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ORIGINAL PAPER

Metabolomics revealed the photosynthetic performance and metabolomic characteristics of *Euglena gracilis* under autotrophic and mixotrophic conditions

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Abstract

Photosynthetic and metabolomic performance of *Euglena gracilis* was examined and compared under autotrophic and mixotrophic conditions. Autotrophic protozoa (AP) obtained greater biomass (about 33% higher) than the mixotrophic protozoa (MP) after 12 days of growth. AP maintained steady photosynthesis, while MP showed a remarkable decrease in photosynthetic efficiency and dropped to an extremely low level at day 12. In MP, low light absorption and photosynthetic electron transport efficiency, and high energy dissipation were reflected by the chlorophyll (chl *a*) fluorescence (OJIP) of the protozoa. The values of Ψ_O, Φ_{Eo}, and ET_O/RC of MP decreased to extremely low levels, to 1/15, 1/46, and 1/9 those of AP, respectively, while DI_O/RC increased to approximately 16 times that of AP. A total of 137 metabolites were showed significant differences between AP and MP. AP accumulated more monosaccharide, lipids, and alkaloids, while MP produced more amino acids, peptides, and long-chain fatty acids including poly-unsaturated fatty acids. The top nine most important enriched pathways obtained from KEGG mapping were related to ABC transporters, biosynthesis of amino acids, purine metabolism, and carbohydrate metabolism. There were significant differences between AP and MP in photosynthetic activity, metabolites, and metabolic pathways. This work presented useful information for the production of high value bioproducts in *E. gracilis* cultured under different nutritional conditions.

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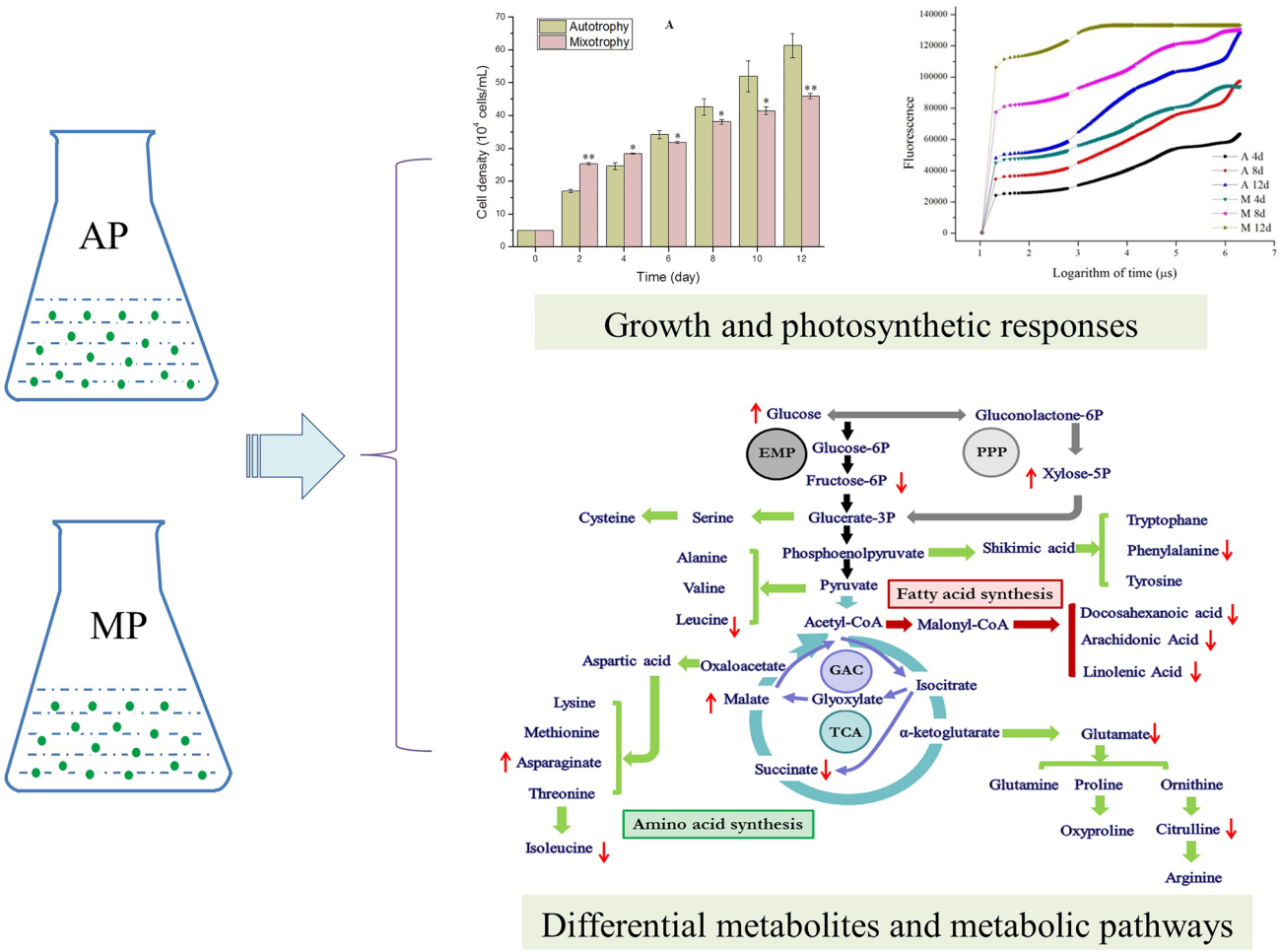
Graphical abstract



Keywords *Euglena gracilis* · KEGG metabolic pathway · Metabolomics · Nutritional conditions · Photosynthesis

Introduction

Euglena gracilis is a microalga-like protozoan that possesses both animal and plant characteristics (Zakryś et al. 2017). It can utilize various organic compounds as its carbon and energy source, and grows well under different nutritional modes, such as autotrophic (using sunlight), heterotrophic (using an external carbon source), and mixotropic (combining both modes) conditions (Kempner 1982; Rodríguez-Zavala et al. 2010). *E. gracilis* has the potential to produce plenty of metabolites, such as polyunsaturated fatty acids (PUFAs) (Barsanti et al. 2000), essential amino acids (Schantz et al. 1975), vitamins (Takeyama et al. 1997), and β-1,3-glucan paramylon (Gissibl et al. 2019), making it one of the few microorganisms that can simultaneously produce high value-added bioproducts as food and nutritional supplements for human consumption (Kottuparambil et al. 2019). Different culture conditions of *E. gracilis* resulted in diverse bioproducts (Gissibl et al. 2019; Schwarzhans et al. 2015; Wang et al. 2018), therefore, the physiological-biochemical characteristics of *E. gracilis* under different culture conditions needed to be understood in order to acquire valuable bioproducts, such as PUFAs, amino acids and paramylon as food supplement.

