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Nuclear mutagenesis and adaptive evolution improved photoautotrophic growth of *Euglena gracilis* with flue-gas CO_2 fixation

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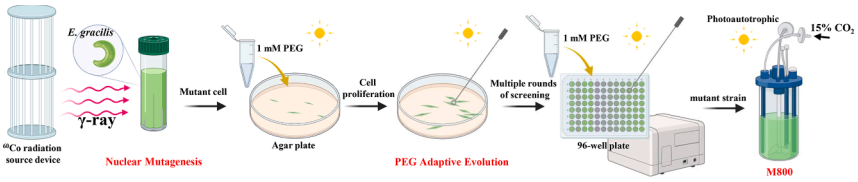
HIGHLIGHTS

- *E. gracilis* was modified with ^{60}Co γ-ray and PEG adaptive to obtain strain M800.
- Biomass and maximum CO_2 fixation rate of M800 were both 47% higher than wild strain.
- Biomass of M800 with 1 mM PEG was 2.31 g/L, which was 79.1 % higher than wild strain.
- M800 alleviate lipid peroxidation damage due to their higher antioxidant activities.
- Cell proliferation of M800 was promoted, the apoptosis and necrosis rates decreased.

GRAPHICAL ABSTRACT



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ABSTRACT

To effectively improve biomass growth and flue-gas CO_2 fixation of microalgae, acid-tolerant *Euglena gracilis* was modified with cobalt-60 γ-ray irradiation and polyethylene glycol (PEG) adaptive screening to obtain the mutant strain M800. The biomass dry weight and maximum CO_2 fixation rate of M800 were both 1.47 times higher than that of wild strain, which was attributed to a substantial increase in key carbon fixation enzyme RuBisCO activity and photosynthetic pigment content. The high charge separation quantum efficiency in PSII reaction center, efficient light utilization and energy regulation that favors light conversion, were the underlying drivers of efficient photosynthetic carbon fixation in M800. M800 had stronger antioxidant capacity in sufficient high-carbon environment, alleviating lipid peroxidation damage. After adding 1 mM PEG, biomass dry weight of M800 reached 2.31 g/L, which was 79.1 % higher than that of wild strain. Cell proliferation of M800 was promoted, the apoptosis and necrosis rates decreased.

1. Introduction

Microalgal carbon fixation is a CO_2 bio-storage technology that has attracted much attention in recent years, because some microalgae have high photosynthetic efficiency, rapid growth, and can efficiently and continuously absorb and convert CO_2 (Cheah et al., 2015; Khan et al., 2018), providing a potentially important method for the reduction of carbon emissions (Wang et al., 2018a). Among the many algal species that have been cultivated on a large scale, *Euglena gracilis* are receiving increasing interest due to their ability to tolerate low pH environments

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and produce the unique product, paramylon. In many algal cells, low pH conditions cause the inactivation of some key enzymes involved in CO_2 assimilation, such as RuBisCO, resulting in the loss of some photosynthetic functions (Liu et al., 2019; Miyachi et al., 2003). Paramylon is a high value product, as in addition to being used as a food and feed additive, it also plays a major pharmaceutical role in the treatment of HIV (Wang et al., 2018c) and some tumors (Quesada et al., 1976).

The commonly used approaches for promoting microalgal growth can be divided into two main areas: improvement of the algal strain itself or optimization of the external environment. The main methods currently applied to improve the CO_2 fixation capabilities of algal strains are genetic engineering, domestication and mutagenesis. Genetic engineering has successfully been used to sequence parts of some algal genomes, revealing the underlying gene expression for certain extrinsic traits (Guiheneuf et al., 2016), the complexity of the genetic engineering process, the demanding experimental conditions and high analytical cost, has limited the development and application of microalgae in the field of carbon fixation. Domestication can be a targeted and effective means of microalgal improvement, with previous studies having reported CO_2 gradient domestication of *E. gracilis* (Xin et al., 2023). The microalgal domestication process focuses more on improving tolerance to high CO_2 concentrations, than on biomass production. In contrast, mutagenesis is a relatively simple process, with a wide range of potential results achievable through the regulation of different mutagens, depending on the specific traits required (Cheng et al., 2019a). Nuclear mutagenesis has a high energy density and mutation rate, avoiding the possibility of microalgal contamination by chemical mutagen mixing. Gamma rays have the widest application potential for microalgal nuclear mutagenesis due to their strong capacity to penetrate and interact with cellular molecules (Cheng et al., 2016). While it is clear that nuclear irradiation mutagenesis can provide a useful method for the promotion of microalgal growth, it has not previously been applied and studied in the field of photosynthetic carbon fixation in *E. gracilis*.

Adding CO_2 absorbents to the algal solution is an effective way of promoting microalgal growth by optimizing the external environment, resulting in the improved conversion and utilization of flue gas CO_2 by microalgal cells. The addition of $NaHCO_3$ and NaOH to the medium has been found to increase the CO_2 fixation rate of *Chlorella* sp. (Nayak et al., 2018) and *Scenedesmus* sp. (Nayak et al., 2013), respectively, although $NaHCO_3$ was continuously consumed with cell growth and NaOH interferes with the pH of the medium. Monoethanolamine (MEA) (Choi et al., 2012) and triethanolamine (TEA) (Kim et al., 2013) have also been used as CO_2 absorbers to promote microalgal growth, although the carbamate intermediate products formed during the process were found to be toxic to microalgae. Polyethylene glycol (PEG) not only effectively increases CO_2 solubility in a cost-effective manner, but also has the advantages of being non-toxic to most organisms with only negligible levels of absorption by intact plants (Lee & Yeh, 2014). However, to date, PEG has mainly been utilized as an osmotic agent in studies on water relationships between plants and fungi, with little information available on its application in microalgal systems (Chen et al., 2003; Hohl & Schopfer, 1991). Kumar et al. first reported the effects of salinity and PEG on the growth of *Dunaliella salina* (Sunil Kumar & Dharmaraj, 2003). Lee and Yeh investigated the effect of PEG individually as a non-ionic osmoticum, on the growth of *Nannochloropsis oculata* (Lee & Yeh, 2014). Zhu et al. studied the promotive effect of PEG200(MW (g/mol) = 190–210) on the growth of *Nannochloropsis oceanica* and found that the promotion occurred due to an increase in dissolved inorganic carbon in the culture medium with 15 vol% CO_2 from coal-fired flue gas (Zhu et al., 2020). However, to the best of our knowledge, the effect of PEG on the photoautotrophic growth of acid-tolerant *E. gracilis* has not yet been studied and it remains unclear whether the promotive effect can be enhanced by mutation screening.

