Bioresource Technology 244 (2017) 1281–1293

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

RNA-Seq and metabolic flux analysis of *Tetraselmis* sp. M8 during nitrogen starvation reveals a two-stage lipid accumulation mechanism

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HIGHLIGHTS

- *Tetraselmis* RNA-Seq was performed at early lipid accumulation after N deprivation.
- qRT-PCR analyses revealed distinct phases for lipid biosynthesis and catabolism.
- Gene expression data was combined with metabolic reconstruction modeling.
- *Tetraselmis* shifts from reduced lipid consumption to active lipid production.
- This process appears independent from *DGAT* expression.

ARTICLE INFO

Article history:
Received 30 March 2017
Received in revised form 31 May 2017
Accepted 1 June 2017
Available online 6 June 2017

Keywords:
Beta-oxidation
Metabolic engineering
Microalgae
RNA-Seq
TAG biosynthesis

ABSTRACT

To map out key lipid-related pathways that lead to rapid triacylglyceride accumulation in oleaginous microalgae, RNA-Seq was performed with *Tetraselmis* sp. M8 at 24 h after exhaustion of exogenous nitrogen to reveal molecular changes during early stationary phase. Further gene expression profiling by quantitative real-time PCR at 16–72 h revealed a distinct shift in expression of the fatty acid/triacylglyceride biosynthesis and β-oxidation pathways, when cells transitioned from log-phase into early-stationary and stationary phase. Metabolic reconstruction modeling combined with real-time PCR and RNA-Seq gene expression data indicates that the increased lipid accumulation is a result of a decrease in lipid catabolism during the early-stationary phase combined with increased metabolic fluxes in lipid biosynthesis during the stationary phase. During these two stages, *Tetraselmis* shifts from reduced lipid consumption to active lipid production. This process appears to be independent from *DGAT* expression, a key gene for lipid accumulation in microalgae.

1. Introduction

Currently, microalgae are considered as one of the most promising feedstocks for oil production. Under the appropriate conditions (e.g. nutrient deprivation), oleaginous microalgae can be induced to accumulate neutral lipids or triacylglycerides (Hu et al., 2008), which, among other applications, can be converted into biodiesel via transesterification. Microalgae can produce significantly more lipids than oil palms (Ahmad et al., 2011), and without competing for precious arable land, biodiverse landscapes (e.g. rainforests) or freshwater resources. Despite their potential, the high cost of large-scale production still needs to be reduced in order for microalgal biofuel to achieve full commercialization and wide-scale use. Currently, algal strain development remains one of the most important aspects of microalgae-for-biofuel development. Research efforts are continuously advancing bioprospecting (Nascimento et al., 2013), selective breeding (Zayadan et al., 2014) and genetic engineering (Gimpel et al., 2013) of microalgae in an effort to maximize growth and lipid accumulation of the highest performing strains. Importantly, several lipid induction techniques have been identified in microalgae (Rodolfi et al., 2009; Sharma et al., 2012). Microalgae typically reduce cell divi-

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http://dx.doi.org/10.1016/j.biortech.2017.06.003
0960-8524/

sion during adverse conditions, such as nutrient starvation or UV radiation, but are still able to accumulate starch or lipids during photosynthesis as a survival mechanism (Timmins et al., 2009; Wang et al., 2009; Sharma et al., 2014).

Metabolic engineering via genetic modification or modulation of cultivation techniques provides a promising area for increased lipid accumulation. For example, recently the overexpression of diacylglycerol acyltransferase (DGAT)-encoding genes in *Tetraselmis chui* and *Phaeodactylum tricornutum* has resulted in 2-fold increases in triacylglyceride content (Úbeda-Mínguez et al., 2017; Dinamarca et al., 2017). Metabolic pathway engineering can be greatly assisted by comprehensive genomic, transcriptomic, proteomic and metabolomic knowledge. For example, key lipid-related pathways must be mapped out, and important bottleneck enzymes and their genes identified as targets for manipulation. To that effect, global transcriptional profiling of microalgal cells during lipid accumulation enables the identification of the underlying transcriptional networks. Even without pre-existing reference genomes, comparative transcriptomics analyses have been used in microalgae to successfully identify pathways and observe changes during induced lipid accumulation (Rismani-Yazdi et al., 2011; Radakovits et al., 2012; Sun et al., 2013). In most studies, the focus has been on metabolic pathway reconstruction and gene discovery at a single time-point, usually 48–96 h into starvation phase when lipid accumulation is at its peak (Guarnieri et al., 2011; Rismani-Yazdi et al., 2012; Sun et al., 2013). While this approach successfully allowed for the reconstruction of fatty acid (FA), triacylglyceride (TAG), β-oxidation and other metabolic pathways, the limited scope of these studies restricts our understanding how the expression of these pathways change, particularly during early stationary phase as cells transition from growth phase into starvation phase. The few studies that have monitored the transcriptional profile of microalgae at various growth stages have observed more transcriptional changes during early-stationary phase compared to stationary phase. These changes occur particularly in photosynthesis, carbon and lipid synthesis pathways, and can be linked to physiological changes (e.g. reduced cell division and increased lipid synthesis) observed during that phase (Valenzuela et al., 2012; Lv et al., 2013). The appropriate lipid induction conditions and time point of RNA sampling are crucial in obtaining distinct expression profiles between control and treatments cultures. Nitrogen depletion is a commonly used method to induce lipid accumulation in microalgae (Hu et al., 2008; Rodolfi et al., 2009; Miller et al., 2010).