Under different culture conditions, the *E. gracilis* demonstrated varying degrees of light acquisition and photosynthesis, which was closely related to its metabolic pathway. Matsuda et al. (2011) investigated the effects of light on metabolite compositions of *E. gracilis* and found that light-grown protozoa showed high level of pyruvate and low level of UAP-glucose, glycerate-1,3-bisphosphate, 2-phosphoglycerate, and adenosine triphosphate (ATP) compared with dark-grown protozoa. Zeng et al. (2016) reported that *E. gracilis* cultured under light (mixotrophy) grew fast than those

cultured under dark (heterotrophy) condition. Photosynthesis could still support mixotrophic protozoa growth during periods of nutrient depletion. The authors also found that fatty acid types under different culture conditions showed significant differences. The photosynthetic characteristics of *E. gracilis* had been determined and used in toxicological studies (Ahmed and Häder 2010a; Sun et al. 2021). The relationship between photosynthesis and metabolism of *E. gracilis* is crucial to be better understood.

Metabolomics plays an important role in biology and toxicology, revealing changes in the metabolic state and in elucidating related metabolic pathways and mechanisms of various organisms (Fiehn et al. 2000; Mashego et al. 2007). Metabolomics technology has been employed to explore metabolites from various metabolic pathways of microalgae (Kato et al. 2021; Mishra et al. 2019). However, few reports have used metabolomics to evaluate the metabolic characteristics and difference of metabolites for *E. gracilis* under different culture conditions. Zeng et al. (2016) used metabolomics to determine metabolites of *E. gracilis* under heterotrophic and mixotrophic culture conditions, while Wu et al. (2021) reported changes in paramylon production in *E. gracilis* by metabolomic analysis, using a new pilot-scale fermentation mode by combining mixotrophic and heterotrophic conditions. It is important to examine the differential metabolites and related metabolic pathway via metabolomics, and this may provide useful data for the selection of cultures to obtain target metabolites.

The objectives of the present study were: (1) to investigate growth characteristics and photosynthetic performance of *E. gracilis* under autotrophic and mixotrophic culture conditions, (2) to evaluate the metabolic characteristics of *Euglena gracilis* under autotrophic and mixotrophic culture conditions via liquid chromatography tandem mass spectrometry (LC-MS/MS), and (3) to determine the metabolic pathways related to different metabolites of *Euglena gracilis* in different culture conditions. Our work could provide evidence for the production of high value-added bioproducts and biological research using the protozoan *Euglena gracilis*.

Materials and methods

Protozoan strain and culture conditions

Euglena gracilis (FACHB-848) culture was purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, China. The protozoa were previously cultured in heterotrophic HUT medium (Table S1) and maintained in this medium under light (85–90 μmol/m^2 s) in our laboratory for the mixotrophic protozoa (MP) culture. A portion of the protozoa was transferred into an autotrophic medium (AP) (Checcucci et al. 1976) (Table S1), and acclimated for 5 months. The protozoa were cultured in an illuminating incubator (GZP-450 S, Shanghai, China) with a controlled environment, under cool-white LED light with a photosynthetic photon flux density of 85–90 μmol/m^2 s, and a light-dark cycle of 12 h: 12 h at 25 ± 0.5 °C. The only difference between light and dark conditions was zero photon flux density in the dark. 50% of the culture was discarded and replaced with an equal volume of fresh medium each week to maintain vigorous vitality of *E. gracilis*.

E. gracilis growth

Both MP and AP were diluted to a same initial cell density (5×10^4 cells/mL) with the corresponding medium, and then 150 mL culture was transferred into 250 mL flasks. Both autotrophic and mixotrophic cultures were performed in six replicates. The protozoa were then cultured under these conditions for 12 days. The flasks were hand shaken three times daily (10 s at regular time: 9:00 am, 2:00 pm and 6:00 pm).

The cell density of the protozoa was monitored every two days by testing the optical density at 680 nm assisted by cell counting, and used for biomass estimation. A standard curve ($y = 80.876\ x$) was used to transfer optical density x to cell density y (10^4 cells/mL). In addition, the specific growth rate was used to evaluate the growth and was calculated according to the following equation (OECD 2006):

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i}$$

where μ_{i-j} is the average specific growth rate from time i to j (d); X_i (10^4 cells/mL) is the cell density at time i (d); X_j (10^4 cells/mL) is the cell density at time j (d).

OJIP test

Chl *a* fluorescence (OJIP) of the protozoa was measured using an Aquapen system (Photon Systems Instruments, AP-C 100, Brno, Czech Republic). Briefly, the protozoan culture was dark-adapted for 5 min at 25 °C, and then 3 mL algal sample was transferred into a 4 mL cuvette in the Aquapen system. After adaptation, fluorescence rise OJIP curves were induced by 2 s pulses of red light (650 nm, 3500 μmol/m^2 s) and recorded in a time span ranging from 20 microseconds to 2 s. O refers to the initial fluorescence level, J (~ 2–3 ms) and I (~ 30 ms) are intermediate level, and P is the peak level (Strasser 1992; Sun et al. 2020). The data was then plotted on a logarithmic time scale. After 6 and 12 days of exposure, twelve selected OJIP parameters, specifically F_v/F_m, Ψ_O, Φ_{Eo}, Φ_{Do}, ABS/RC, TR_O/RC, ET_O/RC, DI_O/RC, RC/CS, S_m, N, and M_O, were determined at

9:00 am (Table S2) (Chen et al. 2007, 2016; Strasser et al. 2000, 2004).

LC-MS/MS-based metabolomic analysis

After 12 days of cultivation, 30 mL of protozoan samples were collected and centrifuged (10,000×*g*) for 10 min at 4 °C. The cells were rinsed with 8.5% NaCl solution three times and then frozen with liquid nitrogen for metabolomic analysis. Both autotrophic and mixotrophic cultures were performed in six replicates.

