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Fatty Acid Accumulations and Transcriptome Analyses Under Different Treatments in a Model Microalga *Euglena gracilis*

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With the continuous growth of the world's population and the increasing development of industrialization, the demand for energy by human beings has been expanding, resulting in an increasingly severe energy crisis. Microalgae are considered the most potential alternatives to traditional fossil fuels due to their many advantages, like fast growth rate, strong carbon sequestration capacity, and low growth environment requirements. *Euglena* can use carbon sources such as glucose, ethanol, and others for heterotrophic growth. Moreover, *Euglena* is highly adaptable to the environment and has a high tolerance to various environmental stresses, such as salinity, heavy metals, antibiotics, etc. Different treatments of *Euglena* cells could affect their growth and the accumulation of bioactive substances, especially fatty acids. To expand the industrial application of *Euglena* as a potential biodiesel candidate, we determine the physiological responses of *Euglena* against environmental stresses (antibiotics, heavy metals, salinity) or carbon resources (glucose and ethanol), and evaluate the potential for higher quality and yield of fatty acid with a high growth rate. Adding glucose into the culture media increases cell biomass and fatty acid production with high-quality biodiesel characters. The transcriptome analysis helped explore the possible regulation and biosynthesis of fatty acids under different treatments and exploited in the improvement of biodiesel production. This study provides insights for further improvement and various culture treatments for *Euglena*-based biodiesel and jet fuels.

Keywords: ***Euglena gracilis*, fatty acids, biodiesel, transcriptome, environmental stresses, carbon sources**

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1 INTRODUCTION

Environmental problems and energy crises are becoming increasingly severe; therefore, it is imperative to discover novel, renewable, and environmentally friendly energy sources (Xia et al., 2019; Luo et al., 2021). Many microalgae, such as *Chlorella*, *Spirulina*, and *Dunaliella*, are cultured on a large scale and used in industrial applications (Gilmour, 2019). The microalga *Euglena gracilis* has characteristics of both plants and animals, with secondary endosymbiotic chloroplasts without cell

walls. Its cells contain many high-value bioproducts, including vitamins, amino acids, unsaturated fatty acids, and paramylon (Grimm et al., 2015; Zakryś et al., 2017). *E. gracilis* is of considerable environmental importance and biotechnological value: it can survive a variety of carbon resources, toxic chemicals, and adverse environmental conditions such as heavy metals, antibiotics, acid, salinity, and high levels of ionizing radiation, and can be observed in most waters such as ponds, fish farms and small rivers (Rodríguez-Zavala et al., 2007; Kottuparambil et al., 2012; Mukaida et al., 2016; Moreno-Sánchez et al., 2017; He et al., 2021). Indeed, *E. gracilis* exhibits remarkable metabolic diversity, blooming as photosynthetic autotrophy, heterotrophy, and photoheterotrophy (Zakryś et al., 2017). Thus, *Euglena* species are highly flexible to different nutrients and tolerant to adverse environments, which makes *Euglena* a model microorganism for environmental assessments, wastewater remediation, and sources of numerous bioproducts.

Environmental factors such as temperature, nutrients, carbon source, heavy metals, trace elements, antibiotics, and organic matter affect the growth of microalgae (Béchet et al., 2013; Gao et al., 2021; Maltsev et al., 2021). In this study, biomass, fatty acid (FA) contents, compositions, and biodiesel quality under the addition of paromomycin (PRM), ethanol, glucose, $CdCl_2$, and NaCl with continuously light cultivation were evaluated.

In recent years, with the development of next-generation sequencing platforms, transcriptome sequencing (RNA sequencing, RNA-seq) has been gradually applied to microalgae research, with significant results (Khan et al., 2018). The first *de novo* transcriptome study revealed unexpected metabolic capabilities for carbohydrate and natural product biochemistry in *Euglena* (O'neill et al., 2015). Another comparative transcriptome analysis investigated *Euglena*'s response to anaerobic conditions, focusing on paramylon and wax ester metabolic pathways (Yoshida et al., 2016). Five available *E. gracilis* transcriptome data, under fermentative, mixotrophic, heterotrophic, and phototrophic culture conditions, were well summarized (Geimer et al., 2009; Ebenezer et al., 2017, 2019; Cordoba et al., 2021). Thus, research on microalgae transcriptomes helps us characterize microalgal diversity better. The application of this technology will enable us to understand the mechanisms by which *E. gracilis* responds to different carbon sources and environmental stresses. However, no data were collected for transcriptome investigation in *Euglena* under environmental stresses.

In this study, we used RNA-seq technology and *de novo* assembly to perform analysis of the *E. gracilis* transcriptome under selected treatments as addition of ethanol (E1.0, 1.0% vol/vol), glucose (G1.0, 1.0% weight), NaCl (NaCl1, 1.0%) Cd0.5 ($CdCl_2$ 0.5 mM), and PRM5.0 (5 μg/ml). Differential gene expression and related metabolic pathways of *E. gracilis* in response to various treatments were investigated. *Euglena* cells showed both shared and distinct responses to these treatments at the transcriptional level. $CdCl_2$ stress significantly down-regulated thousands of genes, some of them related to amino acid metabolism and N-glycan biosynthesis. NaCl inhibited cell growth but did not change gene expression much at the transcriptional level. Different carbon sources such as ethanol and glucose promoted cell growth and altered the expression of genes related to photosynthesis, carbon fixation, nucleotide biosynthetic processes, component of the plasma membrane, ABC transporters, and the mRNA surveillance pathway. GO and KEGG pathway enrichments provide insights into the mechanisms of differential responses of *E. gracilis* to different treatments, especially the FA contents and composition changes.