In this study, 60Co-γ-ray irradiation mutagenesis and PEG adaptive evolution were used to identify a high-performance mutant *E. gracilis* strain by screening, which could not only fully adapt to high CO_2 concentration culture environments, but also had the capacity for rapid photoautotrophic growth and carbon fixation. Subsequently, the effects of various concentrations of PEG on the photosynthetic growth of this algal strain were investigated under high carbon conditions. This will provide a useful supplement to the optimization of *E. gracilis* species and accelerate the large-scale practical application of *E. gracilis* for industrial scale CO_2 fixation.

2. Materials and methods

2.1. Mutation and PEG adaptive screening

The wild-type strain of *E. gracilis* used in this study was CCAP 1224/5Z. The mutant samples were obtained by nuclear irradiation mutagenesis of wild-type samples in logarithmic growth phase. Irradiation was performed at the Academy of Agricultural Science (Zhejiang, China), using 60Co γ-rays as the radiation source at a source intensity of 32 000 Ci. The samples were irradiated with 200, 300, 400, 600, 800, 1100 and 1500 Gy at a dose rate of 5 Gy/min. Three samples (30 mL each) were used for each irradiation dose. After standing overnight in the dark, mutant samples were transferred to a light incubator (TES-1334-a, Taiwan, China) for culture recovery and maintained for one month at 24 °C, with 60 $\mu mol \cdot m^2 \cdot s^{-1}$ light applied in a 12 h: 12 h light–dark cycle. Multiple rounds of screening for all mutant samples irradiated with all radiation doses (200, 300, 400, 600, 800, 1100 and 1500 Gy) were conducted on agar plates and 96-well plates, all of which contained 1 mM PEG as the screening condition, allowing the selection and isolation of the surviving mutant strain with highest growth rate and good PEG adaptation.

2.2. Culture conditions

E. gracilis was grown in 500-mL conical flasks containing 400 mL of autoclaved Cramer–Myers (CM) medium (Cramer & Myers, 1952) and varying concentrations of PEG (0, 1 or 2 mM). Cultures were maintained in an artificial greenhouse with continuous light exposure at 80 $\mu mol \cdot m^2 \cdot s^{-1}$ and a constant temperature of 24 ± 1 °C. PEG 200 was supplied by Aladdin Chemistry Co. Ltd. (Shanghai, China). The optical density of the initial *E. gracilis* inoculum at 750 nm (OD_{750}) was 0.3. Each conical flask was aspirated continuously using 15 vol% CO_2 (85 vol % N_2), with the flow rate controlled at 8 mL min^{-1} using a flow meter (SevenStar CS-200, China).

2.3. Measurement of e. Gracilis biomass dry weight and pH

The biomass dry weight (DW) of *E. gracilis* samples was measured daily using the drying method, as reported previously (Xin et al., 2023). The microalgal liquid (10 mL) was harvested and then dewatered via centrifugation (Beckman Avanti J26-XP, USA) for 10 min at 3000 × g. The cell pellet was washed three times with deionized water, dried at 80 °C for 72 h and then collected for mass measurement. The pH of the growth medium was measured daily using a pH meter (Mettler Toledo, FE20, Switzerland).

2.4. Measurement of pigment content and photosynthetic fluorescence

Chlorophyll and carotenoids were extracted and determined using previously reported methods (Xin et al., 2023). Pigment concentrations were measured by spectrophotometry following ethanol extraction (He et al., 2021).

Photosynthetic fluorescence parameters (Fs, Fm′, Fo, Fm and Fv/Fm) were measured via pulse modulation fluorometry (FMS-2, Hansatech, UK), allowing ΦPSII and non-photochemical quenching (NPQ) to be calculated according to Eqs. (1) and (2):

$$\Phi PSII = \frac{Fm' - Fs}{Fm'} \quad (1)$$

$$NPQ = \frac{Fm - Fm'}{Fm'} \quad (2)$$

2.5. Measurement of the expression of key enzymes

Enzyme extracts were prepared according to a previously described method (Xin et al., 2023), with crude enzyme extracts obtained for enzyme assays using an ultrasonic cell disruptor (UH-S2, AUTO Science, China) (Cheng et al., 2018).

Total protein concentrations were measured according to the bicinchoninic acid (BCA) method using a total protein assay kit. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) was determined using a RuBisCO assay kit, with spectrophotometric analysis. Malondialdehyde (MDA) was measured using a MDA assay kit, according to the thiobarbituric acid (TBA) method. Superoxide dismutase (SOD) was determined via the WST-1 method using a SOD assay kit (Yin & Yang, 2019).

2.6. Determination of cell cycle and apoptosis

The cell cycle of *E. gracilis* was determined using a cell cycle detection kit. Cells were collected by centrifugation at 1500 × g for 10 min and then washed twice with PBS. The cells were re-suspended in 0.3 mL PBS, then 1.2 mL anhydrous ethanol (−20 ℃) was added and the mixture was maintained overnight at −20 ℃ for fixation. Following the fixation phase, the mixture was centrifuged at 1500 × g for 5 min and the pellet was resuspended in 100 μL RNase A solution before being incubated for 30 min at 37 ℃. Next, 400 μL of propidium iodide was added and the reaction was carried out in the dark for 30 min at 4 ℃. The cell cycle of the sample was established using a flow cytometer (FACSCalibur, BD, USA).

Apoptosis of *E. gracilis* cells was identified using an Annexin V-EGFP/PI double staining apoptosis detection kit, following previously reported methods for sample preparation and detection by flow cytometry (Xin et al., 2023).

All kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.7. Measurement of paramylon content

Paramylon was extracted and measured according to a previously reported method (Xin et al., 2023). Cultured *E.gracilis* cells collected by continuous centrifugation were washed with deionized water. Followed by acetone enrichment and SDS precipitation, paramylon was dried and dissolved in 0.5 M NaOH. The paramylon determined using glucose as a standard(Zeng et al., 2016).

3. Results and discussion

3.1. Selection and isolation of mutant strain M800

In this study, low- to medium-dose gamma (γ) radiation treatments were used to induce mutations in *E. gracilis* cells, resulting in a large number of different non-directional mutations (Wang et al., 2018b). In order to obtain a high-performance mutant strain from the various mutant cells, that could be stably enriched with an efficient carbon fixation capacity, multiple rounds of selection were carried out along with PEG adaptive evolution, using high photosynthetic growth rates and PEG adaptation as the screening conditions. The growth of *E. gracilis* samples with air in CM medium supplemented with 1 mM PEG, is shown in Fig. 1, including samples treated with various irradiation doses and the wild-type strain. The biomass (DW) first increased and then decreased with increasing irradiation dose. The highest level of biomass production was achieved 0.57 g/L by the mutant strain which was