LC-MS/MS analysis was performed by using the UPLC system (Agilent 1290 Infinity) with an UPLC BEH Amide column (1.7 µm, 2.1 mm × 100 mm, Waters) coupled to Triple TOF 5600+ (AB SCIEX). The mobile phase consisted of 25 mM NH_4OAc and 25 mM NH_4OH in water (pH 9.75) (A) and acetonitrile (B) was carried out with an elution gradient as follows: 0–0.5 min 95% B; 0.5–7 min, 95−65% B; 7–9 min, 65−40% B; 9–10 min, 40% B; 10–11.1 min, 40–95% B; 11.1–16 min, 95% B, which was delivered at 0.3 mL/min. The injection volume was 1 µL. At the same time, a quality control (QC) sample product was used to monitor and evaluate the stability of the system and the reliability of the experiment. The triple TOF mass spectrometer was used for its ability to acquire MS/MS spectra on an information-dependent acquisition (IDA) during the LC/MS experiment. Electron spray ionization (ESI) source conditions were set as follows: Ion source gas1 (Gas1): 60 psi; ion source gas2 (Gas2): 60 psi; curtain gas (CUR): 30 psi; source temperature: 600 °C, IonSpray Voltage Floating (ISVF) ± 5500 V; TOF MS scan m/z range: 60–1200 Da; product ion scan m/z range: 25–1200 Da; TOF MS scan accumulation time 0.15 s/spectra; product ion scan accumulation time 0.03 s/spectra; mass spectrometry information dependent acquisition (IDA), and used high sensitivity pattern, declustering potential (DP): ± 60 V, collision energy: 30 eV.

Statistical analyses

The results of the cell density and photosynthetic parameters of *E. gracilis* were presented as arithmetic means with their corresponding standard deviations ($n = 6$). The data were analyzed using a parametric two-way analysis of variance (ANOVA) with treatment (autotrophy and mixotrophy) as the sources of variation. All data fulfilled the assumptions of the parametric test, and no data transformation was needed. The statistical analysis was carried out by SPSS 17.0 for Windows. $*p < 0.05$ and $p < 0.01$ were considered as significant and highly significant, respectively.

For the metabolomic analysis, after the LC-MS/MS analysis, the raw MS data were converted to XCMS data by MSDIAL software. For data processing, in-house MS2 database was used to identify metabolites. Peak areas of the annotated metabolites were normalized to the total peak area. The data were log2-transformed and then processed by using the Partial Least Squares. The Student's *t*-tests were utilized for univariate analysis, while principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) were used for multivariate analysis, with a false discovery rate (FDR) of < 0.05 and variable influence on projection (VIP) of > 1. In-house database and online databases (HMDB and Metlin) were applied in metabolite identification. Metabolites which were found to be statistically significantly different in different samples were then qualitatively analyzed based on relevant KEGG pathway.

Results

Growth profiles of *E. gracilis* under autotrophic andmixotrophic conditions

Euglena gracilis cell density was monitored 12 days, and specific growth rates were calculated. Auxotrophic protozoa (AP) showed steady growth and the biomass increased from 5.0×10^4 cells/mL to 6.1×10^5 cells/mL on day 12 (Fig. 1A). The maximum growth rate was observed on day 2 (Fig. 1B). The AP growth rate decreased from 4 day and maintained at approximately 0.1 from day 8 to day 12. Compared with AP, mixotrophic protozoa (MP) obtained a significantly higher cell density ($p < 0.01$) at day 2. The specific growth rate of MP reached 0.85 at day 2 and dropped to 0.02 at day 4. The cell density of MP was lower than AP from day 6 to day 12, and a significantly lower ($p < 0.01$) biomass, approximately 75% of AP, was obtained at day 12.

Photosynthetic performance of *E. gracilis* under autotrophic andmixotrophic conditions

Based on the OJIP test, twelve selected parameters were obtained, and each parameter was compared between AP and MP. The photosynthetic index F_v/F_m (the maximum quantum yield of primary photochemistry) of AP ranged from 0.584 to 0.627, which remained relatively stable during the 12-day cultivation period (Fig. 2A). However, the F_v/F_m value of MP showed a decline and decreased to 0.144 at day 12, although it was close to that of AP at day 2. Variation of four other photosynthetic parameters M_o (slope at the origin of the relative variable fluorescence), S_m (the working integral of the energy needed to close all reaction centers), N (number of plastoquinone A (Q_A) reduction events between time 0 to t_{Fm}), and RC/CS (the number of active PSII RCs per cross section) are shown in Fig. 2B–E. The values of M_o, S_m, and N of AP remained steady during the 12-day cultivation period, and RC/CS exhibited a linear increase. In MP,

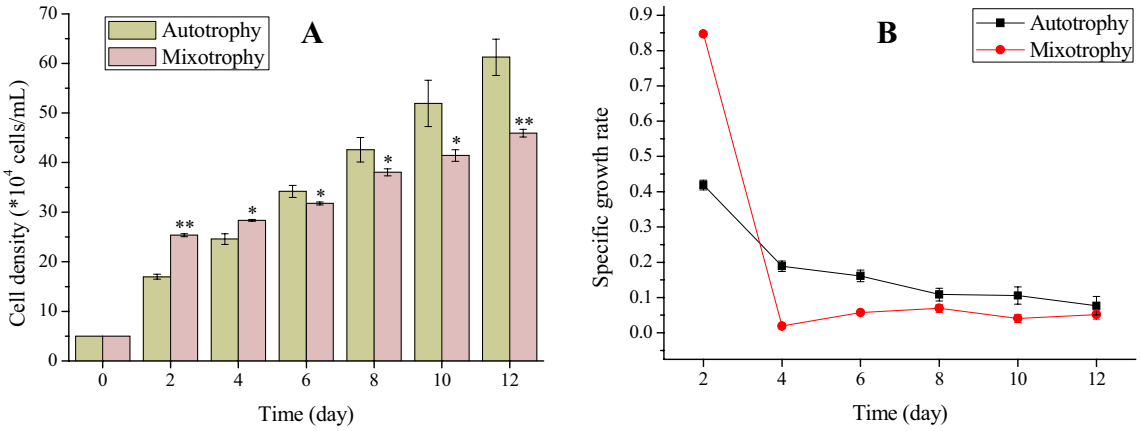


Fig. 1 Cell density (**A**) and specific growth rate (**B**) of *Euglena gracilis* under autotrophic and mixotrophic culture conditions. $*p < 0.05$ and $p < 0.01$ were considered as significant and highly significant between AP and MP on the same day, respectively

the turning point came on day 8: M_o increased from day 8 to day 12, but S_m, n, and RC/CS decreased.

