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Metabolomic profiles of tropical *Chlorella* and *Parachlorella* species in response to physiological changes during exponential and stationary growth phase

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ABSTRACT

Chlorella species are known to be potential algal candidates for biodiesel production due to their ability to store high lipid content and their natural metabolic versatility. An understanding of physiology and metabolic capacity of indigenous *Chlorella* strains is potentially useful for future biodiesel production in the tropical environment. The primary aim of this study was to assess the photosynthetic performance, biochemical content and fatty acid composition of *Chlorella* and *Parachlorella* species grown and harvested during exponential (EX) and stationary (STA) growth phases in batch culture. Physiological data suggested that the cells responded to these conditions by initiating lipid accumulation when growing from EX to STA phase. An increase of lipid and saturated fatty acids (SFA) contents was observed in STA, although this trend was not consistent across the different strains of *Chlorella* and *Parachlorella* species. To gain further insights into metabolomic adaptation at different growth phases, metabolites were extracted from selected *Chlorella* and *Parachlorella* strains at EX and STA phase. These metabolic profiles were analysed resulting in identification of 74 metabolites. Metabolomic profiles of *Parachlorella* showed that there was an increase in recycling of amino acids and nucleic acids at STA phase. The metabolites associated with photosynthesis and chlorophyll biosynthesis were also repressed while carbon sources were channelled into lipid biosynthesis. Meanwhile, *Chlorella* species showed a similar response in carbon allocation for lipid accumulation with lesser influence on amino acid and chlorophyll degradation. Therefore, *Chlorella* and *Parachlorella* species exhibit different changes in metabolic responses at different growth phases, which may be the result of metabolic adaptations arising from their evolutionary plasticity. Overall, our results expand the current understanding of metabolomics of *Chlorella* and *Parachlorella* species and provide valuable insights into their lipid accumulation during different stages, which is important for optimization of lipid productivity for biodiesel production.

1. Introduction

The green algae in the genus *Chlorella* are a large group of eukaryotic, unicellular, and photosynthetic microorganisms that are widely distributed in freshwater and marine environments. *Chlorella* is a photoautotroph and has been used as a model system in the early research on photosynthetic CO_2 fixation. They are also among the very few algal groups capable of using organic carbon for heterotrophic growth, which endows *Chlorella* with the metabolic flexibility to respond to environmental perturbation. Because of its robustness and various metabolic capacities, *Chlorella* has aroused a widespread interest as a potential feedstock for industrial biomass production [1],

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biodiesel [2] and value-added chemicals. *Chlorella* species are among the best oil feedstock microorganisms for the production of biodiesel in the tropical environment [3]. *Chlorella* strains isolated from tropical waters of Malaysia would likely adapt better to local changing environmental conditions and provide a more stable and productive culture with the perspective of outdoor mass cultivation. According to Zhu, et al. [4], carbon overflow (*e.g.* nitrogen depletion or organic carbon feeding) allows *Chlorella* to accumulate a high percentage of neutral lipids that can be processed for biodiesel production.

Chlorella is very efficient in the use of available nutrients. The changes in nutrient availability influence the metabolism and the composition of the cell constituents. Previous reports had used different *Chlorella* species for biodiesel exploration [5]. For instance, the *Chlorella sorokiniana*, grown under the photoautotrophic, mixotrophic and heterotrophic conditions, has been proved to be a potential industrial microalga for biofuel production [6]. This is because *C. sorokiniana* accumulates more neutral lipids or triacylglycerols (TAG) than phospholipids as it reaches stationary (STA). In most of the *Chlorella* species examined, TAGs are composed primarily of C14 – C18 fatty acids that are saturated or monounsaturated [7]. Saturated fatty acids (SFA) are very useful precursors for biodiesel production. In comparison, the polyunsaturated fatty acids (PUFA) have an important role in the function of cells through their important effect on cell membrane fluidity [8].

The total lipid content and fatty acid composition may vary according to species, environmental conditions, nutritional status, as well as growth stages. In some algae, saturated fatty acids (SFA) are accumulated during the stationary (STA) phase suggesting that the storage lipids are differently regulated during different growth phases. Su, et al. [9] showed that there were significant differences in the intercellular lipid metabolites of algae during exponential (EX), early STA and late STA phases. Liang, et al. [10] found that the fatty acid composition of four marine diatom species, *Chaetoceros gracilis*, *Cylindrotheca fusiformis*, *Phaeodactylum tricornutum*, and *Nitzschia closterium*, changed when they were cultivated at the EX growth phase, the early STA phase, or the late STA phase. The controlling factors that enable production of large amounts of storage lipid in the form of SFA in a single, specific cell type are still fragmentary. Compared with transcriptomics and proteomics studies, metabolomics studies provide a systematic analysis of the dynamic responses to stimulation and small molecule metabolite changes in an organism at a specific time and condition [11]. Many previous metabolomics studies of algae were mostly focused on the identification of lipids and their derivatives [11–14] in addition to selected secondary metabolites involved in defence reactions, such as mycosporine like amino acids [15] and isoflavones compounds [16,17]. However, broad large-scale comparative metabolomics studies are lacking for microalgae. This is in contrast to terrestrial plants, where abundant consolidated information on metabolites is available.