2 MATERIALS AND METHODS

2.1 Strain and Cultural Conditions

E. gracilis 1224/5Z was obtained from the Culture Collection of Algae and Protozoa (https://www.ccap.ac.uk/). Aliquots of cells ($1–3 \times 10^5$) were incubated in 5 ml of fresh culture medium. The algal cells were grown in photosynthetic medium (1.8 g/L NH_4Cl, 0.6 g/L KH_2PO_4, 0.6 g/L $MgSO_4$, 60 mg/L urea, 0.02 g/L $CaCl_2$, 0.48 mg/L Na_2EDTA, 2 mg/L $Fe_2(SO_4)_3$, 60 μL HCl, 0.01 mg/L Vb_1, 0.0005 mg/L Vb_{12}, 20 mg/L $CuSO_4{\cdot}5H_2O$, 0.4 g/L $ZnSO_4{\cdot}7H_2O$, 1.3 g/L $Co(NH_3){\cdot}H_2O$, and 1.6 g/L $MnCl_2{\cdot}4H_2O$) under a light intensity of approximately 100 μmol/m^2/s in an illuminating incubator at 26 °C until algal cells reached the stationary phase (Afiukwa et al., 2007; Wang et al., 2018).

2.2 Cultivation and Stress Treatments

E. gracilis cells were cultured for 6 days in a photosynthetic medium, then $1{\times}10^6$ cells/mL were centrifuged at 5,000×g for 3 min and transferred into an equal medium volume. Treatments were applied, including supplementation with PRM (PRM1, 5, 25 as 1, 5, 25 μg/ml), ethanol (E0.5, 1.0, 1.5 as 0.5, 1, 1.5% vol/vol), glucose (G0.5, G1.0, G1.5 as 0.5, 1.0, 1.5% weight), $CdCl_2$ (Cd0.5, 1, 1.5 as $CdCl_2$ of 0.5, 1.0, 1.5 mM), or NaCl1.0 (NaCl as 1% weight); After 6 days, samples were collected and used for future experiments. The concentrations were selected based on previous references (Kirk, 1962; Gonzalez-Moreno et al., 1997; Sánchez-Thomas et al., 2016; Ji et al., 2018) and our primarily experimental results.

2.3 Growth Biomass

The dry weight of $10^7–10^8$ cells was measured using the oven-drying method (Edmunds, 1965). The total algal chlorophyll was extracted with 95% ethanol, and the content was spectrophotometrically assayed according to the method (Harris, 2009). Triplicates of each treatment were conducted.

2.4 Fatty Acids and FAME Analyses

After 6 days' growth, *E. gracilis* samples were taken separately and centrifuged at 3,000 × g for 10 min to collect algal cells. The samples are freeze-dried for 48 h and weighed. Take 10 mg dry algae powder, add C19: 0 fatty acid methyl ester as internal standard, 0.01% butylated hydroxytoluene (BHT) methanol solution as an antioxidant, and refer to Zeng et al. (2016) for processing. The composition and content of fatty acids are detected by gas chromatography-mass spectrometry GC-MS (United States, 7890A-5975C). The column model is VF-23 ms (0.32 mm × 0.15 μm × 60 m), high-purity helium with a purity greater than 99.999% is used as the carrier gas, the injection

volume is 1 μL, the solvent is delayed for 5 min, the inlet temperature is 240°C, constant flow mode, and the split ratio is 10: 1. For calculating the fatty acid content, the internal standard curve method is used to calculate the absolute content and then convert it into the percentage content.

One-way ANOVA is used for the statistics of different treatment samples. The statistical software is SPSS20. The quality of *Euglena* fatty acids derived biodiesels, key indexes like saponification (SN), iodine values (IN), and cetane number (CN) were also investigated (Lu et al., 2012). The saponification value (SN) is the mass of sodium hydroxide required for the complete saponification of 1 g grease, which can reflect the relative molecular weight of grease, and SN = Σ(560 × Pi)/MWi. The iodine value (IN) indicates the degree of grease unsaturation. The iodine value reflects the number of double bonds in FA and IN = Σ(254 × D × Pi)/MWi. CN is a standard to measure the combustion performance of biodiesel in engines, and CN = 46.3 + 5458/SN - 0.225 × IN. This study combines GC-MS component analysis and gets three main performance parameters of FA in *E. gracilis* as biodiesel under different treatments (Lu et al., 2012).

2.5 RNA Isolation and RNA-Seq Analyses

The cDNA library construction and double-ended PE125 Illumina sequencing were performed by Real Omics (Biotech) Co., Ltd (Shenzhen, China). Approximately 10^6–10^7 cells were harvested by centrifugation at 5,000×g for 5 min at 4°C and used for total RNA isolation with a Trizol Kit (Invitrogen, United States). For RNA-seq, triplicates of each sample were used.

Because *E. gracilis* has no reference genome, Trinity software was used to assemble the clean reads, Corset was used to perform hierarchical clustering, and Benchmarking Universal Single-Copy Orthologs (BUSCO) was used to evaluate the completeness and integrity of the transcriptome assembly. Seven NCBI databases were used to annotate the assembled transcripts: NCBI non-redundant protein sequences (Nr), NCBI nucleotide sequences (Nt), Protein family (Pfam), euKaryotic Ortholog Groups (KOG) Clusters of Orthologous Groups of proteins (COG), Swiss-Prot manually annotated and reviewed protein sequence database, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO). The input data for the differential expression analysis were read counts obtained from the analysis of gene expression data. The analysis of samples with biological replicates was performed in DESeq2 based on a negative binomial distribution.

The KEGG Orthology Based Annotation System (KOBAS) was used to perform KEGG pathway enrichment analysis for each comparison with an automatic annotation server e-value of $1e^{-10}$. GO annotations were obtained from the annotations of the top 10 Nr blast hits using Blast2GO (Conesa et al., 2005) and from the Pfam database using InterPro scan (Anders and Huber, 2010; Kimbrel et al., 2011). The Seq method was used to identify pathways enriched in up-regulated and down-regulated differential gene sets for each group. When more genes are up-regulated than down-regulated in a selected pathway, the pathway would be marked up-regulated under this condition (Campanaro et al., 2007).