Seven photosynthetic parameters of *E. gracilis* were examined at a 4-day interval (Table 1). At day 4, both Ψ_O (probability that an electron moves further than Q_A^-) and Φ_{Eo} (quantum yield for electron transport) of MP were significantly lower ($p < 0.05$) than those of AP. Furthermore, MP showed higher energy dissipation than AP, with a significant higher Φ_{Do} 25% (quantum yield (at t = 0) of energy dissipation) ($p < 0.05$). MP exhibited a relatively low photosynthetic efficiency. ABS/RC (absorption flux per reaction center), TR_O/RC (trapped energy flux per RC), and ET_O/RC (electron transport flux per RC) of MP were significantly higher ($p < 0.05$) than AP, while the utilization efficiency of the absorbed photon and transported electron decreased. At day 12, the end of the culture period, the differences between MP and AP became more obvious: Ψ_O, Φ_{Eo}, and ET_O/RC of MP decreased to extremely low levels, to 1/15, 1/46, and 1/9 those of AP, respectively. With the increase in ABS/RC (eight times that of AP), DI_O/RC (dissipated energy flux per RC) also increased to approximately 16 times that of AP. The ratio of DI_O/ABS reached 0.86, indicating a high dissipation ratio of energy absorbed by the cells. In contrast, the ratio of DI_O/ABS in AP remained steady, with a value close to 0.39.

Identification of differential metabolites of *E. gracilis*

The untargeted metabolomics were used to obtain the differential metabolites of *E. gracilis* under different cultivation conditions. In this study, a total of 626 metabolites of *E. gracilis* were detected and identified in two different cultivation conditions. Metabolites with VIP > 1 and $p < 0.05$ were considered as differential metabolites between the two culture groups. After a 12-day cultivation period, 137 metabolites were identified as passing these criteria (Table S3). Among these significantly differentially expressed metabolites, 38 metabolites were up-regulated and 99 metabolites were down-regulated in AP compared with MP.

Multivariate and univariate analyses

PCA plots of the metabolomic profiles of AP and MP after 8-day cultivation are shown in Fig. 3A. In all spectra, QC samples clustered in a small area, indicating minor variation in sample stability and good reproducibility of the instrument analysis. The two sample groups showed significant differences in the chemical composition of metabolites. OPLS-DA scores plots are shown in Fig. 3B. The two groups of samples could be distinguished significantly, and both are within the 95% confidence ellipses. The AP and MP groups are distributed along the first principal component [1] axis, with no crossover or overlap, indicating significant differences between AP and MP. A volcano plot of differential metabolites (Fig. 3C; VIP > 1 and $p < 0.05$) shows that the differences between AP and MP are significant: 61 metabolites were significantly up-regulated and 76 were significantly down-regulated in AP compared with MP.

Significant differential metabolites

Relative levels of identified differential metabolites from LC-MS/MS are presented in Table S3. The identified metabolites were classified into the following groups based on their chemical structure (Fig. 4): fatty acids, lipids, amino acids, peptides, carbohydrates, organic acids, purines and adenosines, terpenoids, glycosides, alkaloids, and others (not included in Fig. 4). Among these metabolites, most lipids (7 out of 9) and alkaloids (9 out of 10) were up-regulated,

