. quadricauda* (Zhao et al., 2012), *Micractinium pusillum* (Li et al., 2012), diatoms (Heterokontophyta, class Bacillariophyceae): *Nitzschia closterium* f. *minutissima* (Liu et al., 2012), *Skeletonema marinoi* (Bertozzini et al., 2013), Heterokontophyta, class Eustigmatophyceae: *Nannochloropsis* sp. (Pal et al., 2011; Rodolfi et al., 2009).

Of the nine species tested for lipid production under nitrogen starvation, four of them (*C. vulgaris*, *Chlorella zofingiensis*, *Neochloris oleoabundans*, and *S. obliquus*) attained a lipid content of more than 35% of their dry weight. In two strains (*S. obliquus* and *C. zofingiensis*), the duration of biomass productivity remained high for several days in the absence of nitrogen and showed the highest reserve lipid productivity (322 and $243 \text{ mg l}^{-1} \text{ day}^{-1}$ (Breuer et al., 2012)).

For biofuel production from algae, neutral lipids (triacylglycerols) produced under nitrogen deficiency are the most convenient. Generally, microalgae used for lipid production can generally be divided into two categories: (1) high lipid content but low cell growth; (2) high cell growth but low lipid content. Much of the early research on oil production from photosynthetic microalgae focused on the search for species with a high oil content. However, the species with the highest oil content grew most slowly, resulting in low rates of oil production (Lv et al., 2010). The main weakness of the nitrogen starvation treatment is that growth rate is greatly decreased (Bertozzini et al., 2013; Hsieh and Wu, 2009; Lv et al., 2010; Pruvost et al., 2011; Rodolfi et al., 2009).

To overcome this bottleneck, a two-step procedure for lipid production was developed. In the first step, optimal growth conditions for production of biomass are maintained (complete mineral medium, light, temperature, and CO_2). Then the culture conditions are changed to introduce sufficient stress to induce over-accumulation of lipids. This

approach was tested in two strains of *Chlorella* sp. and one strain of *Scenedesmus*, using different sources of nitrogen (NaNO_3 , KNO_3 and urea) for growth and their limitation for lipid production (Ratha et al., 2013). For *C. vulgaris*, nutrient rich conditions, followed by nitrogen starvation, induced overproduction of lipids to 53% of DW (Mujtaba et al., 2012), which was also similar to the effect of the procedure on increased lipid production in *N. oleoabundans* (Klok et al., 2013) and in *P. kessleri* growing in complete mineral medium of different concentrations followed by starvation for nitrogen, phosphorus or sulfur, or a combination of all nutrients in depleted diluted media (Li et al., 2013) (Fig. 4). The cost of the procedure could be further decreased by omitting CO_2 supply in the depletion phase when biomass increase ceased (Li et al., 2013).

In spite of the overwhelming body of evidence on the induction of over-accumulation of storage lipids by nitrogen limitation, there are several reports showing that lipids can accumulate just after repletion of a high concentration of nitrogen but not under nitrogen starvation conditions. This was the case in the marine oleaginous microalgae *Isochrysis zhangjiangensis* (Chrysophyta), which accumulated lipids to 53% of DW at a high concentration of nitrate (9 g L^{-1}); such a strain, grown in nitrogen rich wastewaters, could be ideal for the production of lipids for biodiesel (Feng et al., 2011). Overproduction of store lipids without nutrient limitation was also found in two mutated strains of *P. kessleri* produced by heavy-ion mutagenesis (Ota et al., 2013). A mutant of *Desmodesmus* sp. produced by the same method accumulated much more lipid than wild type, but for lipid accumulation, nitrogen limitation was required (Hu et al., 2013).

6.2.2. Phosphorus

Phosphorus starvation can induce starch or lipid accumulation in a fashion similar to limitations of other macroelements, depending on the specific species or strain, and in some cases, on the length of the starvation period. In oleaginous algae, only lipids accumulate. In *Nannochloropsis* under phosphorus starvation conditions, lipids increased to 50% DW (Rodolfi et al., 2009).

A study of the effect of phosphorus deficiency on cell cycle progression in a synchronized culture of *C. reinhardtii* showed that during the first cell cycle RNA synthesis was already inhibited, and protein synthesis slowed down, leading to a reduced number of DNA replication rounds, nuclear divisions, and released daughter cells (Lien and Knutsen, 1973). Similar effects of phosphorus deficiency were observed during the first cell cycle of synchronized *S. quadricauda*. During the second cell cycle, practically all macromolecular syntheses (DNA, RNA, protein), except starch, ceased and no nuclear or cellular division occurred (Zachleder et al., 1988). Phosphorus limitation in *C. vulgaris* slowed dry weight increases and stopped cell division completely after 12 h of growth. The relative content of starch in phosphorus-starved cells (in % of DW) was lower than in sulfur-starved *C. vulgaris* cells (Brányiková et al., 2011), similar to the marine alga *T. subcordiformis* (Yao et al., 2012; Yao et al., 2013).