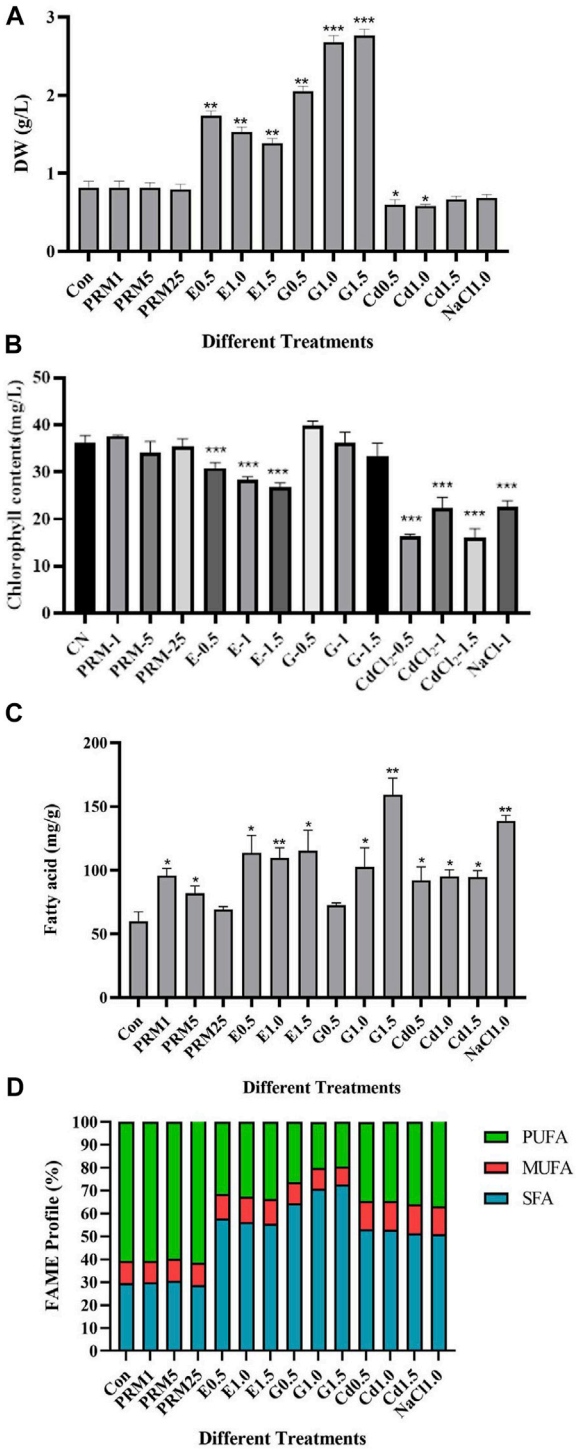

FIGURE 1 | Physiological changes of *E. gracilis* under different treatments. Dried weights **(A)**, chlorophyll contents **(B)**, variations of fatty acids contents **(C)** and FAME profile **(D)** of *E. gracilis* under different treatments. Asterisks "*", "**" indicate significant differences between treatments and control treatment (Con) at $p < 0.05$ and $p < 0.01$, respectively. Treatments include supplementation with paromomycin (PRM1, 5, 25 as 1, 5, 25 μg/ml), ethanol (E0.5, 1.0, 1.5 as 0.5, 1, 1.5% vol/vol), glucose (G0.5, G1.0, G1.5 as 0.5, 1.0, 1.5% weight), CdCl2 (Cd0.5, 1.0, 1.5 as CdCl2 of 0.5, 1.0, 1.5 mM), or NaCl1.0 (NaCl as 1% weight).

2.6 Statistical Analysis

Significant differences in growth biomass, fatty acids, and biodiesel fuel properties were tested using Dunnett's *t*-test. All data were obtained and averaged from at least three independent experiments, and standard errors were calculated and displayed as error bars.

3 RESULTS

3.1 Biomass and Chlorophyll Contents Under Different Treatments

Biomass differed significantly under different environmental treatments (**Figure 1A**). The PRM treatment caused no significant change in biomass, and NaCl and $CdCl_2$ treatment significantly reduced biomass ($p < 0.001$) by 18.18 and 18.72% relative to the control, respectively. All ethanol and glucose concentrations significantly increased biomass ($p < 0.001$) relative to the control by 64.75–107.66% and 216.47%, respectively. Glucose treatments showed higher biomass increases under all current concentrations in this case.

Chlorophylls in microalgae are sensitive to environmental stress. The addition of ethanol (all concentrations) $CdCl_2$ (all doses), and NaCl decreased the chlorophyll content of *E. gracilis* significantly (**Figure 1B**), whereas PRM and Glucose had no significant effect.

3.2 Fatty Acid Contents, Compositions, and FAME

The total fatty acid (TFA) content of *E. gracilis* under all treatments increases to varying degrees compared with the control, and some treated groups showed concentration-dependent manners, such as paromomycin (PRMs) and glucose (G) treatments (**Figure 1C**). The TFA content in PRM groups tends to decrease as the concentration increases. A low concentration of PRM (PRM1) has a higher effect on accumulating total fatty acids. However, in glucose-treated groups (G) TFA tends to increase with concentration, with the highest in G1.5 by about 132% increases compared to the control (Con). The second high TFA content was detected in the NaCl group, around 140 mg/g. The addition of ethanol (E0.5, E1.0, E1.5, as 0.5–1.5% vol/vol) nearly doubled the TFA compared to Con, with no significant difference among different concentrations. This study also showed a similar TFA increase by $CdCl_2$ at different concentrations.

Investigation of FA composition, namely SFA, MUFA, and PUFA, indicated interesting differences under treatments (**Figure 1D** and **Supplementary Table S1**). Compared with the control, the FA composition of *E. gracilis* under paromomycin treatments (PRM1, -5, -25) changed little. In contrast, the FAME profile under $CdCl_2$, ethanol, glucose, and NaCl treatment changed significantly.