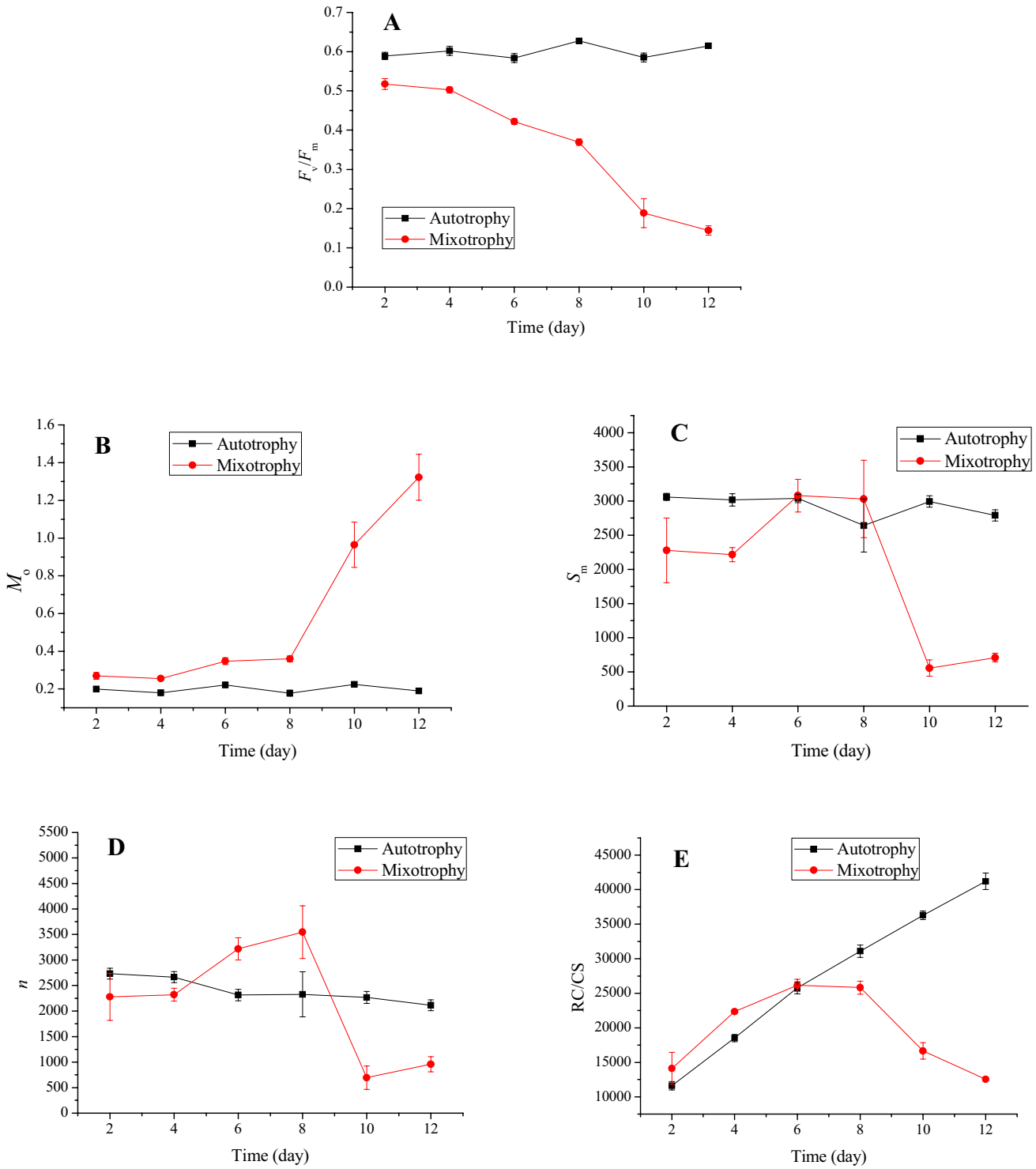


Fig. 2 The photosynthetic parameters, F_v/F_m (**A**), M_o (**B**), S_m (**C**), N (**D**) and RC/CS (**E**) of *Euglena gracilis* during 12-day period under autotrophic and mixotrophic culture conditions. The error bar means standard deviation

while most long-chain fatty acids (7 out of 8), amino acids (13 out of 15), peptides (4 out of 5), and organic acids (3 out of 4) were down-regulated. MP accumulated more polyunsaturated fatty acids (PUFAs), such as arachidonic acid, docosahexanoic acid, and linolenic acid in the cells than AP (Table S3).

Metabolites with significant differences (FC > 5 or < 0.2) were (S)-1-carbamoylpyrrolidine-2-carboxylic ccid (FC

Table 1 Photosynthetic indices of *Euglena gracilis* in autotrophic (AP) and mixotrophic (MP) cultures

Parameters	4 day		8 day		12 day	
	AP	MP	AP	MP	AP	MP
Ψ_O	0.80 ± 0.01^a	0.76 ± 0.01^b	0.80 ± 0.03^a	0.70 ± 0.02^c	0.75 ± 0.02^b	0.05 ± 0.04^d
Φ_{Eo}	0.48 ± 0.02^a	0.38 ± 0.01^b	0.50 ± 0.02^a	0.26 ± 0.01^c	0.46 ± 0.02^a	0.01 ± 0.01^d
Φ_{Do}	0.40 ± 0.01^a	0.50 ± 0.01^c	0.37 ± 0.00^b	0.63 ± 0.01^d	0.39 ± 0.01^a	0.85 ± 0.02^e
ABS/RC	1.47 ± 0.05^a	2.08 ± 0.04^c	1.39 ± 0.07^a	3.19 ± 0.09^d	1.23 ± 0.04^b	9.44 ± 1.45^e
TR_O/RC	0.88 ± 0.02^a	1.05 ± 0.02^c	0.87 ± 0.04^a	1.18 ± 0.02^d	0.76 ± 0.03^b	1.35 ± 0.09^e
ET_O/RC	0.70 ± 0.02^a	0.79 ± 0.01^c	0.70 ± 0.06^a	0.82 ± 0.03^c	0.57 ± 0.03^b	0.06 ± 0.05^d
DI_O/RC	0.58 ± 0.03^a	1.04 ± 0.04^c	0.52 ± 0.03^{ab}	1.98 ± 0.10^d	0.48 ± 0.02^b	8.09 ± 1.37^e
DI_O/ABS	0.39	0.50	0.37	0.62	0.39	0.86

Within each index, data with the same superscript indicates no significant differences ($p < 0.05$, n = 6)

0.03), glutamic acid (FC 0.06), indole-3-acetyl-L-alanine (FC 0.10), citrulline (FC 0.02), phosphatidylglyceride (FC 0.01), guanine (FC 5.27), sorbitol (FC 6.74), putative heterocyst glycolipid (FC 0.06), ganoderic acid A (FC 10.29), ganoderic acid D2 (FC 11.14), kirenol (FC 0.08), hydrangenoside C (FC 8.38), MGMG (monogalactosylmonoacylglycerol) 18:3 (FC 8.53), camptothecin (FC 0.05), argatroban (FC 0.01), leupeptin Pr-LL (FC 0.12), and alamethicin (FC 13.61).

KEGG analyses

In addition to multivariate and univariate analyses, metabolites were mapped to KEGG metabolic pathways for enrichment analysis, and the top nine most important pathways are listed in Fig. 5. Pathway enrichment analysis showed that "ABC transporters," "Biosynthesis of amino acids," "purine metabolism," and "aminoacyl-tRNA biosynthesis" were the top four pathways statistically significantly enriched, with 9, 6, 5, and 5 differential metabolites, respectively. Five other pathways were related to carbohydrate, amino acid, and lipid metabolism. Both glutamate and phenylalanine were down-regulated (AP versus MP) and involved in the four metabolic pathways. Glucose was involved in three metabolic pathways (ABC transporters, pentose phosphate pathway, and galactose metabolism) and up-regulated in AP compared with MP.

Figure 6 illustrates some of the differential metabolites in carbohydrate, amino acid, and lipid metabolism. Glucose is the initial substance of the Embden–Meyerhof pathway (EMP) and one of the metabolites in the ABC transporter pathway. Glucose was up-regulated and fructose was down-regulated in EMP in AP compared with MP. In the pentose phosphate pathway (PPP), xylose was up-regulated. It is worth noting that malate was up-regulated in the tricarboxylic acid cycle (TCA), while succinate was down-regulated in AP compared with MP. Malate is also an intermediate of the glyoxylic acid cycle (GAC). Docosahexanoic acid, arachidonic acid, and linolenic acid were down-regulated. Most of the amino acids, including phenylalanine, leucine, isoleucine, and citrulline were down-regulated, while asparaginate was up-regulated.

Discussion

Growth profiles of *E. gracilis* under autotrophic andmixotrophic conditions

Understanding the physiological characteristics of *E. gracilis* under different culture conditions is beneficial to biological research as well as for the production of high value added bioproducts. In the present study, the only carbon source for AP came from photosynthesis, while the carbon sources of MP were sodium acetate in the HUT medium, and glucose that is derived from photosynthesis. However, the two carbon sources did not help MP achieve higher productivity. AP grew steadily and reached a high biomass (6.1×10^5 cells/mL) at day 12, while MP experienced high growth rate in the first 4 days and lower growth rate on the remaining 8 days, and finally obtained biomass of 75% compared with AP.