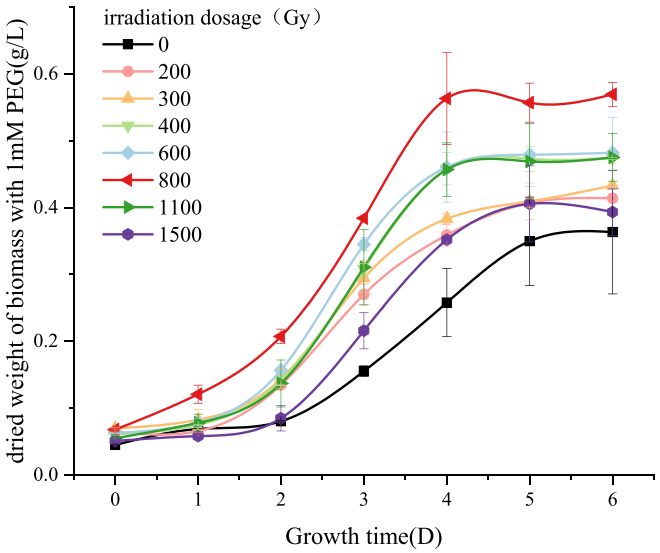

Fig. 1. Growth of *E. gracilis* in air after nuclear irradiation.

treated with a gamma ray dose of 800 Gy, exhibiting a 1.57-fold increase in biomass as compared to the wild-type strain (0.36 g/L). Gamma rays have very short wavelengths and high frequencies, resulting in the release of massive amounts of energy which is highly penetrative and can interrupt or damage DNA and proteins within cells, triggering genetic mutations and even cell death (Cheng et al., 2017). The induction of high-quality gene mutations that result in the development of stable and effective algal strains, is more probable using high-dose irradiation than low-dose. However, high-dose irradiation can cause severe cell damage and increase mortality due to the excess of photon energy. The experiment in Fig. 1 was intended to preliminarily evaluate the macroscopic performance of microalgae samples under different radiation doses, and was not a basis for the subsequent mutant screening process. In fact, multiple rounds of screening for all microalgal cells under all radiation doses (200, 300, 400, 600, 800, 1100 and 1500 Gy) were conducted during the experiment. the mutant algae strain with the highest biomass production was selected among all the cells, because the goal was to obtain the mutant strain with the highest CO_2 fixation capacity. The PEG-adapted mutant strain of *E. gracilis* finally selected was named M800 because it came from the sample with an initial radiation dose of 800 Gy. M800 exhibited the fastest photosynthetic autotrophic growth ability and therefore, was selected for further investigation.

3.2. Growth efficiency and carbon fixation capacity of M800

The photosynthetic growth and carbon fixation capacity of the wild-type strain and M800 strain in environments containing various PEG concentrations, are shown in Fig. 2. The wild-type strain reached 1.288 g/L and 1.293 g/L biomass (DW) after 6 days of incubation with 1 mM and 2 mM PEG, respectively, exhibiting an increase by 9.0 % and 9.4 % over the wild-type strain (with 0 mM PEG). The maximum rates of CO_2 fixation achieved were 0.57 and 0.79 g/L/d for wild-type strain with 1 mM and 2 mM PEG, respectively, exhibiting an increase by 4 % and 43 % as compared to the wild-type strain without the addition of PEG. When microalgae are cultured in an open system with a continuous supply of CO_2 gas, the rapid rate of CO_2 escape and the low rate of CO_2 utilization have been important factors preventing an increase in microalgal photosynthetic carbon fixation rates (Chisti, 2007). PEG can physically adsorb CO_2 and effectively increase CO_2 solubility in the culture medium. However, as shown in Fig. 2(a–b), the addition of PEG caused a slight decrease in the biomass production and CO_2 fixation rates of *E. gracilis* during the first 4 days of incubation, with the scale of decrease being inversely proportional to the PEG concentration added. During the

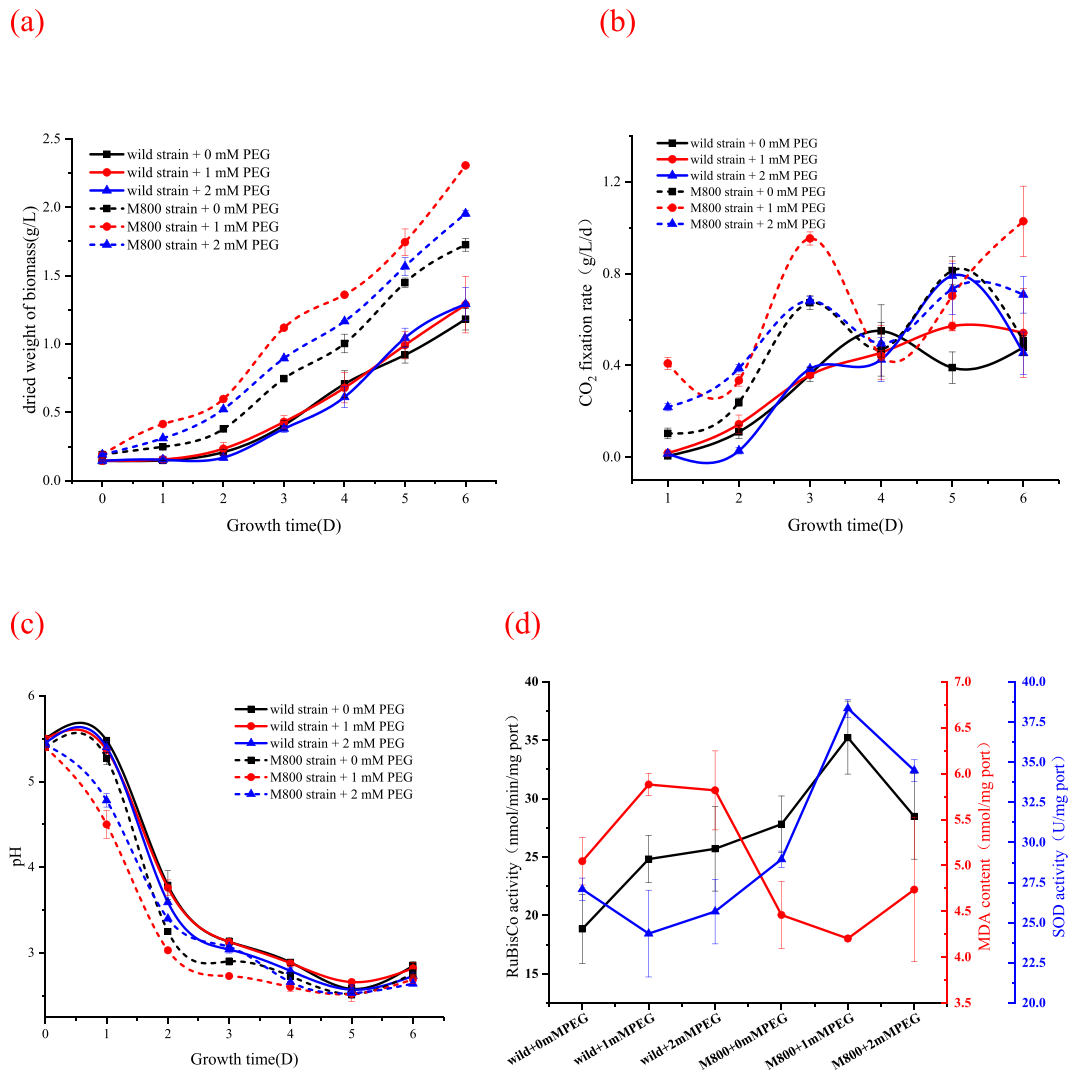

Fig. 2. Growth and carbon fixation capacity of M800 and wild strain with various PEG concentrations. (a) Dried weight of biomass; (b) CO_2 fixation rate; (c) pH; (d) Enzyme activity involved in photosynthetic and antioxidants.