Several species (*Scenedesmus* sp., *C. ellipsoidea*, *C. vulgaris*, *C. sorokiniana*, *C. pyrenoidosa*, *Dunaliella primolecta*, and *Haematococcus pluvialis*) accumulated lipids instead of starch under conditions of phosphorus starvation (Wu et al., 2013). *Scenedesmus* sp. was shown to be particularly suitable for scaled-up production of biofuels because the highest potential biomass yield of 6100 kg biomass per kg P and lipid accumulation to about 30% of biomass DW were obtained with the lowest phosphorus content (Wu et al., 2013). Elimination of phosphorus also induced lipid accumulation in *P. kessleri*, and the culture accumulated lipid to nearly the same extent as under nitrogen limitation (Li et al., 2013) (Fig. 4).

6.2.3. Sulfur

Comparison of six oleaginous algal species (*Thalassiosira pseudonana*, *O. aurita*, *N. oculata*, *Isochrysis galbana*, *Chromulina ochromonoides*, and *D. tertiolecta*) grown at 10°C and 20°C in complete and sulfur-depleted

mineral media showed that growth rate and fatty acid content were negatively correlated, suggesting that nutrient limitation was the most important factor affecting lipid metabolism (Breuer et al., 2013).

A specific role for sulfur in cell reproductive processes has been proposed by several authors, who found that cellular division in *C. ellipsoidea* is blocked in the absence of sulfur (Hase et al., 1958; Hase et al., 1959; Hase et al., 1960a; Hase et al., 1960b; Hase et al., 1960c; Tamiya, 1964). In spite of the fact that these authors did not follow starch or lipid synthesis in their experiment, it was later repeatedly proven that it was particularly the inhibition of DNA replication-division events that caused overproduction of energy reserves under nutrient limitation. Specific analysis of the effect of sulfur absence on the time course of macromolecular syntheses, and nuclear and cellular division events, for two cell cycles in synchronized cultures of *S. quadricauda* showed that macromolecular syntheses (RNA, protein) were arrested during the second cell cycle concomitantly with blocked nuclear and cellular division (Šetlík et al., 1988). However, photosynthesis continued and starch accumulated as it did in cells grown in complete medium. Because normal starch synthesis was accompanied by very poor cell growth, the ratio of starch to protein (or dry matter) was much higher than in the control culture, as was later confirmed in *C. vulgaris* (Brányiková et al., 2011) (Figs. 2 and 3). Due to the presence of reserves, starved cells that were refed with sulfur commenced RNA, protein, and DNA synthesis immediately, and were able to pass the entire cell cycle in the dark while dividing into eight daughter cells (Šetlík et al., 1988). This not only suggests the importance of sulfur for cell cycle progression but also indicates yet another way that starch reserves are important for cell cycle events as well as for the entire metabolism.

Of all the nutrient (nitrogen, phosphorus) limiting treatments, sulfur starvation yields a maximum relative starch content up to 60% of DW. Moreover, the high starch content was maintained for a long period (Brányiková et al., 2011; Šetlík et al., 1988), which suggests that sulfur limitation was the most effective method of enhancing starch productivity and content. A similar enhancing effect of sulfur deprivation on starch synthesis was described in *C. reinhardtii*, where a nearly 10-fold increase in cellular starch content occurred (Zhang et al., 2002) and in *Dunaliella salina* where a 10-fold increase in starch-to-protein ratios was induced under sulfur-deprived conditions (Cao et al., 2001). Regarding differences between sulfur and nitrogen or phosphorus starvation effects, it is apparent that the amount of sulfur required for cell growth and reproduction is not so high, and its limitation does not have such severe consequences as in the case of phosphorus and particularly nitrogen limitation. This is also supported by the fact that under sulfur deprivation, starch content did not decrease for at least 15 h (Brányiková et al., 2011), which is crucial for scaled-up culturing for industrial production of starch-enriched algal biomass, providing sufficient time for high yields of algae in large bioreactors. Large-scale cultivation usually takes place in outdoor solar photobioreactors under an illumination regime of alternating light (day) and dark (night) periods. In *C. vulgaris*, with cultures grown in complete mineral medium under alternating light/dark intervals, decreased biomass concentration during the dark period was caused mainly by extensive degradation of starch (the net production of starch during light was degraded in the dark). The sulfur-starved cultures grew slower than control cultures, but the biomass concentration did not decrease during the dark interval. Sulfur limitation stopped cell division, preventing bulk starch degradation during the dark interval. Consequently, in sulfur-starved cultures, both absolute and relative contents of starch increased markedly (from about 0.02 to 0.7 g L⁻¹ and from 7% to 60% of DW respectively) (Brányiková et al., 2011).