Mixotrophic or heterotrophic cultures were thought to obtain more biomass than autotrophy. Glucose was frequently used as exogenous carbon source for *E. gracilis*. Grimm et al. (2015) reported that the maximum dry weight of *E. gracilis* was achieved under heterotrophic conditions, followed by mixotrophic and autotrophic. Jung et al. (2021) demonstrated that *E. gracilis* achieved more dry mass under mixotrophic conditions than autotrophic after 7-day cultivation, with biomasses of 5.16 ± 0.13 g/L and 2.16 ± 0.06 g/L (dry weight), respectively. However, Wang et al. (2018) reported that the specific growth rate was slightly lower when *E. gracilis* was grown in mixotrophic conditions than when grown in autotrophic conditions, although the former experienced a longer exponential duration. Glucose was employed as the organic carbon source for mixotrophic culture in all these studies.

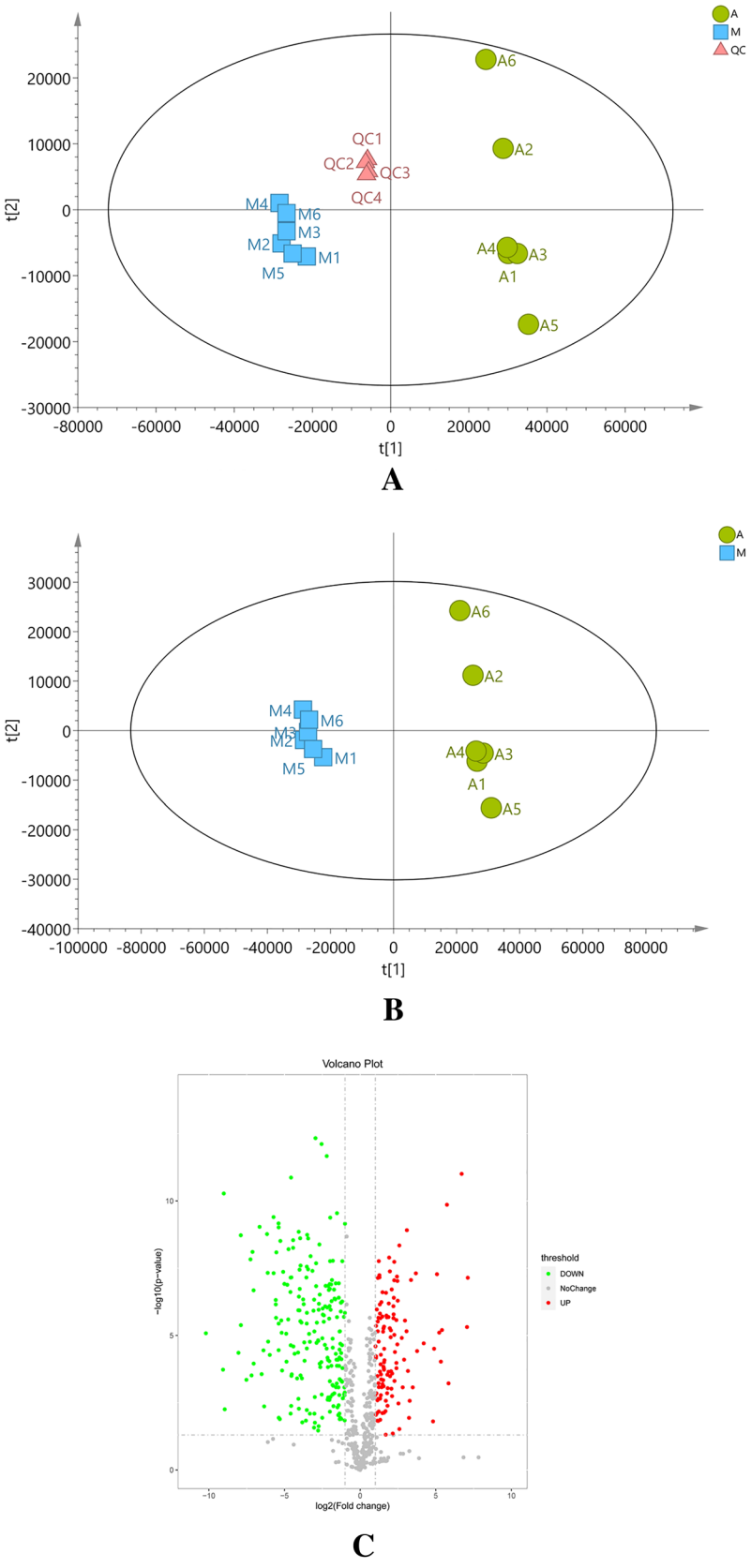


Fig. 3 PCA and OPLS-DA score plots and volcano plots of metabolomic profiles. **A** PCA score plots (OC: $R^2X = 0.766$, $Q^2 = 0.676$; autotrophic (AP) versus mixotrophic (MP) cultures: $R^2X = 0.814$, $Q^2 = 0.711$); **B** OPLS-DA scores plots ($R^2X = 0.811$, $R^2Y = 0.999$, $Q^2 = 0.996$); **C** volcano plots ($FC > 2$ or $FC < 0.5$, $p < 0.05$)

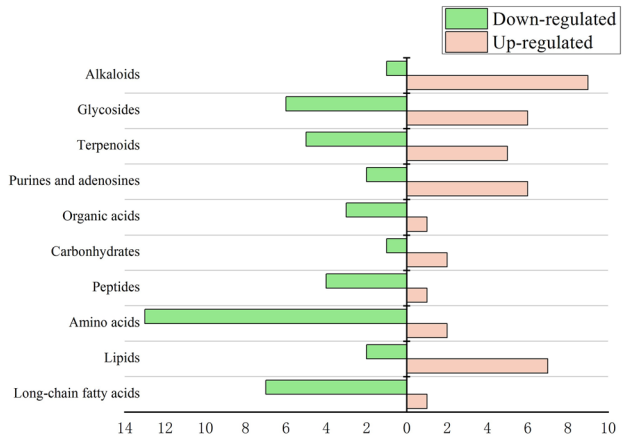


Fig. 4 Ontology of differential metabolites in *Euglena gracilis* (autotrophic [AP] versus mixotrophic [MP] cultures)