4th to 6th day of incubation, the average carbon fixation rate of the wild-type strain was shown to increase in the presence of PEG, with the magnitude of improvement positively correlated to the increase in PEG concentrations. Maintaining the inorganic carbon concentration in the medium can promote the photosynthetic growth of microalgae, but only if cells have the capacity for efficient carbon conversion and utilization. Otherwise, excessively high carbon stress can induce lipid peroxidation damage, inhibit microalgal growth or induce apoptosis and necrosis (Wang et al., 2022). A previous study confirmed that 15 % CO_2 is a sufficiently high concentration to support the photoautotrophic growth of *E. gracilis* (Xin et al., 2023). When the concentration of CO_2 dissolved in the medium is increased, cells self-regulate by undergoing a growth lag during the early stage of culturing, allowing their adaption to the sudden increase in inorganic carbon availability. Therefore, the effect of PEG on *E. gracilis* growth was not significant in the early stages of culturing, while the later stages exhibited a significant increase in the carbon fixation rate and specific growth rate. However, it is noteworthy that the biomass (DW) of the wild-type strain cultured with 1 mM or 2 mM PEG, did not exhibit significant differences. The promotion of cell growth did not increase in accordance with increasing PEG concentrations, suggesting that the higher concentration of carbon adsorbed by 2 mM PEG provided a surplus of support for photosynthetic growth in the wild-type strain, which may have had a negative effect on cell growth.

For M800 cultured without PEG, the biomass (DW) and the maximum CO_2 fixation rate on the 6th day were 1.73 g/L and 0.81 g/L/d, respectively, both of which were approximately 1.47-fold higher than the wild-type strain without PEG. The biomass (DW) of M800 cultured with 1 mM and 2 mM PEG reached 2.31 g/L and 1.95 g/L, respectively, exhibiting an increase by 79.1 % and 51.2 % as compared to the wild-type strain cultured with 1 mM and 2 mM PEG, respectively. These results indicate that nuclear irradiation mutagenesis effectively produced a stable algal strain with the capacity for high growth and carbon fixation efficiency. In addition, as the mutant strain had already undergone PEG adaptive evolution during the strain screening process, it was capable of self-regulation in response to sudden increases in inorganic carbon concentrations in the culture medium. This was demonstrated by the fact that the addition of PEG, at both 1 mM and 2 mM concentrations, had a significantly greater effect on the growth of M800 than it did on the wild-type strain. Overall, The greatest increase in M800 growth was achieved by the addition of 1 mM PEG, achieving a maximum CO_2 fixation rate of 1.03 g/L/d, which was 27 % higher than that of M800 without the addition of PEG. In addition, the higher specific growth rate and CO_2 fixation rate in the early stages of culture of M800 in the presence of PEG, suggests that the growth lag observed in the early stages of wild-type strain culturing with PEG, was effectively mitigated. This allows the mutant strain to grow rapidly within a short period,

making *E. gracilis* suitable for application in large-scale industrial flue gas CO_2 fixation facilitates, as they require short harvesting cycles.

To further investigate the effects of nuclear irradiation and the addition of PEG on cellular photosynthetic carbon fixation, the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) was measured in *E. gracilis* cells after three days of incubation under different conditions. As shown in Fig. 2d, the RuBisCO activity of the mutant strain was higher overall than that of the wild-type strain, reaching 27.81 nmol/min/mg port in M800 (without PEG), corresponding to a 48 % increase over the wild-type strain (without PEG). The addition of 1 mM or 2 mM PEG to the wild-type strain increased RuBisCO activity by 32 % and 36 %, respectively. RuBisCO activity reached 35.23 and 28.47