The general trend of FA composition changes was almost the same, both which SFA proportion increases and PUFA proportion decreases, indicating more saturated fatty acids under these treatments. MUFA proportion changes little, and the most significant change was observed in the G1.5 treatment, whose SFA proportion increased from 29.51% of the control group to 72.72%. The PUFA proportion decreases from 60.85% of the control group to 19.49%. The change patterns in the other groups, Cd0.5, 1.0, 1.5, E0.5, 1.0, 1.5, and NaCl, were similar with no apparent dose-dependent pattern.

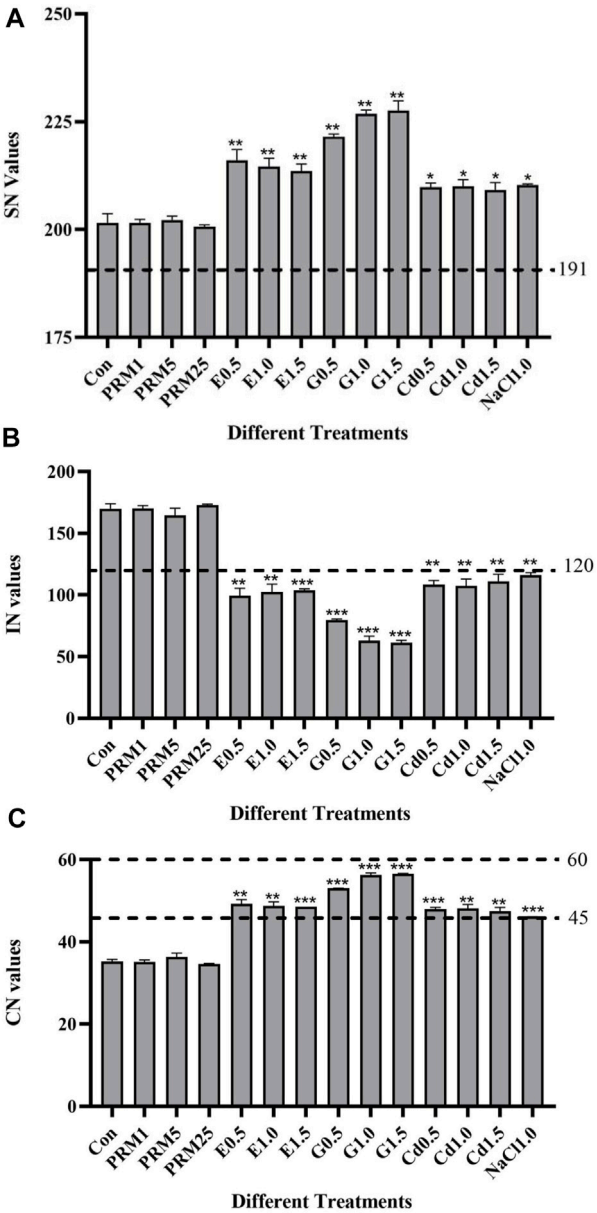

FIGURE 2 | Biodiesel fuel properties of *E. gracilis* TFA under different treatments at the sixth day. **(A)** saponification value (SN), **(B)** iodine value (IN), **(C)** cetane number (CN). Asterisks "*", "**" indicate significant differences between treatments and control treatment (Con) at $p < 0.05$ and $p < 0.01$, respectively. Lines indicate the standard threshold values for each biodiesel fuel property (SN, IN, or CN).

3.3 Evaluation of Biodiesel Fuel Properties of *E. gracilis* TFA

Biodiesel fuel characters were evaluated for *E. gracilis* TFA under different consumption methods. All the data are obtained and averaged from at least three independent experiments. As shown in the figure, compared with the control, the changes of SN (a), IN (b), and CN (c) of *E. gracilis* under different treatments were generally different (**Figure 2**).

The SN value of *E. gracilis* TFA slightly or significantly increased under different treatments than the control, except for no change under antibiotics treatments. The SN value of the G-1.5 treatment is the largest compared with the control, which increases by about 13%. The German EN 14214 standard requires that the SN value of biodiesel should be 187–191 mg/g. The SN value of each group in this study was larger than the standard (**Figure 2A**).

The minimum IN value standard in various countries' biodiesel production standards is <125 g/100 g (Arguelles and Martinez-Goss, 2021). The IN values of *E. gracilis* TFA in the control and paromomycin treated group are higher than the minimum standard by 35.92–36.02%, which is not suitable for direct use as biodiesel feedstock. In addition, the IN values of *E. gracilis* TFA under $CdCl_2$, ethanol, glucose, and NaCl treatments are significantly lower than those in control (**Figure 2B**). With the highest IN level (116.15), NaCl treated group has already met the standards of China, Australia, and the European Union (<120 g/100 g) and could be used as an ideal choice for biodiesel feedstock. The IN values of $CdCl_2$, ethanol, and glucose treatments (107.44–111.03, 99.22–103.62, 61.21–79.63) all reach the strict standards of biodiesel production in various countries, like the biodiesel production standards <115 g/100 g in Germany (EN 14214) and France, with excellent combustibility (Lu et al., 2012; Arguelles and Martinez-Goss, 2021). Among the low IN value treatments, the G1.5 treatment has the lowest compared with the control, with a significant reduction of about 64%.

Compared with the control, CN was improved under all treatments except for PRM (**Figure 2C**). The minimum requirement of CN in biodiesel production standards of various countries is higher than 45. The CN value of control and PRM groups is still 22.87–22.96%, respectively, lower than the minimum production standards of biodiesel. It could not be used directly as the raw material for biodiesel. The CN value of the E0.5 treatment is already 0.04% higher than the Chinese GB/T 20,828 standard and has the potential as a biodiesel feedstock. The CN values of G0.5, G1.0, and G1.5 treatment groups (53.02, 56.22, 56.51) have all reached the most demanding EU 14124 standard (> 51). The ideal treatment is the addition of glucose under different concentrations, about 51–60% higher than the control.