The flagellate green microalga *Tetraselmis* sp. is widely mentioned in the literature, but little functionally annotated sequence information is available on this genus in public databases, apart from a recent study on temperature tolerance that used *de novo* transcript assembly (Shin et al., 2016). *Tetraselmis* sp. (Prasinophyceae) presents a good model organism, based on its reported ability to accumulate high lipid content as well as its robustness to tolerate a range of environmental conditions (Chini Zitelli et al., 2006; Rodolfi et al., 2009). *Tetraselmis* sp. M8 cells accumulate approximately 20–30% lipids and it has been shown that they lose their flagella during stressful conditions, resulting in rapid settling, a feature that can significantly reduce harvesting/dewatering costs and provide an avenue for commercial production (Lim et al., 2012; Sharma et al., 2014). The growth characteristics of *Tetraselmis* sp. M8 strain and its lipid accumulation capability and composition were previously found suitable, in principle, for biodiesel production under both laboratory and outdoor cultivation conditions (Lim et al., 2012; Sharma et al., 2014; Narala et al., 2016).

Even in the absence of a fully sequenced and annotated genome, transcriptomic analysis by microarrays or RNA-Seq can provide a powerful tool to improve our understanding of the underlying physiological networks that allow microalgae to respond to environmental changes (Rismani-Yazdi et al., 2012; Valenzuela et al., 2012; Sun et al., 2013). In the present study, we gain insights into the lipid accumulation mechanism of the genus *Tetraselmis*, particularly the expression of genes in the FA synthesis, TAG synthesis and β-oxidation oxidation pathways, as cells transition from growth phase into stationary phase. Physiological observations such as growth, lipid accumulation and FA profiles were linked to transcriptional data obtained first by global transcriptomic sequencing, followed by quantitative reverse transcriptase PCR (qRT-PCR) time-course analysis of each of the aforementioned pathways, and metabolic reconstruction modeling.

2. Materials and methods

2.1. Culture growth conditions

To detect changes in lipid-related pathways as cells transition from continuous exponential growth in log phase to stationary phase, it was important that RNA sampling was carried out on concurrently-grown control cultures that were maintained in log phase. Therefore, semi-continuous cultures of *Tetraselmis* sp. M8 were first established to maintain cells under constant nutrient-replete conditions and exponential growth phase before the start of each experiment. This way, cells could be maintained in constant growth phase and cell density by feeding and dilution in a constant cycle until the start of experiment. Three 1 L-master cultures were maintained by replacing half the culture (500 mL each) with autoclaved 25 PSU artificial seawater (Aquasonic) supplemented with F/2 medium (Guillard and Ryther, 1962; enriched with an additional 100 μM of phosphate) every 48 h for 2 weeks. The cultures were grown in 1 L-Schott bottles with constant bubbling at 24 °C under 16:8 light/dark photoperiod of fluorescent white lights (80 μmol photons $m^{-2}s^{-1}$). For RNA-Seq, semi-continuous cultures were maintained with a regime as above. At the start of the experiment, master cultures were mixed and distributed to nine cultures (three cultures per treatment). Nitrogen-deprived and phosphate-deprived cultures had media replaced with nitrogen-deficient or phosphate-deficient F/2 medium to induce lipid production, while control cultures received N/P-replete medium. For the time course experiment, the semi-continuous cultures were maintained by diluting to 5×10^5 cells/mL and feeding with F medium (enriched with an additional 100 μM of phosphate) every 48 h. Full strength F medium was used as larger differences in nutrient levels between treatments and were expected to lead to more pronounced lipid induction. At the start of the experiment the nitrogen-starvation treatment was supplied by replacing with nitrogen deficient F-medium. In both experiments, the nitrogen and phosphate concentration of the cultures were measured daily to ensure nutrient-deplete conditions only occurred at 48 h after feeding (Supplementary Fig. 1). Nitrogen use of the medium is part of algae cultivation and therefore also occurred in the control cultures, but not to a point that cells had run out of nitrogen reserves. The dilution and feeding regime was altered in the time course experiment to reduce the duration in which cultures experienced nutrient-deplete conditions at 48 h before feeding.

2.2. Physiological parameter analysis

During the course of the experiments, various physiological parameters such as cell density, Nile red fluorescence, nitrate and phosphate concentration, chlorophyll *a* and *b*, and fatty acid (FA) content were measured. Total nitrate and phosphate contents in the media were measured as described by Adarme-Vega et al. (2014) using API Aquarium pharmaceutical Nitrate NO^{3-} and Phos-