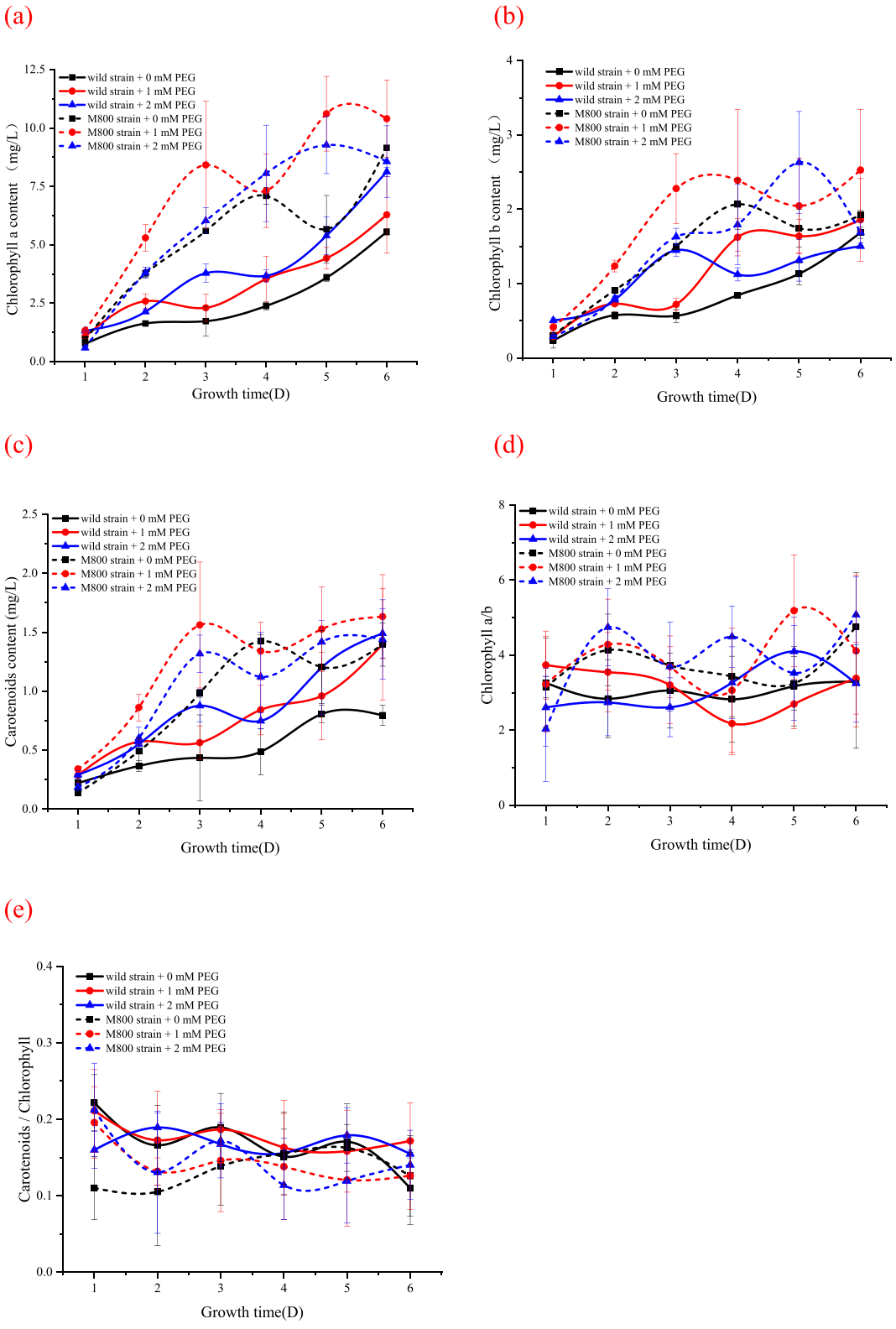

Fig. 3. Photosynthetic pigment of M800 and wild strain with various PEG concentrations.

nmol/min/mg port in M800 with 1 mM and 2 mM PEG, respectively, exhibiting an increase by 42 % and 11 % as compared to the wild-type strain supplemented with the same PEG concentrations, respectively. RuBisCO catalyzes the initiation of CO_2 reduction and plays a crucial role in the Calvin cycle (Vuppaladadiyam et al., 2018), serving as both a key carbon-fixing enzyme and a rate-limiting enzyme for photosynthesis and therefore, directly affects the rate of photosynthetic growth (Mistry et al., 2019). The mutant strain M800 was functionally enriched for differentially expressed photosynthetic genes, through a process of mutation with multiple rounds of selective screening, resulting in a more active RuBisCO transcriptional synthesis process. The high level of RuBisCO expression significantly enhanced the ability of the M800 mutant strain to convert and utilize carbon, fundamentally raising the threshold for photosynthetic growth of *E. gracilis* at 15 % CO_2. On this basis, PEG was added to the growth media to provide a more abundant and stable release of CO_2 near the RuBisCO active site. An increase in reaction substrate contributed substantially to the efficiency of RuBisCO-catalyzed CO_2 reduction, resulting in the accelerated synthesis of *E. gracilis* biomass. The constant supply of sufficient CO_2 allows RuBisCO to actively catalyze CO_2 reduction reactions rather than the oxidation reactions of photorespiration, facilitating efficient intracellular energy allocation (Michelet et al., 2013). PEG had a more sensitive and significant effect on the mutant strain than the wild-type, as the increased growth threshold alleviated the inhibitory effect of high carbon stress on *E. gracilis* cells due to high PEG concentrations. In addition, the PEG adaptive evolution process beneficially reinforced the capacity of mutant cells to self-regulate growth in high carbon environments.

3.3. Light energy conversion capability of M800

Photosynthetic pigments are required for the absorption and conversion of light energy by microalgal cells. As shown in Fig. 3a–c, the chlorophyll *a* (chl a), chlorophyll *b* (chl b) and carotenoid contents of M800 were higher than those of the wild-type strain under all three working conditions(0, 1 or 2 mM PEG). The maximum chl a, chl b and carotenoid contents of M800 without PEG were 9.15 mg/L, 2.07 mg/L and 1.42 mg/L, respectively, exhibiting an increase of 64.6 %, 22.5 % and 75.3 % as compared to the wild-type strain without PEG. Chl a is a core pigment and is involved in capture light energy, which then can be used for carbon fixation, while chl b is involved in antenna light energy absorption, to assist cells in capturing a broad spectrum of light wavelengths (Baer et al., 2016; Teo et al., 2014). Nuclear irradiation mutagenesis effectively promoted the synthesis of chl a and b in M800 cells, resulting in light energy absorption and photochemical reactions being simultaneously promoted. As shown in Fig. 3d, the chlorophyll *a*/b ratios of M800 under all three working conditions were higher than those of the wild-type strain, suggesting that the mutant M800 cells had a greater capacity for light energy conversion, directly enhancing photosynthesis. Carotenoids supplement the absorption of light energy wavelengths that chlorophyll cannot utilize, but more importantly protects photosynthetic apparatus against oxidative damage (Varela et al., 2015). Therefore, the carotenoid/chlorophyll ratio reflects the light utilization efficiency of cells (Filella et al., 2009). The Carotenoid/chlorophyll ratio of M800 was generally lower than that of the wild-type strain, indicating that nuclear mutagenesis enabled M800 cells to survive in high CO_2 concentration environments without consuming too much energy to quench reactive oxide species for light protection. Therefore, nuclear mutagenesis retained the photosynthetic reaction focus of algal cells and improved their photosynthetic carbon fixation capacity. As shown in Fig. 3(a, b), the addition of 1 mM PEG increased chlorophyll concentration in both M800 and the wild-type strain. The addition of appropriate amount of PEG can effectively increases CO_2 solubility in the culture medium, which can promote the growth of microalgae (Zhu et al., 2020). The increased availability of inorganic carbon as a photosynthetic substrate provides positive feedback to the cellular Calvin cycle (Vuppaladadiyam et al., 2018). This change may lead to upregulated levels of chlorophyll synthesis to match increased carbon fixation in cell growth, since chlorophyll is an indispensable mediator of light energy absorption and conversion[37]. Although the chlorophyll *a*/b ratio and the carotenoid/chlorophyll ratio fluctuated in the wild-type strain after the addition of PEG, the average ratios were comparable to those of the wild-type strain without PEG addition and did not increase significantly. The chlorophyll *a*/b ratio of M800 was upregulated by the addition PEG, indicating that the PEG adaptive evolution process amplified the effect of PEG on the promotion of light energy conversion. Unlike the wild-type strain, carotenoid concentrations did not increase consistently after the addition of PEG to M800, with a lower carotenoid/chlorophyll ratio observed at the end of the culture period than in the absence of PEG. This indicates that PEG also leads to a rapid increase in the carotenoid content of cells in the early stages of culture, which acts as an antioxidant in response to changes in the external inorganic carbon environment. However, as M800 had previously undergone PEG self-adaptation, the process of self-regulation was shortened and cells rapidly regained their light utilization efficiency and photochemical reaction rates.