3.4 RNA-Seq Analyses

3.4.1 Summary of RNA-Seq and Differentially Expressed Genes

Our biomass and TFA results showed that treatments of E1.0, G1.0, NaCl1.0, Cd0.5, PRM25 caused significant differences compared to control samples, thus, these conditions were used for further transcriptome analyses. An average of 47,452,424 reads was generated for each sample. After removing low-quality reads, an average of 46,542,906 clean reads was obtained for each sample. The average GC content was less than 60%, the Q20 and Q30 values were 97 and 92%, respectively, and the sequencing error rate was 3%.

TABLE 1 | Differentially expressed genes (DEGs) based on RNA-seq under different treatments compared with the control. Treatments include supplementation with paromomycin (PRM25, 25 μg/ml), ethanol (E1.0, 1.0% vol/vol), glucose (G1.0, 1.0% weight), $CdCl_2$ (Cd0.5, 0.5 mM), or NaCl1.0 (NaCl as 1.0% weight).

Treatments	Total DEGs	Up-regulated	Down-regulated
NaCl1.0	490	280	210
PRM25	209	128	81
Cd0.5	4,324	3,040	1,284
G1.0	1,080	37	1,043
E1.0	1,040	56	984

Compared with the control, overall gene expression changes were the most obvious in the $CdCl_2$ treatment, with 3,040 and 1,284 up-regulated and down-regulated genes. The heavy metal Cd^{2+}, therefore, appeared to have a significant effect on the transcriptome of *E. gracilis* (**Table 1**). The gene expression changes were less evident in the ethanol and glucose treatments. Under ethanol addition (E1.0), there were 56 and 1,040 up- and down-regulated genes, i.e., about 20 times more down-regulated genes than up-regulated ones. Similarly, in the glucose treatment (G1), there were 1,043 down-regulated genes and only 37 up-regulated genes.

Compared with thousands of genes altered under the addition of Cd^{2+}, glucose, and ethanol treatments, PRM and NaCl treatments had relatively modest changes on the overall transcriptome, causing up-regulation of 128–280 genes and down-regulation of 81–210 genes (**Table 1**).

3.4.2 Gene Expression Patterns Related to Reactive Oxygen Species, Fatty Acid Metabolism and Chlorophyll Biosynthesis

Reactive oxygen species (ROS) scavenging genes, such as *APX*, L-ascorbate peroxidase; *FTRC*, Ferredoxin-thioredoxin reductase catalytic chain, chloroplastic; *GPX*, glutathione peroxidase; *SOD1*, Superoxide dismutase [Fe]; *SOD2*, Superoxide dismutase [Mn]; *SOD3*, Superoxide dismutase [Zn]; *CAT1,2*, catalase1, 2; *trxB*, Thioredoxin reductase, were screened for differential gene expression analysis (**Table 2**). Very obviously, 13 of 18 selected genes were induced by addition of $CdCl_2$, while most genes were down-regulated with PRM25 (13/18) and NaCl (11/18) (**Table 2**).

According to FA metabolism, total 31 genes were identified and no significant changes with more than 1.5 or 2 fold. With slightly up-regulation, most FA metabolism related genes were correlated with E1.0 (26/31 genes) and G1.0 (26/31 genes). NaCl treatment did not cause much up-regulation instead (9/31 genes) (**Supplementary Table S2**).

Based on chlorophyll synthesis and metabolism genes, mostly down-regulated gene numbers were identified in treatments of NaCl1.0 (17/24 genes) and Cd0.5 (10/24 genes) (**Table 3**). Again, most changes of these genes were less than 1.0 fold.

TABLE 2 | The Log2FC (fold change) of selected Reactive Oxygen Species (ROS) scavenging genes under different treatments, treatment vs. control.

Gene_ID	KO name	Cd0.5	G1.0	E1.0	PRM25	NaCl1.0
Cluster-31172.42963	*APX*	0.15196	0.25317	-0.051678	0.010221	0.13602
Cluster-31172.42961	*APX*	0.16485	-0.051658	-0.047102	0.038122	-0.084889
Cluster-31172.21483	*FTRC*	0.088081	-0.018337	-0.0071269	0.008834	0.23297
Cluster-31172.21140	*GPX*	-0.067412	0.10687	0.13296	-0.19666	0.17218
Cluster-31172.31278	*GPX*	0.052787	0.25853	0.26442	-0.14531	0.041538
Cluster-31172.24294	*GPX*	0.084417	0.023608	0.0184	-0.16479	-0.11895
Cluster-31172.15664	*GPX*	0.36104	0.13766	0.16633	0.037672	0.067972
Cluster-31172.30627	*SOD1*	-0.23118	0.1078	0.24022	-0.21941	-0.12951
Cluster-31172.19490	*SOD1*	-0.093043	0.13826	-0.11919	-0.014011	-0.094739
Cluster-31172.16822	*SOD1*	0.020231	-0.12859	-0.20322	-0.20549	0.14619
Cluster-31172.17975	*SOD2*	0.089971	-0.013959	-0.19817	-0.38083	-0.24051
Cluster-31172.36115	*SOD2*	0.10199	0.19635	-0.024988	-0.10232	0.21592
Cluster-31172.26831	*SOD3*	0.15103	0.17146	0.30485	-0.12909	-0.0087302
Cluster-31172.22853	*CAT1*	0.17912	0.18002	0.29535	-0.21605	-0.23217
Cluster-31172.31814	*CAT2*	0.63894	0.30887	0.20949	0.12308	-0.088459
Cluster-31172.26383	*trxB*	-0.57939	-0.39629	-0.55989	-0.44473	-0.090589
Cluster-31172.33966	*trxB*	-0.070243	0.12997	0.025197	-0.13855	-0.037955
Cluster-31172.37823	*trxB*	0.077437	0.065051	0.22527	-0.11915	-0.13544

Gene_ID Cluster-31172.XXs were assigned based on our RNA-seq data. APX, *L-ascorbate peroxidase;* FTRC, *ferredoxin-thioredoxin reductase catalytic chain, chloroplastic;* GPX, *glutathione peroxidase;* SOD1, *superoxide dismutase [Fe];* SOD2, *superoxide dismutase [Mn];* SOD3, *superoxide dismutase [Zn];* CAT1,2, *catalase1, 2;* trxB, *thioredoxin reductase. Red color fonts in Cd0.5 indicate up-regulated expression, and green color font in other treatments mean down-regulated expression.*

TABLE 3 | The Log2FC (fold change) of selected chlorophyll related genes under different treatments, treatment vs. control. Gene_ID Cluster-31172.XXs were assigned based on our RNA-seq data.