The same approach of sulfur starvation, when applied to algal strains suitable for overproduction of lipids, was found to be less effective when compared to nitrogen starvation conditions (Brányiková et al., 2011; Li et al., 2013); this explains why so few reports deal with the production of lipids under sulfur starvation. However, the clear effect of sulfur limitation on inducing starch and lipid synthesis can be used to study

mutual relationships in those algal strains and species that can accumulate both starch and lipids under starvation conditions (Mizuno et al., 2013). Such studies on different algal species confirm the findings regarding nitrogen limitation, for example, the transient accumulation of starch followed by a steady increase in lipid storage in the form of lipid droplets (Mizuno et al., 2013; Takeshita et al., 2014).

Similar to nitrogen starvation, sulfur deficiency also altered the fatty acid composition in *Chlorella lobophora*, *P. kessleri* (Takeshita et al., 2014), and *C. ellipsoidea* (Otsuka and Morimura, 1966).

6.2.4. Nutrient depletion

Growth of algal cultures in scaled-up bioreactors requires relatively expensive mineral medium containing soluble salts of several macro- and microelements. It can be assumed that removing all elements, rather than individual ones, could induce the same or possibly higher hyper-accumulation of lipids with additional beneficial savings. Indeed, over-accumulation of lipids in *P. kessleri* cultures grown in 5- and 10-fold diluted medium led to a higher accumulation of lipids when compared with nitrogen starvation, as well as with less efficient sulfur or phosphorus starvation (Fernandes et al., 2013; Li et al., 2013). The same effect was confirmed for 10 different strains of *C. sorokiniana*, *C. vulgaris*, and *P. kessleri* (Přibyl et al., 2012) as well as in large-scale, outdoor, open, solar photobioreactors used for commercial purposes (Fernandes et al., 2013; Li et al., 2013). Thus, depletion of elements from mineral medium by growth of algal biomass seems to be the most effective way for achieving maximum lipid over-accumulation and for cost savings. However, it should be kept in mind that an important condition for efficient overproduction of storage lipids by nutrient depletion is sufficient light energy (mean light intensity) at the time of depletion. Therefore, it is necessary to use the two-step process described above: grow cell cultures in an appropriately concentrated nutrient medium to attain a sufficiently low biomass concentration under sufficiently high mean light intensity, as required for successful lipid production, followed by the period of depletion.

6.3. Lipid bodies

6.3.1. Description, localization, appearance, visualization

While starch reserves in green algae are located exclusively in chloroplasts in the form of starch grains, the reserve neutral lipids (triacylglycerols) are stored mostly in the cytoplasm in the form of specialized structures called lipid or oil bodies, or lipid droplets, and only some of them are located in chloroplasts. Lipid bodies are round organelles with a basic function of energy and carbon storage. The neutral lipids, being the main component of lipid bodies, are surrounded by a monolayer of phospholipids and a number of proteins. These proteins carry out distinct functions, making lipid bodies highly active organelles that not only govern the storage and turnover of lipids but also regulate the synthesis and transport of lipids, protein storage and degradation, and interactions with other organelles (Moellering and Benning, 2010; Shibata et al., 2009; Shibata et al., 2010; Yang et al., 2012).

The lipid bodies were found practically in all algae studied up to now, in a wide range of taxonomic groups. For visualization of lipid bodies (even within live algal cells) and the quantification of the content of lipids in them, the fluorescent stain Nile Red (9-diethylamino-5H-benzo (α)phenoxazine-5-one) has been used for more than 20 years (Chen et al., 2009; Cooksey et al., 1987; de la Jara et al., 2003). Lipid bodies of various sizes were described after Nile Red staining of *C. vulgaris* (Přibyl et al., 2012) and *C. reinhardtii* (Siaut et al., 2011; Wang et al., 2009; Work et al., 2010).