3.4. Photosystem II photochemical efficiency of M800

Chlorophyll fluorescence in *E. gracilis* was analyzed in conjunction with the photosystem II photochemical efficiency parameters ΦPSII and Fv/Fm (Fig. 4a, b), as well as the fluorescence quenching parameter NPQ (Fig. 4c). The ΦPSII and Fv/Fm of M800 were higher than in the wild-type strain under all three conditions, maintaining the higher levels throughout almost the entire culture period. The maximum ΦPS II and Fv/Fm values of M800 without PEG were 0.558 and 0.734 respectively, which were 14 % and 13 % higher than in the wild-type strain without PEG. This indicates that nuclear irradiation can increase the actual quantum efficiency of charge separation in the PS II reaction center, facilitating non-cyclic electron transfer and carbon assimilation processes. Furthermore, the increased the maximum quantum efficiency of PS II in M800 implies that the energy capture efficiency of the open PS II reaction center was also increased. The addition of PEG can also increase ΦPSII and Fv/Fm values, promoting intracellular electron transfer, increasing the number of cellular light reaction centers and the activity of the quinone pool, and enhancing photosynthetic carbon sequestration in *E. gracilis*. Furthermore, due to the sensitivity of Fv/Fm to changes in external environmental factors such as light, temperature and nutrient conditions, the ratio is often used to assess the magnitude of cellular self-regulation in response to external experimental conditions. As shown in Fig. 4, with the addition of PEG a slightly smaller change in Fv/Fm was observed in M800 than the wild-type strain, which may be due to the PEG adaptive process providing M800 with the capacity to rapidly adjust its PS II system in response to increased inorganic carbon concentrations. The basic site of NPQ is the complementary antenna, with the process being largely independent of the redox state of the QA. NPQ can reliably reflect changes in the fluorescence fraction and thermal dissipation of non-photochemical quenching. The mutant strain M800 possessed a lower capacity for NPQ during cultivation, which may allow a more active cellular photochemical response. Furthermore, NPQ is an important tool for cells to regulate their photosynthetic quantum efficiency, with its down-regulation usually associated with an increase in cellular light utilization efficiency (Goncalves et al., 2016). The substantially higher light utilization efficiency of M800, as compared to the wild-type strain, was attributed to the difference in NPQ capacity caused by nuclear irradiation increasing PsbS protein expression. The functional enrichment of differentially expressed genes by nuclear irradiation and selective screening in the mutant strain of *E. gracilis*, efficiently amplified the expression of elements related to cell response processes. The NPQ of M800 with the addition of 1 mM PEG was lower than that of M800 without PEG, with the low level of fluorescence quenching being beneficial for efficient photosynthetic carbon fixation. However, the fluorescence quenching of M800 remained at a much higher level for

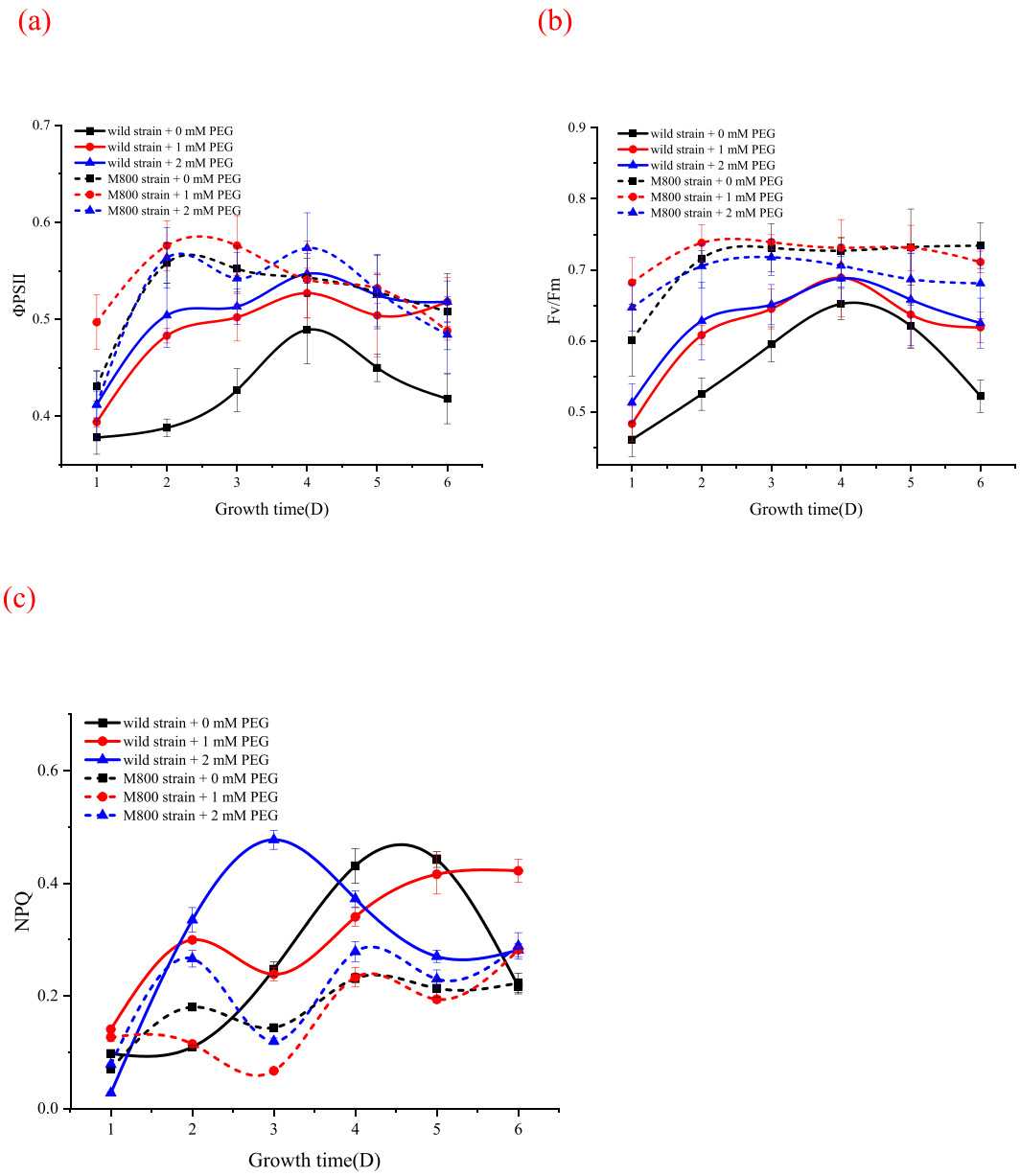

Fig. 4. Photochemical parameters of (a) ΦPSII, (b) Fv/Fm, and (c) non-photochemical quenching (NPQ) of M800 and wild strain with various PEG concentrations.

most of the culturing period with the addition of 2 mM PEG. The effect of PEG concentration on fluorescence quenching appeared to be more stable in the mutant strain, but in the wild-type strain, PEG concentration had no clear similar effect on NPQ. The fact that *E. gracilis* growth promotion was not positively correlated with PEG concentration, provides a novel insight from a cellular perspective and an important focus for further research.