Gene_ID	KO name	Cd0.5	G1.0	E1.0	PRM25	NaCl1.0
Cluster-31172.33879	CAO	-0.2102	0.1121	0.36737	-0.061843	-0.0086368
Cluster-31172.38450	chlG, bchG	-0.0035863	0.087771	0.036195	-0.68488	-0.10191
Cluster-31172.24557	chlG, bchG	0.20533	0.19798	0.20092	0.11515	0.074355
Cluster-31172.34067	DVR	0.49806	0.22756	0.41184	0.08435	0.22081
Cluster-31172.12419	E1.3.1.33, por	-0.085459	0.31578	-0.039392	-0.1063	-0.2928
Cluster-31172.30226	E1.3.1.33, por	0.36629	-0.016723	0.18033	-0.09283	-0.27796
Cluster-31172.41935	HCAR	0.034584	0.18931	0.19967	-0.078673	-0.16876
Cluster-31172.22139	LHCA1	-0.32772	0.14507	-0.070321	-0.13334	-0.14353
Cluster-31172.34648	LHCA9	0.48159	0.19422	0.47038	0.55464	0.39359
Cluster-31172.32063	LHCB1	-0.50215	0.043473	0.16147	0.26066	0.057025
Cluster-31172.35839	LHCB1	-0.46686	-0.028961	0.13009	0.43923	0.26772
Cluster-31172.27457	LHCB1	0.1813	0.075637	0.24588	0.14815	-0.15394
Cluster-31172.32089	LHCB1	0.23691	0.14142	0.42049	0.24979	-0.041671
Cluster-31172.35597	LHCB1	0.34805	0.11932	0.29077	0.21118	-0.13149
Cluster-31172.28680	LHCB1	0.50319	-0.49634	-0.22636	-0.10685	-0.1687
Cluster-31172.31624	LHCB1	0.98218	0.10297	0.30361	0.3717	-0.097532
Cluster-31172.32503	LHCB1	1.03	0.17016	0.42601	0.34549	-0.097739
Cluster-31172.35322	LHCB2	0.07938	0.13249	0.32385	0.14945	-0.15313
Cluster-31172.33766	LHCB4	-0.46828	-0.098655	0.042181	0.080384	-0.33116
Cluster-31172.31649	LHCB4	-0.2596	0.094778	0.11783	0.092226	-0.2668
Cluster-31172.21282	NOL, NYC1	-0.5075	0.21729	0.036893	-1.1132	-0.18871
Cluster-31172.45925	NOL, NYC1	0.10415	0.1881	-0.008742	0.034387	0.22995
Cluster-31172.24011	PAO, ACD1	-0.35873	0.1644	0.049772	-0.10193	-0.51182
Cluster-31172.21287	psaA	0.85456	-0.84092	-0.65994	0.031673	0.26759

3.4.3 GO and KEGG Pathway Enrichments

GO Pathways enriched under different treatments showed various and similar patterns under our selected treatments. Pathways down-regulated in response to 1.0% ethanol (E1) included several nucleotide biosynthetic or metabolic processes, signal transduction, and cell communication; integral and intrinsic component of the plasma membrane (**Supplementary Figure S1** E1.0 GO). Similarly, adding glucose reduced nucleotide biosynthetic or metabolic processes, signal transduction, and the component of plasma membranes (**Supplementary Figure S1** G1.0 GO). NaCl treatment caused up-regulation of genes associated with the arginine biosynthetic process, argininosuccinate synthase activity, and down-regulation of genes in the monosaccharide binding pathway (**Supplementary Figure S1** NaCl GO). In response to the antibiotic PRM, genes in the pyruvate, proline,

and arginine metabolic processes, sodium ion transmembrane transport, and sodium ion export were up-regulated. Still, genes involved with oxaloacetate decarboxylase activity were down-regulated (**Supplementary Figure S1** PRM GO). The heavy metal treatment is an exception in this case: more pathways were up-regulated than down-regulated. Most significantly, Na + transport, organic transmembrane transports, non-membrane-bounded and plasma membranes, and heterocyclic and organic cyclic compound binding pathways were increased against Cd ions (**Supplementary Figure S1** Cd GO).

KEGG pathways enriched under E1 are involved in the down-regulation of photosynthesis, carbon fixation in photosynthetic organisms, glyoxylate and dicarboxylate metabolism, and ABC transporters. Upregulated pathways like spliceosome, cysteine, and methionine metabolism were detected under E-1 (**Supplementary Figure S2** E-1 KEGG). Photosynthesis, glyoxylate and dicarboxylate metabolism, glycerophospholipid, fatty acid degradation, and lipid metabolism pathways were down-regulated with glucose (G1). In contrast, sulfur metabolism, N-glycan biosynthesis, mRNA surveillance pathway, endocytosis, and cysteine and methionine metabolism were up-regulated (**Supplementary Figure S2** G1 KEGG). Similar changes were also detected in NaCl treatment. Under NaCl treatment, photosynthesis, oxidative phosphorylation, glycerophospholipid, glycerolipid, and ether lipid metabolisms were down-regulated. In contrast, selenocompound metabolism, plant-pathogen interaction, plant hormone signal transduction, endocytosis, and cysteine and methionine metabolism were up-regulated (**Supplementary Figure S2** NaCl KEGG). There are relatively few pathways changed by PRM treatment, with down-regulated purine, porphyrin, chlorophyll metabolisms, and up-regulated glycolysis/gluconeogenesis and endocytosis (**Supplementary Figure S2** PRM KEGG). More complicated changes were disturbed by $CdCl_2$, with many pathways such as down-regulated spliceosome, protein processing, nucleotide excision repair, N-glycan biosynthesis, basal transcription factors, and up-regulated ribosome, glycolysis/gluconeogenesis, and fatty acid biosynthesis and ether lipid metabolism pathways (**Supplementary Figure S2** Cd KEGG).

