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IFAC-PapersOnLine 49-26 (2016) 330–335

Metabolic modeling of *C. sorokiniana* diauxic heterotrophic growth

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Abstract: Microalgae are promising microorganisms for the production of numerous molecules of interest, such as pigments, proteins or triglycerides that can be turned into biofuels. Heterotrophic growth on wastes represents an interesting approach to achieve higher biomass concentrations, while reducing cost and improving the environmental footprint. Wastes generally consist of a mixt of diverse molecules. It is crucial to understand microalgal metabolism in such conditions, where switching between substrates might occur. Metabolic modeling has proven to be an efficient tool for understanding metabolism and guiding the optimization of biomass or target molecule production. Here, we focused on the metabolism of *Chlorella sorokiniana* growing heterotrophically on acetate and butyrate. The metabolism was represented by 163 metabolic reactions. The DRUM modeling framework, with a mildly relaxed quasi-steady-state assumption, was used to account for possible intracellular accumulation during switching between substrates. Six experiments were used to calibrate the model and eight experiments for the validation. The model efficiently predicted the experimental data, including the transient behavior. To the best of our knowledge, this is the first study to describe the dynamic metabolic fluxes of microalgae during heterotrophic and diauxic growth. It shows that an accurate model of metabolism can now be constructed, even in dynamic conditions, with the presence of several carbon substrates. It also opens new perspectives for the heterotrophic use of microalgae, especially for biofuel production from wastes.

Keywords: Systems biology, Metabolic modeling, Microalgae, Heterotrophy, Diauxic growth

1. INTRODUCTION

Microalgae are promising microorganisms for the production of numerous molecules of interest in several industrial fields, such as pharmaceutics or cosmetics (antioxidants, pigments, unsaturated long-chain fatty acids), agricultural products (food supplements, functional food, colorants) and animal feed (aquaculture, poultry or pig farming) (Mata et al. 2010). They are also promising organisms for green chemistry (bioplastics), the environment (wastewater treatment, CO2 mitigation), and even energy production (biodiesel, bioethanol, hydrogen) (Mata et al. 2010). Microalgae are unicellular eukaryote microorganisms that can grow autotrophically using light energy and CO_2. Many species can also grow heterotrophically, in the dark, on various organic carbon sources, including glucose (Perez-Garcia et al. 2011).

Autotrophic growth of microalgae is limited by light distribution to all the cells, constraining the cell concentration to below 10 g/l (for the thinnest and most concentrated cultivation systems). Heterotrophic growth does not have this limiting factor and higher biomass density can be achieved (Perez-Garcia et al. 2011), drastically reducing the harvesting costs. In addition, heterotrophic growth is usually faster, reducing the cultivation time (Doucha & Lívanský 2011). However, industrial production of heterotrophic microalgae is hampered by the high economic and environmental costs of glucose, commonly used as a substrate. One solution is to use the waste from other processes, such as glycerol, acetate or butyrate, which represent low cost carbon substrates. For instance, dark anaerobic fermentation produces an effluent mainly composed of acetate and butyrate (Rafrafi et al. 2013). However, some substrates in waste, such as butyrate, can be inhibitory (V. Turon et al. 2015). Moreover, the successive metabolic switches between different substrates are not well understood and are likely to significantly affect growth. Therefore, this bioprocess still needs to be mastered and optimized to produce microalgae and extract the targeted byproducts on an industrial scale and at a competitive price, with consistent quality and in a sustainable way.

In this context, mathematical modeling of the metabolism has proven to be an efficient tool for optimizing growth and increasing the production of target molecules. To date, no models exist for heterotrophic microalgal metabolism dynamically switching between several substrates. So far, only static fluxes have been predicted under constant substrate consumption (Boyle & Morgan 2009; Chang et al. 2011; Dal'Molin et al. 2011; Chapman et al. 2015). Representing the dynamic shifts for a blend of substrates typical of real wastewater is a major challenge, since some intracellular accumulation might occur, either during the transition between substrates, or due to the varying nature of the light. As a consequence, the quasi-steady state assumption (QSSA) required by most of the existing metabolic approaches may be an invalid hypothesis in this case (Baroukh et al. 2014). The DRUM modeling framework recently proposed in (Baroukh et al. 2014) was used here to handle the non quasi-steady state (QSS). It allowed the development of a dynamic metabolic model for *Chlorella*

10.1016/j.ifacol.2016.12.148

sorokiniana grown on a single-substrate culture and a mixed-substrate (acetate and butyrate) culture. The model is thus designed to represent heterotrophic growth mode. To the best of our knowledge, this is the first metabolic modeling of microalgae growing heterotrophically under diauxic conditions.

2. MODEL SET-UP

2.1 Experimental conditions

The goal of the experiments was to grow *Chlorella sorokiniana* on a synthetic medium imitating the digestate composition produced by a dark fermenter processing organic waste. At this stage, the composition of the medium was kept simple, with only the two main organic components – acetate and butyrate (Rafrafi et al. 2013) – to gain a clear understanding of their effects on microalgae growth. *Chlorella sorokiniana* was grown in the dark, in axenic conditions at 25°C and constant pH (6.5) in triplicate batches with different initial concentrations of acetate and butyrate. Nitrogen (ammonium) and phosphorus were provided in non-limiting concentrations in order to focus solely on carbon metabolism. To ensure that no substrate was favored because of acclimation, the inoculum was grown autotrophically beforehand. See Turon et al. (2015) for more details of the experimental protocols.