3.5. Antioxidant capacity and the stability of photosynthetic apparatus in M800

The effects of nuclear mutagenesis and PEG addition on antioxidant activity and the extent of cell damage, were analyzed based on the measurement of MDA concentration and SOD activity in *E. gracilis* cells under each working condition. As shown in Fig. 2d, the MDA content of M800 was consistently lower than that of the wild-type strain in all cases. The MDA content of M800 with the addition of 0 mM, 1 mM and 2 mM PEG were 4.46, 4.20 and 4.74 nmol/mg port, respectively, which accounted for only 88 %, 71 % and 81 % of the levels in the corresponding wild-type strain, respectively. The SOD activities were also consistently higher in M800 than in the wild-type strain, 28.9, 38.3 and 34.5 U/mg, with the addition of 0 mM, 1 mM and 2 mM PEG, respectively, exhibiting an increase by 7 %, 58 % and 34 % as compared to the wild-type strain under the same conditions. Both enzymatic and non-enzymatic cell systems are capable of producing oxygen radicals, which can trigger lipid peroxidation through the peroxidation of polyunsaturated fatty acids in the membrane tissue. In addition, the breakdown of lipid hydroperoxides caused by lipid peroxidation can also impair cellular metabolism and function, leading to cell damage and even death. High carbon concentration environments can induce an increase in microalgal photorespiration, leading to an increase in the degree of peroxidation and affecting the growth of microalgae (Cheng et al., 2019b). The MDA content increased and the SOD activity decreased in the wild-type strain following the addition of 1 mM PEG, while the MDA content decreased and the SOD activity increased when the PEG concentration was increased to 2 mM. This indicates that *E. gracilis* cells are able to self-regulate to some extent in response to PEG-induced lipid peroxidation, by increasing carbon sequestering and

the activity of antioxidant enzymes, mitigating damage from oxygen radicals. However, it may be speculated that the self-regulation capability of cells is relatively modest and therefore, the growth promoting effect of PEG did not increase in a concentration-dependent manner in either the wild-type strain or the mutant M800 strain. The high performance mutant strain was selected due to its abilities for efficient RuBisCO carboxylation and rapid carbon conversion, alleviating damage to the cell biofilm from lipid peroxidation. In addition, the higher level of intracellular SOD activity in M800 indicates a higher antioxidant capacity, protecting biofilm systems such as the cystoid and cell membranes, to maintain the expression of key functions, such as photosynthesis, and the overall stability of the cell itself. In particular, when M800 was exposed to the same concentration of PEG (1 mM) as during the PEG adaptive evolution process, both of these advantages were maximized, resulting in a substantial increase in the process of carbon fixation into biomass.

3.6. M800 Cell cycle, apoptosis and necrosis

Flow cytometry was used to detect changes in the cell cycle, apoptosis and necrosis of *E. gracilis* cells under six working conditions. As shown in Fig. 5a, the eukaryotic cell cycle incorporates processes of replication and division, which usually consists of four phases: G1 phase (gap 1), S phase (DNA synthesis), G2 phase (gap 2) and M phase (mitosis)(2011). As shown in Fig. 5a, compared to the wild-type strain under the three conditions, the mutant M800 strain with the

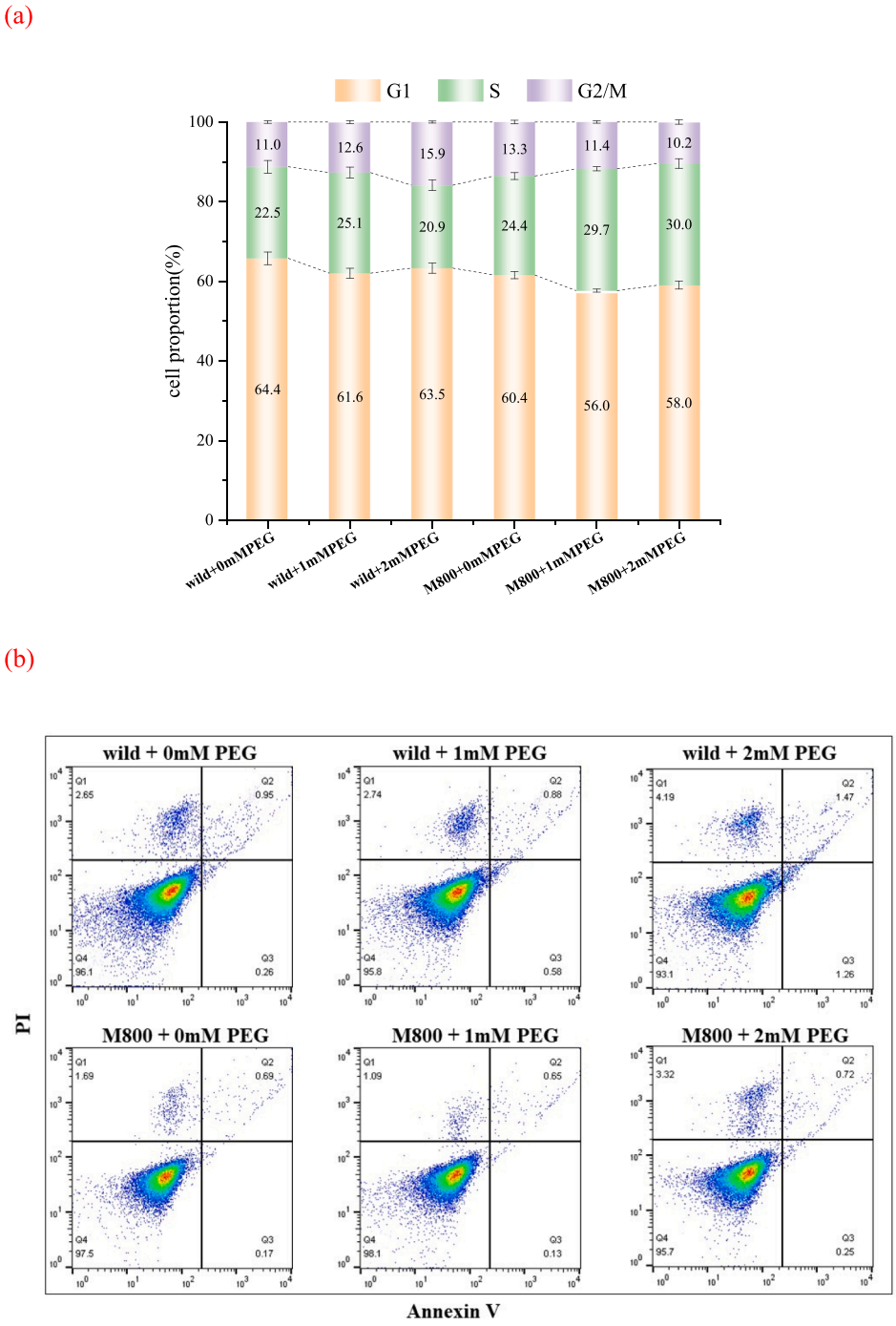

Fig. 5. (a)Cell cycle and (b) apoptosis of M800 and wild strain with various PEG concentrations.