4 DISCUSSION

Cd^{2+} is a serious environmental pollutant. It is highly toxic to living organisms because it is an unnecessary ion, even at low concentrations. Not only Cd^{2+}accumulates in *Euglena*, but other heavy metals such as Hg^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Tc^{7+}, and Cr^{6+}, thereby contributing to the remediation of waste and polluted water (Mendoza-Cózatl et al., 2006). *E. gracilis* is considered an efficient and reliable bioremediation microorganism with the potential to remediate heavy metal environments (Sánchez-Thomas et al., 2016). In this study, *E. gracilis* growth was slightly reduced due to a low concentration of 0.5 mM $CdCl_2$. A previous study showed that *Chlorella* also had some tolerance to cadmium. Its growth was unaffected by 0.5 and 1 mg/L $3CdSO_4 \bullet 8H_2O$, although concentrations of 3 and 5 mg/L inhibited growth, and 7 mg/L completely blocked growth (Cheng et al., 2016). Based on the thousands of genes differentially expressed in response to $CdCl_2$ treatment and the observed reductions in biomass, we can conclude that $CdCl_2$ has a significant impact on *Euglena* cells.

NaCl treatment produced similar results, reducing biomass by 18.18%, consistent with previous studies of NaCl in *Scenedesmus* and *E. gracilis* (Ji et al., 2018). This may reflect that NaCl promotes the degradation of chlorophyll a, reduces photosynthetic capacity, and ultimately inhibits microalgae growth. This is verified through our chlorophyll assessments and related gene expression analysis.

The biochemical effects of antibiotics paromomycin (PRM) have seldom been studied in *E. gracilis* and other microalgae. PRM and streptomycin are aminoglycoside antibiotics, and streptomycin has a known inhibitory effect on *E. gracilis* growth (Kirk, 1962). However, this study showed that low concentrations (1–25 μg/ml) of PRM had little effect on *E. gracilis* growth, suggesting that *E. gracilis* is tolerant to low PRM concentrations, consistent with previous reports on *Euglena* antibiotic tolerance (Kirk, 1962; He et al., 2021). *E. gracilis* could grow mixotrophically; that is, it can first use organic carbons in the medium or field waters instead of CO_2 via photosynthesis under the light. Transcriptome analyses indicated down-regulated photosynthesis with the addition of ethanol or glucose. Ethanol has been reported to induce cell division and promote the growth of *E. gracilis* (Horrum and Schwartzbach, 1980; Venugopal et al., 2006). Likewise, studies have shown that adding glucose to the culture medium can promote the growth of *E. gracilis* and *Scenedesmus quadriceps* (Taylor, 1960; Nicolas et al., 1980). Here, we also found out that ethanol and glucose significantly promoted the growth of *E. gracilis*, and the effect of glucose was significant under selected concentrations in this study.

All treatments with low doses of ethanol (all doses), glucose (1.0, 1.5%), $CdCl_2$, NaCl, and PRM (1–5 μg/ml) can promote the accumulation of fatty acids of *E. gracilis*. Also, FA compositions showed some interesting patterns under different treatments.

Coleman's research shows that the fatty acid synthesis rate of *E. gracilis* with an additional carbon source is higher than that of photosynthetic carbon fixation. In this study, adding ethanol and glucose significantly increases the fatty acid contents of *E. gracilis* compared with the control, of which G1.5 treatment has the highest FA content and the total lipid content of *E. gracilis* can reach 16.59% ($p < 0.001$). When *E. gracilis* is cultivated in ethanol-containing media, its total lipid content increases (Coleman et al., 1988; Thuillier-Bruston et al., 1990). When treated with different ethanol concentrations, the total lipid content can also be increased by 82.59–146.96% ($p < 0.01$). The glucose-rich medium can increase the lipid content in *Scenedesmus quadricauda* (Kandimalla et al., 2016), and glucose (35 mM) as a carbon source can promote the accumulation of lipid in *Chromochloris zofingiensis* (Zhang et al., 2019). The addition of carbon sources into *E. gracilis* culture medium alters the FA compositions a lot. The transcriptome data indicated very similar responses to ethanol and glucose. For instance, nucleotide biosynthetic or metabolic processes such as signal transduction, integral and intrinsic components of plasma membrane pathways were down-regulated under both treatments. Characters of plasma membrane depend on the composition of membrane lipids based on the saturated ratio. With the concentrations in this study, glucose increased significantly higher FA contents than ethanol. The reduced fatty acid degradation

detected in the G1.0 treatment may contribute partially to higher FA production under glucose addition.

Our transcriptome analysis showed up-regulated ROS related genes in $CdCl_2$ treatment group. Alho's research shows that Cd has a more significant impact on the biochemical composition of *Raphidocelis subcapitata*, increasing total lipid content (7.2 times), total carbohydrates (3.5 times), and ROS production (3.7 times) (Alho et al., 2019). Addition of $CdCl_2$ into *E. gracilis* culture medium caused the up-regulation of sodium ion and organic transmembrane transports to pump out Cd^{2+}. At the same time, $CdCl_2$ treatment increased glycolysis, fatty acid biosynthesis, and ether lipid metabolism changed non-membrane bound and plasma membranes with increased SFA and reduced PUFA. Thus, increased FA content and changed FA composition of *E. gracilis* might be the defense mechanism of *E. gracilis* when exposed to Cd^{2+}, which helps reduce the metal damage to photosynthesis (Alho et al., 2019).