More recently (Cooper et al., 2010), the fluorescent stain Bodipy 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4diazas-indacene), has begun to be used for visualization of algal store lipids and their quantification. The effective staining of lipid bodies with Bodipy is possible even in those algae with thick cell walls and, more specifically, bound lipid bodies. Bodipy-stained lipid bodies were visualized in

taxonomically remote algal species, such as dinoflagellate *Prorocentrum* sp., *Ophiocytium maius* (Xanthophyceae) with an elongated cylindroid cell, *Chrysochromulina* sp. (Haptophyceae) with naked cells, and *Mallomonas splendens* (Synurophyceae), an alga covered with silica scales (Cooper et al., 2010). Lipid bodies of various sizes and numbers were described in *C. vulgaris* (Přibyl et al., 2013), *C. reinhardtii* (Siaut et al., 2011; Wang et al., 2009; Work et al., 2010), *P. kessleri* (Li et al., 2013), and in the starchless mutant strain, *C. pyrenoidosa* STL-PI, cultivated under nitrogen limitation conditions (Ramazanov and Ramazanov, 2006). Detailed electron microscopic studies of lipid body ultrastructure were described in *Chlorella* (Přibyl et al., 2013) and *C. reinhardtii* (Goodson et al., 2011).

Detailed analyses of the accumulation of lipid bodies in chloroplasts and the cytoplasm of both wild type and the starchless mutant, *sta6 C. reinhardtii*, revealed that production of lipid bodies under nitrogen starvation was markedly increased in mixotrophic conditions in the presence of exogenous acetate. The lipid bodies filled nearly the entire volume of the cell, and the cells floated, failing to settle with centrifugation. Biochemical pathways and genes involved in the overproduction of lipids were also described (Goodenough et al., 2014; Goodson et al., 2011). A similar increase in the size of lipid bodies and their fusion into one huge lipid body, occupying most of the cell volume, was also observed in *Chlorella* and *Parachlorella*, under nutrient limitation (Přibyl et al., 2012).

6.3.2. Function of lipid bodies

The most important function of lipid bodies is to serve as a source of energy and carbon in case the supply from photosynthesis is not sufficient (e.g., low light intensity or darkness), or during recovery in replenished mineral medium following nutrient starvation (Fernandes et al., 2013). After replenishment of the nutrient medium, the large lipid bodies were fragmented into smaller ones, and the lipid level decreased rapidly to nearly zero; similar to the starch reserves; such mobilization of the cellular lipid store occurred even in darkness (Fernandes et al., 2013; Přibyl et al., 2013), suggesting shared functions between both types of energy reserves.

However, the role of lipid bodies in algae is not limited to their storage function but plays an important role in adaptation to environmental conditions: 1) lipid bodies supply long-chain fatty acids necessary for rearrangement of the photosynthetic apparatus and cellular membranes; 2) production of lipids and their storage in lipid bodies serve as energy sinks, consuming photo-assimilates produced under stress when their production could lead to photo-oxidative damage; and 3) lipid bodies can serve as a depot for lipophilic carotenoids, creating an optical screen, which protects the cell grown at high light intensities against photo-damage. Accumulation of carotenoids, particularly under stress conditions, was observed in *H. pluvialis* (Chlorophyta, Volvocales) (Peled et al., 2012; Wayama et al., 2013), *Dunaliella bardawil* (Rabbani et al., 1998), *D. salina* (Chlorophyta, Volvocales) (Lamers et al., 2012), *Parietochloris incisa* (Chlorophyta, Trebouxiophyceae) (Solovchenko and Merzlyak, 2008), and two species of *Trentepohlia* (Chlorophyta, Trentepohliales) (Mukherjee et al., 2010) (for more detailed review, see Solovchenko (2012)).

7. Conclusion

Microalgae produce two types of carbon-based energy reserves: polyglucans and lipids. The type of polyglucan is group specific, with starch being the staple polyglucan of green algae (Chlorophyta), localized to the chloroplast in the form of starch grains. Lipid composition is, in contrast to polyglucans, variable. Their molecules (triacylglycerols) consist of glycerol esterified with three fatty acids, which can be the same or different, with different lengths and levels of saturation. Their composition varies, depending not only on the species of algae but also the cultivation conditions. Lipids accumulate mainly in the cytoplasm in the form of lipid bodies. The lipid bodies function not only as an energy reserve storage but also as a source of long-chain fatty acids

for the photosynthetic apparatus and cellular membranes, as well as storage of lipophilic carotenoids that serve as efficient photo-damage protectors under high light intensity. Both energy compounds serve primarily as buffers in an extremely variable external energy (e.g. light) and carbon (carbon dioxide) supply. They are used to support both cell growth and division, and also serve as an energy sink, consuming photo-assimilates under strong light or when energy consumption by growth and cell division is blocked by nutrient starvation or other factors.