The primary aim of this study was to assess the metabolome changes that occur under different growth stages in *Chlorella* and *Parachlorella* species by coupling UPLC/Q-TOF-MS with multivariate statistical analysis to reveal the effect of growth phase (EX and STA) on the FA composition and expression of metabolite levels in *Chlorella* and *Parachlorella* species. The significant metabolites were selected and identified, and their biological functions related to different growth phases were discussed. Using metabolomics techniques, this study provides an overview of the metabolomic basis of the growth phase effect in *Chlorella* and *Parachlorella* species.

2. Materials and methods

2.1. Preparation of inoculum and culture condition

Two strains of *Chlorella* sp. (UMACC001 and UMACC187) and two strains of *Parachlorella* spp. (UMACC253 and UMACC254) (hereafter referred to as *Chlorella* and *Parachlorella*) obtained from the University of Malaya Algae Culture Collection (UMACC) were included in this study [18]. The indigenous microalgae were isolated from different habitats in Malaysia (Table 1). The mixed cultures were originally subjected to purification by serial dilution followed by streaking on agar plates (20 gL^{-1} of Bacto™) to obtain individual colonies. The cultures were then isolated onto the agar plates or slants and kept in a culture chamber (25 ± 1 °C and irradiance of 8 $\mu mol\ m^{-2}\ s^{-1}$). The individual colonies were inoculated into Bold's Basal Medium (BBM) and Provasoli medium buffered with 3 mM HEPES (pH 6.8–8.2). The inorganic nitrogen and phosphate of both media were adjusted to 2.94 mM and 1.72 mM respectively, to standardize the N: P ratio to 2:1, which is recommended for growing and maintaining algal cells in batch cultures [18]. An inoculum size of 10% of total culture volume, standardized at an optical density at 620 nm (OD_{620nm}) of 0.5 (equivalent to 5.05×10^5 cells mL^{-1} number of cells and 0.25 mg L^{-1} of chlorophyll *a*, $r^2 = 0.9$) from EX phase cultures was used. For growth studies, the microalgae were cultured in triplicate flasks with a working volume of 600 mL. The flasks were incubated in a controlled-environment chamber set at 25 ± 1 °C, illuminated with cool white fluorescent lamps (40 $\mu mol\ m^{-2}\ s^{-1}$) on a 12:12 h light-dark cycle and supplied with 100 mL min^{-1} of filtered ambient air. All samples for biochemical and metabolome analysis were taken 6 h after onset of illumination. For the growth phase experiment, algal samples were sampled during EX growth when cell number reached an average of 5.86×10^6 cells mL^{-1} between day 3 to 5 and, during STA phase when cell number exceeded 2.54×10^7 cells mL^{-1} on day 12, using the entire 600 mL culture.

Table 1
List of the microalgae strains used in this study and their origin.

Taxa	Strain	Medium	Origin
Chlorella sp.	[a]UMACC 001	BBM[b]	Pond at Institute of Postgraduate Studies and Research Farm, University of Malaya
Chlorella sp.	UMACC 187	BBM	Tin container, Chinese graveyard, Kuantan Pahang
Parachlorella spp.	UMACC 253	Prov50[c]	Sea Bass Pond at Sepang, Selangor
Parachlorella spp.	UMACC 254	Prov50	Sea Bass Pond at Sepang, Selangor

[a] UMACC: University of Malaya Algae Culture Collection.
[b] BBM: Bold's Basal Medium.
[c] Prov50: Provasoli 50 Medium.

2.2. Photosynthetic performance

A series of photosynthetic activity parameters were determined by a Diving-PAM chlorophyll fluorometer (Walz, Effeltrich, Germany). The parameters were the maximum quantum yield of photosystem II (F_v/F_m), rapid light curve (RLC), maximum potential relative electron transfer rate without photoinhibition ($rETR_{max}$), non-photochemical quenching (NPQ). The Diving-PAM was controlled using Win Control, Walz (Walz). Aliquots of culture (5 mL) was placed in the measuring tube and directly incubated for 15 mins. Then, the light response curves of photosynthesis were generated using the Win Control software v.3.2 by applying 10-s intervals of actinic illumination with 8increasing intensities (0–2000 μmol photons $m^{-2}\ s^{-1}$) of photosynthetic active radiation (PAR). The maximum quantum yield (F_v/F_m) of the photosystem II (PS II), which estimates the physiological stress in microalgae, was derived from:

$$F_v/F_m = F_m - F_0)/F_m$$

where F_0 and F_m are the minimum and maximum fluorescence measured in the dark-adapted sample, respectively.

The relative electron transport rate (rETRmax), which is related to the overall photosynthetic performance of microalgae, was determined by fitting rETR *vs*. Irradiance curves, using the equation by Platt, et al. [19].