corresponding PEG concentration exhibited a reduced G1 phase and an increased S phase. After synthesizing RNA and ribosomes in the G1 phase and accumulating the energy necessary for replication, cells enter the S phase to complete DNA replication and histone synthesis, nucleosome assembly and subsequent division (G2/M). The ability of the mutant strain to enter the S phase from the G1 phase in a timely manner and maintain a vigorous capacity for division, indicates that mutant M800 cells had less irreparable DNA damage. Cyclin-dependent kinases (CDKs) regulate cell cycle progression by phosphorylating specific substrates, which then bind to cyclin to form a cyc-CDK complex(2011). Nuclear irradiation mutagenesis ameliorated chromosomal DNA damage in M800 in high carbon concentration environments, enhanced cycE-CDK2 complex activity, increased the transition of cells to the S phase and effectively promoted cell proliferation. The PEG adaptive evolution process also strengthened the promotion effect of low PEG concentrations on *E. gracilis* cell proliferation, while higher PEG concentrations resulted in the mutual adhesion and fusion of cells and thus, impeded the completion of cell division.

The survival, apoptosis and necrosis of *E. gracilis* cells are shown in Fig. 5b, with region Q1, Q2, Q3 and Q4 representing necrosis, late apoptosis, early apoptosis and live cells, respectively. A comparison of the flow cytograms shows that the apoptosis and necrosis rates of M800 in a 15 % CO_2 environment, were lower than those of the wild-type strain. Apoptosis is a programmed death process initiated autonomously by cells to better adapt to their environment (Yin & Yang, 2019), in which cells activate suppressor and effector caspases, leading to cellular shrinkage and chromatin compression (Segovia & Berges, 2009). Cell necrosis is self-destructive form of cell death due to disordered intracellular changes caused by passive stress under pathological conditions. Due to its greater CO_2 absorption and conversion capacity, the M800 mutation reduced the pressure on cells to select apoptosis pathways for environmental adaptation, while also alleviating the irreversible damage caused by external environment stress, e.g., high carbon concentrations. M800 cells contained less irreparable DNA damage than the wild-type strain, which also contributes to the lower rate of apoptosis in M800 cells. A large proportion of cells that fail to enter the S phase due to the presence of irreparable damage, enter an apoptotic state. In the wild-type strain, the addition of low PEG concentrations had little influence on cells, while high PEG concentrations accelerated apoptosis and necrosis. However, this was not the case in M800, where the lowest rates of apoptosis and necrosis were observed in cells in the presence of low PEG concentrations, due to the strong carbon fixation capacity and environmental adaptability obtained during the PEG adaptive evolution process.

3.7. Paramylon content of M800

The contents of paramylon were measured in M800 and the wild-type strain under six different working conditions in the middle (day 4) and the end (day 6) of the culture period. As shown in Fig. 6, the paramylon content of M800 was higher than that of the wild-type strain, with M800 having the highest content of 0.55 g/L with the addition of 1 mM PEG. This was attributed to the fact that paramylon is a storage polysaccharide that is synthesized in large quantities in *E. gracilis* during rapid growth (Grimm et al., 2015; Wang et al., 2018c). Previously reported research has shown that the paramylon synthesis process is influenced by temperature and light (Feuzing et al., 2022). In the present study, it was demonstrated that CO_2 content is also an important environmental factor affecting paramylon production. Establishing methods to promote paramylon synthesis while efficiently fixing carbon is becoming an increasing focus and research issue for industrial production, as currently photoautotrophic culturing processes produce *E. gracilis* with a low paramylon content.

Over the past few decades, microalgal biomass as a natural resource for the production of high-value metabolites has emerged in the global market in the form of various commercial products (Bajhaiya et al., 2017; Fan et al., 2022). The global market of microalgal merchandise has been estimated to attain USD 1143 million by 2024 (Fan et al., 2022; Mehta et al., 2017). Therefore, it can be expected that in large-scale industrial microalgal cultivation with a shorter culture period (around 2–4 days), *E. gracilis* strain with higher biomass yield and paramylon content will be highly competitive. The optimization of algal species by a combined approach of nuclear mutagenesis and PEG-adaption, may offer novel solutions that support the use of *E. gracilis* as a biogenic carbon fixation agent for the development of high value-added products. The optimization of algal species by a combined approach of nuclear mutagenesis and PEG-adaption, may offer novel solutions that support the use of *E. gracilis* as a biogenic carbon fixation agent for the development of high value-added products.

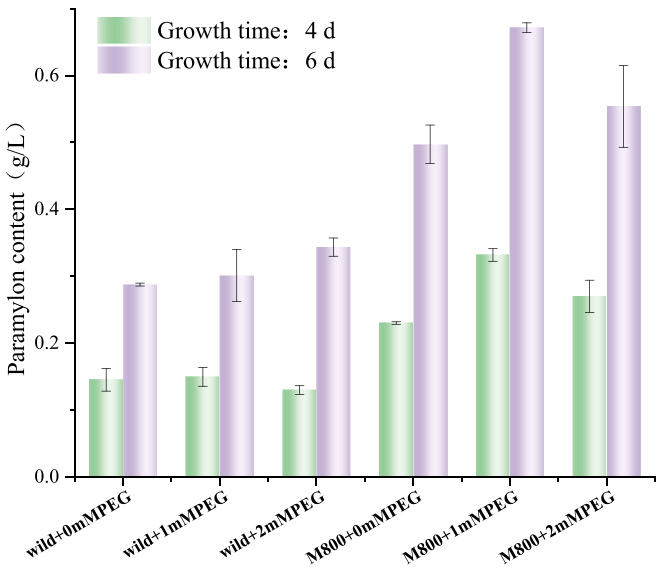

Fig. 6. Paramylon content of M800 and wild strain with various PEG concentrations.

4. Conclusions

^{60}Co-γ radiation mutagenesis remarkably enhanced the photosynthetic carbon fixation growth of the *E. gracilis* by inducing mutations, and the adaptive evolution process further improved the adaptability of the mutant strain to high carbon environments and alleviated the cellular damage caused by high carbon stress. The biomass and maximum CO_2 fixation rate of M800 were 1.47 times higher than that of wild strain. After adding 1 mM PEG, the biomass dry weight of M800 was 79.1 % higher than wild strain. These results are conducive to the application of acid-tolerant *E. gracilis* in the field of biological fixation of industrial flue gas CO_2.

CRediT authorship contribution statement

Kai Xin: Conceptualization, Methodology, Data Curation, Formal analysis, Writing – original draft. **Jun Cheng:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision. **Ruhan Guo:** Writing – review & editing. **Lei Qian:** Writing – review & editing. **Yulun Wu:** Writing – review & editing. **Weijuan Yang:** Supervision.

Declaration of competing 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.

Data availability

No data was used for the research described in the article.

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