2.2 Metabolic Network Construction

Since *Chlorella sorokiniana* has not been sequenced yet, no genome-scale metabolic network (GSMN) reconstruction was possible. However, the core carbon and nitrogen metabolic networks in the GSMN of previously reconstructed microalgae species are relatively similar (Baroukh, Muñoz-Tamayo, Steyer, et al. 2015). Thus, the conserved core metabolic network was used, containing the central metabolic pathways relevant to heterotrophy: glycolysis, pentose phosphate pathway, citric acid cycle, oxidative phosphorylation, and synthesis of chlorophyll, carbohydrates, amino acids and nucleotides. Species-specific pathways such as the synthesis of secondary metabolites were not represented, since these pathways were assumed to represent negligible fluxes compared to the main pathways and thus to have little impact on the metabolism. The reactions involved in macromolecule synthesis (proteins, lipids, DNA, RNA and biomass) were lumped into generic reactions. Our purpose is to propose a relatively generic model, instantiated and calibrated for *C. sorokiniana*. According to Baroukh et al. (2015), such a generic model should be applicable to a wide range of microalga species.

2.3 Definition and reduction of the sub-networks

Generally, metabolic modeling relies on the QSSA of the whole metabolic network, where intracellular metabolites cannot accumulate or be depleted (Mahadevan et al., 2002). The idea of the DRUM approach is to mildly relax this hypothesis, by splitting the metabolic network into a limited number of sub-networks (Baroukh et al., 2014), for each of which the QSS is assumed. Some metabolites, when situated at the junction between the sub-networks, can therefore accumulate. The sub-networks are defined by metabolic functions and take into account cellular compartments. Different ways of splitting the network were tested, but the best results (experimental fitting of estimation data) were obtained when the metabolic network was simply split into two sub-networks corresponding to i) the glyoxyzome and ii) biomass synthesis (Figure 1). Apart from cofactors and inorganic compounds, only succinate (SUC) was assumed to be intermediary metabolites (A) that potentially accumulate.

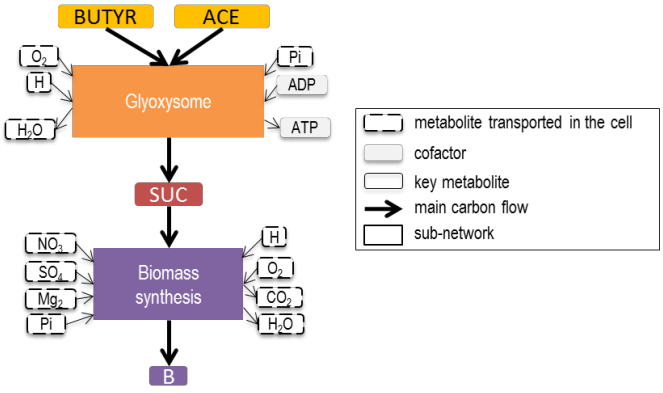

Fig. 1. Central carbon metabolic network of a unicellular heterotrophic microalga decomposed into two sub-networks. In the glyoxysome, fatty acids (including acetate and butyrate) are degraded to Acetyl-CoA, which is then transformed to succinate (SUC) thanks to the glyoxylate cycle. SUC is then used as a primary precursor via the TCA cycle, glycolysis and the pentose phosphate pathway, for protein, DNA, RNA, carbohydrate and lipid synthesis.

Each sub-network was then reduced to macroscopic reactions (MRs) using elementary flux mode analysis (Klamt and Stelling, 2003). To compute elementary flux modes (EFMs), the efmtool program was used (Terzer and Stelling, 2008). For the three sub-networks, the EFM could be computed easily, since their total number was less than 233 (it should be noted that an EFM analysis of the full network results in 984 modes).

2.4 Analysis of the glyoxysome sub-network

Glyoxysomes are specialized peroxisomes found in plants or microalgae (Dal'Molin et al., 2011), in which fatty acids (including acetate and butyrate) can be used as a source of energy and carbon for growth when photosynthesis is not active. Fatty acids are hydrolyzed to acetyl-CoA, and then transformed into succinate via the glyoxylate cycle. Succinate can then be transformed into a variety of macromolecules for biomass growth, through combinations of other metabolic processes taking part in other compartments of the cell.

Reduction of the glyoxysome sub-network yielded two MRs, one for each substrate:

$$3.5\ H + 2\ ACE + 0.5\ Pi + 0.5\ ADP + 0.5\ O_2 \rightarrow 0.5\ ATP + SUC + 0.5\ H_2O \quad (MR1)$$

$$7\ H + 4\ Pi + 4\ ADP + 1.5\ O_2 + 1\ BUTYR \rightarrow 1\ SUC + 4\ ATP + 5\ H_2O \quad (MR2)$$