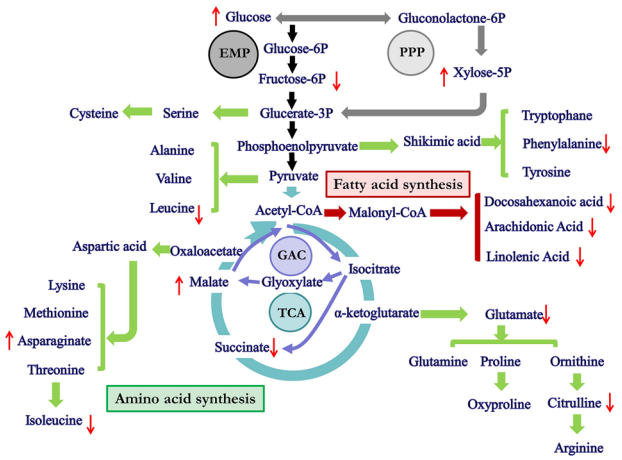


Fig. 6 Metabolic pathways involving most of the differential metabolites in carbohydrate, amino acid, and lipid metabolisms (autotrophic [AP] versus mixotrophic [MP] cultures). The direction of the arrows represent up-regulation or down-regulation in AP compared with MP. The different colors indicate different metabolic pathways. EMP, embden meyerhof pathway; PPP, pentose phosphate pathway; GAC, glyoxylic acid cycle; TCA, tricarboxylic acid cycle; CoA, coenzyme A

In *E. gracilis*, glucose is synthesized from CO_2 through photosynthesis, and broken down to obtain electrons that transfer through a branched respiratory chain to achieve ATP (Buetow 1989). When glucose was available, *E. gracilis* uptook it through the membrane and it was used directly for the synthesis of ATP (Graves 2010). Due to the limited CO_2 in the culture medium (Jensen et al. 2020), growth rates in the mixotrophic conditions increased compared with those in the autotrophic condition. However, Wang et al. (2018) found that *E. gracilis* initially grew exponentially in autotrophic batch cultures, with no apparent lag phase, and the exponential phase lasted approximately 2–4 days, but protozoa in mixotrophic conditions only started to consume glucose after an adaptation phase (up to 8 days at 23 °C).

In the present study, acetate in the HUT medium was used as carbon source for MP. Acetate is a widely used substrate for the growth of *Euglena gracilis* (Bates andHurlbert 1970, Cook 1967). It is regarded as a very good non-carbohydrate carbon source just as glucose is a good carbohydrate carbon source (Nakazawa 2017). However, it was found that a rich organic medium had adverse effects with a fading of *E. gracilis* var. *bacillaris* (Ternetz 1912). Organic carbon sources including glucose and other organic carbon sources could inhibit light triggered chloroplast development in *Euglena* (Schiff 1982; Sumida et al. 2007) found that the presence of the organic carbon source did inhibit the development of the latent capacity for chlorophyll synthesis. These reasons could be responsible for the low productivity of MP in our study.

Photosynthetic performance of *E. gracilis* under autotrophic andmixotrophic conditions

Analysis of photosynthetic performance was used to demonstrate the adaptive strategy of *E. gracilis* under different nutritional conditions. The parameters, including Ψ_O, Φ_{Eo}, Φ_{Do}, ABS/RC, TR_O/RC, ET_O/RC, and DI_O/RC had been used to show the absorption and the use of light energy by

Metabolic pathway	Differential metabolites								
ABC transporters	Glutamate***	Glucose***	Phenylalanine***	Fructose***	Leucine***	Adenosine***	Mannitol***	Isoleucine***	Sorbitol***
Biosynthesis of amino acids	Glutamate***	Phenylalanine***	Leucine***	Asparagine***	Citrulline***	Isoleucine***			
Purine metabolism	Adenine***	Adenosine***	Guanine***	Hypoxanthine***	3'-AMP***				
Aminoacyl-tRNA biosynthesis	Glutamate***	Phenylalanine***	Leucine***	Asparagine***	Isoleucine***				
Fructose and mannose metabolism	Fructose**	Mannitol**	Sorbitol**						
Pentose phosphate pathway	Glucose**	Gluconic acid**	2-Deoxy-D-ribose 5-phosphate**						
Galactose metabolism	Glucose**	Fructose**	Sorbitol**						
Cyanoamino acid metabolism	Phenylalanine**	Asparagine**	Isoleucine**						
Butanoate metabolism	Glutamate**	Succinate**	Malic acid**						

note: green color represents down-regulated, red color represents up-regulated; multivariate analysis based on the VIP and P-value: *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 5 KEGG metabolic pathway enrichment analysis of differential metabolites (autotrophic [AP] versus mixotrophic [MP] cultures)

photosynthetic organisms during the photosynthesis process (Chen et al. 2016; Oukarroum et al. 2007; Strasser et al. 2000, 2004). These results showed that the photosynthetic efficiency of MP dropped to a low level compared with AP, maintaining a stable photosynthetic efficiency. As mentioned above, organic carbon sources were found to inhibit chloroplast development and chlorophyll synthesis in *Euglena*, these may be contributed to the low photosynthetic efficiency in MP. However, these changes took place after 2 days, particularly on day 8, although MP had already acclimated in HUT medium. This might be related to prolonged exposure of MP to organic carbon sources.

M_o, S_m, N, and RC/CS, were the useful indicators for investigating electron transport and the reactive center in photosynthetic systems (PS) (Stirbet 2011; Strasser et al. 2004). S_m was proportional to the number of electrons passing through the electron transport chain. The turnover number N indicated how many times electron acceptor Q_A was reduced to Q_A^- until the maximum fluorescence intensity F_m was reached. In this study, both S_m and N of MP decreased at day 12, which caused the decrease of Φ_{Eo} and ET_O/RC (Table 1). The number of active PSII RCs per cross-section (i.e., RC/CS) also decreased, which was related to low photosynthetic efficiency. M_o was the approximate value of the initial slope of the variable chlorophyll fluorescence curve, reflecting the reduction of Q_A in the first 250 μs (Krüger et al. 1997; Strasser et al. 2000). The increased M_o of MP may be partially responsible for the high ABS/RC level.