As we all know, under environmental pressure, FA accumulates as energy reserves, allowing algae to survive (De Carvalho and Caramujo, 2018). Increased intracellular lipid can maintain redox homeostasis because these biomolecules act as electron absorbers and help resist oxidative damage caused by ROS (Sun et al., 2018). Our transcriptome data showed NaCl also inhibited photosynthesis and changed glycerophospholipid, glycerolipid, and ether lipid metabolism. In the end, NaCl treatment resulted in the FA content increasing and FA composition alternating with a high SFA content ratio.

PRM could promote FA accumulation with no change of FA composition under low concentrations. Based on our transcriptome data, porphyrin and chlorophyll metabolisms were down-regulated while glycolysis was up-regulated under PRM treatment. We propose that the decreased chlorophyll inhibits photosynthesis, similar to NaCl, ethanol, or glucose treatments, and promotes FA accumulation. There is no alternation of any membrane metabolism among GO and KEGG pathways enriched from our RNA-seq data. This observation is consistent with no FA composition change under PRM treatment.

Interestingly, FA compositions showed significantly different and similar patterns under different treatments. Except for PRM treatments, all treatments in this study increased saturated rates 1.7~2.4 fold compared to the control. The lipids in green cells of *E. gracilis* grown in the light differ from those in etiolated cells (with no photosynthesis) grown in the dark. The FA of the photosynthesizing green *E. gracilis* cells is mainly of the unsaturated variety (Rosenberg, 1963). An early study showed a complex lipid pattern of *Euglena*: phosphatidylglycerol sulfolipid, and several glycolipids appeared to be the dominant lipids during photosynthesis (Helmy et al., 1967). *E. gracilis* was cultured under heterotrophic or phototrophic growth conditions using ethanol, glucose, or CO_2 as the primary carbon source. TFA analyses indicated that ethanol produced more highly unsaturated acids than glucose (Reitz and Moore, 1972). In *E. gracilis* synthesis, the more significant part of the cellular unsaturated fatty acids accompanies photosynthesis. Although it is not known whether unsaturated fatty acids participate directly in the photosynthetic mechanism, their synthesis is an essential factor in the economy of the photosynthesizing cells. Comparing the relative proportions of fatty acid species in a chloroplast fraction of green *E. gracilis* cells showed a significantly increased ratio of unsaturated to saturated homologs in the chloroplast (Rosenberg, 1963). The effects of ethanol and glucose on fatty acid profile showed significantly higher SFA accumulation (ΣSFA/(ΣMUFA+ΣPUFA) with glucose compared to ethanol (2.5 vs. 0.9) (Barsanti et al., 2000). In our previous report, in fully green photosynthetic *E. gracilis* cells under light conditions, desaturase activity was enhanced to generate a set of MUFAs and PUFAs (Zeng et al., 2016). Transcriptome analyses provide some clues about FA composition changes under treatments. Down-regulated photosynthesis was detected in E1, G1, and NaCl treatments for KEGG enriched pathways, which correlates with the significantly increased saturated FA in these treated cells.

The increase of SFA and the decrease of PUFA proportion mean that the saturation of biodiesel increases. It helps to change some indicators related to the combustion performance, like increasing the CN and lowering IN of biodiesel. This study finds that using suitable conditions to treat *E. gracilis* could improve not only the yield of *E. gracilis* FA can but alsobiodiesel quality. Based on the SN, IN, and CN, *E. gracilis* FA was evaluated as biodiesel. According to the biodiesel production standard, the performance of *E. gracilis* FA in the glucose treatment group is the best. Among them, both IN and CN of *E. gracilis* FA of G1.5 treatment reach the highest standards for biodiesel production in various countries. However, *E. gracilis* FA in all groups has the same shortcoming. The SN is too high to meet biodiesel standards in many countries.

5 CONCLUSION

The total fatty acids and related components of *E. gracilis* have increased under various treatment conditions with various TFA saturated rates and biodiesel fuel properties. Both iodine value and cetane number have reached the highest standard of current international biodiesel production under some treatments. Among them, TFA with glucose treatment is excellent, which can be used as an in-depth research object in biodiesel. The transcriptome analyses indicate that high-quality biodiesel could be achieved in *E. gracilis* by carefully designed cultivation medium and conditions, andbalancing the growth and photosynthesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/, PRJNA714494.

AUTHOR CONTRIBUTIONS

JW conceived the concept, and JH, YC, YL, JZ conducted the experiments and collected the data. MD analyzed the data. JW wrote the manuscript draft. AL and WF helped to collect materials and finish the manuscript. AL, JW, JZ, and MD offered help to this project and/or revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fceng.2022.884451/full#supplementary-material

Supplementary Figure S1 | Functional categorization of up- (red color) and down-regulated (blue color) genes in E. gracilis under different treatments based on gene ontology (GO) annotations. BP, biological process, CC, cellular components, MP, molecular functions.

Supplementary Figure S2 | Bubble plots of DEGs for visualizing KEGG pathway enrichments. Bubble size indicates the log size of the KEGG term, enrichment of the down-regulated genes (down KEGG), and up-regulated genes (up KEGG).

Supplementary Table S1 | The contents of fatty acids in the unit cell of E. gracilis under different treatment conditions (pg/cell). Treatments include supplementation with paromomycin (PRM1, 5, 25 as 1, 5, 25 μg/mL), ethanol (E0.5, 1.0, 1.5 as 0.5, 1, 1.5% vol/vol), glucose (G0.5, G1.0, G1.5 as 0.5, 1.0, 1.5% weight), CdCl2 (Cd0.5, 1.0, 1.5 as CdCl2 of 0.5, 1.0, 1.5 mM), or NaCl1.0 (NaCl as 1% weight), CN, control.

Supplementary Table S2 | The Log2FC (fold change) of selected fatty acid metabolism genes under different treatments, treatment vs. control.

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