Most green algae (Chlorophyta) can produce both starch and lipids but overproduction of one or another type is distinct in different species or strains and can be regulated by growth conditions. In most of the “starch strains”, starch production is inducible. However, lipid production is inducible only in some strains (“lipid strains”). By mutating starch metabolism, starch species like *Chlamydomonas* and others can be changed into lipid ones; they will be unable to produce starch and will produce lipids instead. In all species, regardless of whether they are the starch or lipid type, starch is intensively accumulated shortly after the beginning of nutrient starvation or other stress treatments, whereas storage lipid accumulation in lipid strains starts within hours or days later, accompanied by degradation of previously accumulated starch. Clearly, the selection of an appropriate algal strain for starch or lipid overproduction is a key step in the selection of appropriate algal species, particularly in scaled-up photobioreactors for industrial purposes.

For any over-accumulation, it should be kept in mind that the energy compound will be stored only if the content of energy reserves exceeds that required for growth, for example, the net content will increase. To attain the maximal accumulation of reserves, three basic conditions require optimization: 1) light intensity; 2) temperature; and 3) carbon dioxide concentration. This is particularly challenging for large-scale outdoor cultivation, where fulfilling each of these conditions brings additional costs to the production of biomass. The ideal location for large-scale cultivation will be in close vicinity to a source of cheap carbon dioxide, such as flue gas, and in areas with stable, high light intensity and temperatures that only change mildly with the season. Moreover, a source of cheap fertilizers, ideally, mineral rich wastewaters, as well as water for cooling, is also required. The number of locations fulfilling all criteria is very limited; therefore, the choice of a particular location is a trade-off offering the most feasible situation economically.

Even under optimized conditions, when the maximum production of energy reserves is reached, the majority of these reserves will be spent during darkness to support cell survival and especially to sustain cell cycle processes, such as DNA replication, nuclear and cellular division, which are the major energy consumers. Thus, if the cell cycle remains undisturbed, the majority of any accumulated energy reserves will, under natural conditions, be spent during the night. Clearly, to maximize energy over-accumulation in biotechnologically relevant outdoor conditions, two conditions must be fulfilled: 1) optimal growth conditions and 2) limitation of energy-requiring cell cycle events.

Blocking the cell cycle can be achieved under laboratory conditions by the application of specific inhibitors of DNA replication or proteosynthesis. The affected cells grow and produce starch normally. Because the starch is not spent by blocked cell cycle processes, it will over-accumulate. Such treatments are advantageous for basic research because the mechanisms by which they affect cellular processes can be studied, but they are not practical for large-scale cultivation because of cost and toxicity of the inhibitors as well as that of subsequent biomass.

Cell cycle events, particularly DNA replication and nuclear and cellular division, can be easily blocked by macroelement (nitrogen, sulfur, or phosphorus) limitation. This is the most widely explored industrial strategy because it is both economically viable and an environmentally friendly strategy for enhancing starch as well as lipid accumulation. The nutrient limitation clearly cuts the cost of adding the limiting macroelement. A better treatment, compared

with particular macroelement limitation, is achieved by using an appropriately diluted mineral medium, leading to the depletion of all elements by growing algae. Such a treatment is not only simple, particularly in large volumes of algal suspension in mass cultivation, but also the most effective way to achieve maximum lipid over-accumulation and save costs.

The main bottleneck of the nutrient limitation approach is that it not only blocks the processes of the cell cycle but also limits growth, particularly protein synthesis, if prolonged as long as is required for lipid accumulation. To overcome this, a two-step procedure for lipid production can be exploited. In the first step, optimal growth conditions for biomass production are maintained (complete mineral medium, optimal light, temperature, and CO₂). Next, the algal cultures are exposed to nutrient stress to induce over-accumulation of lipids or starch. In large-scale outdoor open solar photobioreactors, the first step includes the choice of an appropriate concentration of mineral medium enabling the cell culture to grow to the desirable biomass concentration at which the nutrients in the medium are depleted. The second step, the over-accumulation of energy reserves, naturally follows once the medium is depleted, thereby saving costs for fertilizer and CO₂.

Microalgae have been praised as potential industrial energy compound producers. Here, we explain the relationship between energy reserve accumulation and cell cycle progression, and that basic research predictions of algal behavior can be used in biotechnological processes, and thus improve them without any further trial and error. We strongly believe that this approach is indispensable to speed up the development of algal-based biotechnology. Thus, we argue that not only product-driven research but also basic research will be of benefit to the final goal of sustainable production.

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