Metabolomics characteristics of *E. gracilis* under autotrophic andmixotrophic conditions

In biotechnology and toxicology, the application of metabolomics to microalgae had revealed the changes in their metabolic state in response to environmental change or stress (Gauthier et al. 2020; Kato et al. 2021; Mishra et al. 2019). In *Euglena*, several studies had investigated the metabolites and/or metabolic pathways using metabolomics, and shown that total metabolites and differential metabolites varied considerably based on the culture conditions and detection methods. Zeng et al. (2016) applied gas chromatography–mass spectrometry (GC-MS)-based metabolomics to differentiate heterotrophic and mixotrophic culture conditions, and found that 86 metabolites of *E. gracilis* were obtained, and only one metabolite putrescine was differentiated. Wu et al. (2021) used ultra-high-performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UHPLC-QTOF-MS) for metabolomic analysis of *E. gracilis* cultured under mixotrophic and heterotrophic conditions. The results showed that 815 metabolites were annotated by the KEGG database and 372 candidate metabolites were differentiated (FC > 1.5 or < 0.6, and $p < 0.05$). The citrate cycle, related amino acids, and the glycerophospholipid biosynthetic pathway were up-regulated under mixotrophic conditions, while the carbon skeleton entered the paramylon synthesis pathway under heterotrophic conditions.

As an important carbohydrate, glucose is produced from water and carbon dioxide via photosynthesis in plants, serving as an energy reserve and metabolic fuel (Galant et al. 2015). In this study, AP synthesized more glucose than MP, which may be beneficial for paramylon (a unique β-1,3-glucan derived from glucose) accumulation in the cells. This is consistent with Wang et al. (2018), who found high concentrations of paramylon could be produced by *E. gracilis* in autotrophic conditions. Glucose also entered the pentose phosphate pathway and led to xylose up-regulation, while the down-regulation of fructose was partially responsible for the low amino acid productivity. *E. gracilis* was also found to use acetate as the sole source of carbon for growth (Nakazawa 2017). Acetate taken up from the media is converted to acetyl coenzyme A (acetyl-CoA) by the ATP-dependent reaction of acetyl-CoA synthetase (Ohmann 1964), and then the acetyl-CoA enters the glyoxylate cycle (Kornberg andKrebs 1957). It was reported that addition of exogenous acetate to autotrophic cultures of *E. gracilis* strain Z induces formation of the glyoxylate by-pass, which is the principal pathway for assimilation of acetate in heterotrophically cultivated *Euglena* (Cook andCarver 1966). The acetate in the HUT medium used in our study may lead to up-regulation of malate that belonged to GAC and TCA.

Compared with autotrophic culture, mixotrophic culture facilitated PUFA accumulation in *E. gracilis*. Jeong et al. (2016) reported that the proportional *n*-3 PUFA content in mixotrophic *E. gracilis* culture was much higher than in autotrophic culture, and concluded that mixotrophy was the best cultivation for improving nutritional content. Schwarzhans et al. (2015) found that culture media with higher nitrogen concentration resulted in a higher n3/n6-PUFA-ratio and demonstrated that the inexpensive peptone medium was ideal for lipid production by *E. gracilis*. This study showed that three PUFAs (arachidonic acid, docosahexanoic acid, and linolenic acid) were up-regulated in MP, which is consistent with the previous studies. Sodium acetate in the medium could be transferred to acetyl-CoA (Maloy andNunn 1982), and then be used in TCA or fatty acid biosynthesis. However, mixotrophic culture also resulted in similar or even low PUFA content in *E. gracilis*, compared with autotrophic culture (Gao et al. 2020; Wang et al. 2018).

High nitrogen provided more structural components of nitrogen-containing metabolites and stimulated the production of biomass and amino acids (Sepehri andSarrafzadeh 2019, Yaakob et al. 2021). In this study, the abundant nitrogen in the culture medium of MP resulted in high levels of amino acids in *E. gracilis*. And the result agrees with a recent study, which reported that, in HUT medium

containing peptone for the culture of *E. gracilis*, essential amino acid content was significantly increased by mixotrophic culture (Gao et al. 2020). It demonstrated that peptone containing high amount of glutamic acid enhanced the *E. gracilis* growth rate (Schwarzhans et al. 2015). Glutamic acid may act as good nitrogen source for amino acid biosynthesis. The amino acids accumulated in *E. gracilis* could be the pool of nitrogen sources (Hasan et al. 2017), and also provide high-value amino acids for humans.

Potential application of *E. gracilis* photosynthetic and metabolomiccharacteristics

Euglena gracilis was considered to be a potentially useful cultivated organism for humans, serving as a food and nutritional supplement (Matsuda et al. 2011; Zeng et al. 2016). It could synthesize and accumulate considerable amount of PUFAs, amino acids, paramylon, and antioxidants such as β-carotene and vitamins in response to different culture conditions (Barsanti et al. 2000; Hasan et al. 2019; Takeyama et al. 1997). In this study, we investigated the variation in photosynthetic efficiency and parameters of *E. gracilis*, and then compared the metabolic characteristics of *E. gracilis* under autotrophic and mixotrophic conditions. These results could provide useful information for the selection of culture mode to obtain different target metabolites.

Euglena gracilis had also been used as a biological model in numerous studies on eukaryotic cell biology due to its remarkable metabolic plasticity (Folťnová and Grones 1997, Zimorski et al. 2017). Owing to its high sensitivity to various contaminants, *E. gracilis* had been shown to be a suitable organism in ecotoxicology (Einicker-Lamas et al. 2002; Hu et al. 2015; Navarro et al. 1997). Photosynthesis had been reported to be sensitive to environmental stressors, and was frequently used as a biomarker in evaluating toxicity (Ahmed and Häder 2010a, b; Sun et al. 2021). In these studies, both autotrophic and mixotrophic culture media were used for *E. gracilis* growth. The current study demonstrated that the photosynthetic characteristics of *E. gracilis* under different culture media differed greatly. These results on metabolomic analysis also provided useful information when metabolites were used as biomarkers.

Conclusions

The photosynthetic efficiency of MP cultured in the HUT media decreased to a low level ($F_v/F_m = 0.144$) after 12-day growth. Examination of various photosynthetic parameters revealed low efficiency of light absorption and photosynthetic electron transport, as well as high dissipation of energy for MP. A total of 626 metabolites were detected and identified using metabolomic analysis, and 137 metabolites were identified as being significantly different between AP and MP, illustrated by multivariate and univariate data analyses. Enriched pathways were mainly related to ABC transporters, biosynthesis of amino acids, and purine and carbohydrate metabolism. Our study could provide a foundation for the selection of *E. gracilis* culture conditions to obtain needed bioproducts or realize biological research goals, with overall consideration of the protozoa growth, photosynthesis, and metabolites.

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Data availability The datasets used and analyzed within this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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