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Full Length Research Paper

Optimization of the culture conditions of a *Chlamydomonas* high oil content ultraviolet mutant CC124-M25 and polymorphism analysis by inter-simple sequence repeat (ISSR)

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Microalgal oils have been considered as a promising feedstock for biodiesel production, which can potentially completely substitute fossil fuels. In the present study, 4500 mutants were obtained from *Chlamydomonas reinhardtii* CC124 through ultraviolet (UV) induced mutagenesis. After screening these mutants using HSM and HSM-N media, a mutant CC124-M25 with high oil content was selected under an HSM-N medium condition. Compared with the wildtype CC124, the mutant CC124-M25 has a similar growth rate, but the oil content increased by 10%, reaching up to 42%, which has a good prospect for biodiesel production. Optimization studies showed that the nitrogen-free medium with 20 mmol/L potassium element, called N0P20 medium is the most suitable for oil accumulation in the mutant, in which the oil content can reach a maximum of 0.5 g/g. The oil content of the mutant cells grown in the medium contain 2 g/L sodium acetate also can reach a maximum of 0.5 g/g, suggesting that 2 g/L sodium acetate is optimum for oil accumulation. In addition, ISSR markers were used to analyze the difference between the mutant CC124-M25 and the wildtype CC124. In total, 4 out of 100 primers could amplify distinct bands with good polymorphsim. The results showed that the polymorphism information content or CC124 was 78% and that for the mutant CC124-M25 was 50%, suggesting the genetic stability of the mutant obtained by UV mutagenesis.

Key words: *Chlamydomonas reinhardtii*, UV mutagenesis, orthogonal, inter-simple sequence repeat (ISSR).

INTRODUCTION

Energy crises and environmental problems have become increasingly prominent because of the continued use of unsustainable petroleum-sourced fuels. Scientists have made efforts to look for alternative renewable energy sources. In recent years, biodiesel has gained immense popularity because of its renewable and carbon dioxide-neutral properties. The current feedstock for commercial biodiesel includes waste cooking oil, animal fat, and various oleaginous species such as soybean and rapeseed. However, these raw materials cannot meet the needs of the rapid development of biodiesel production (Deng et al., 2011a; Chisti, 2007; Mata et al., 2009). Microalgae have been considered as a promising feedstock for biodiesel production because of their high oil content, rapid biomass production, and high efficiency photosynthesis (Deng et al., 2009); they have been proved to have the potential to replace fossil fuels completely.

Microalgae-derived biodiesel accounts for only a small portion of the world energy market because it has higher price than today's fossil fuel. Decreasing the cost of biodiesel production from microalgae is most crucial. To address this problem, obtaining microalgal strains with the combination of high oil content and fast growth rate, and developing bio-refining technology and an algal

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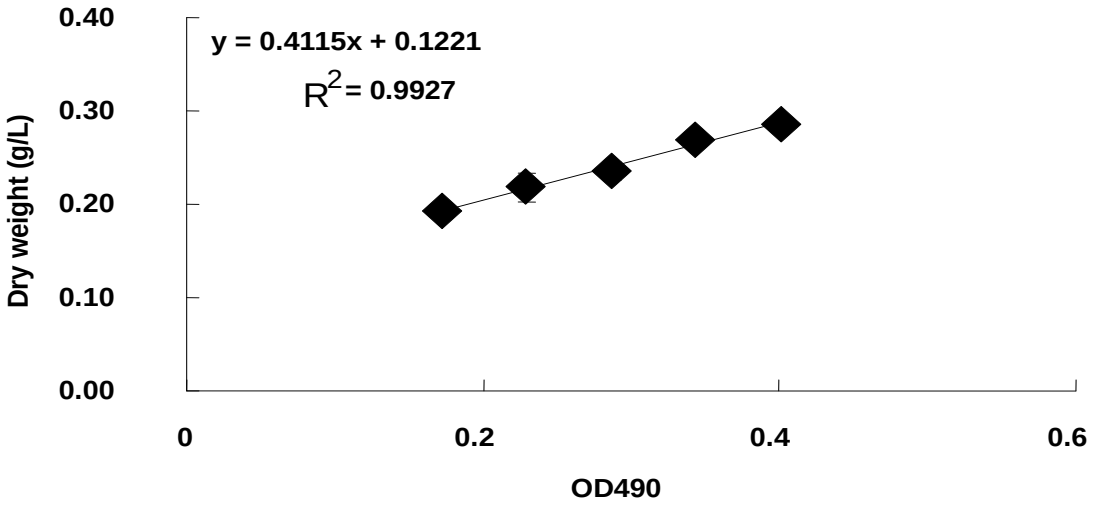

Figure 1. Linear relationship between weight of dry biomass and OD 490 from *C. reinhardtii* CC124.

photo-bioreactor seem to be efficient strategies, as feedstock costs account for approximately 75% of the total production (Fabregas et al., 1989).

Chlamydomonas reinhardtii, a unicellular eukaryotic microalgae, can utilize light energy to grow autotrophically or utilize an organic carbon source, such as sodium acetate and glucose, and organic nitrogen (N), such as urea, to grow heterotrophically (http://www.chlamy.org/ 2010.09.18) (Klinkert et al., 2004). It appeared about 10 million years ago, and currently it has developed into a model organism to study various life processes, including photosynthesis, flagellar assembly, phototaxis, and circadian rhythms, because of its clear genetic background. *C. reinhardtii* is a unique biological material that contains three genetic systems located in the nucleus, chloroplast, and mitochondria (Merchant et al., 2007). In addition, it has rapid growth, a short breeding cycle, and low-cost cultivation (Huang, 2010; Tan et al., 2007). Owing to its various advantages, oil accumulation in *C. reinhardtii* has attracted interest from many scholars (Siaut et al., 2011; Li, 2010). Xue et al. (2005) measured oil content by the weight of dry biomass in *C. reinhardtii*, *Spirulina* sp., *Saccharomyces cerevisiae*, and *Rhodotorula glutinis* using soxhlet extraction method. The results shows that the highest oil content was obtained in *C. reinhardtii*, accounting for 0.38 g/g by weight of the dry biomass.

Medium composition affects the growth of algae cells, the content and compositation of fatty acids (Fabregas et al., 1986, 1989; Hikfors et al., 1986). The changes in pH value and salinity of medium vary with medium composition. The weight of biomass and oil content of miciroalgae strains can be dramatically enhanced by optimizing the medium composition. Zhang et al. (2008) reported that, by optimizing the composition of medium, the biomass weight of *Chlorella pyrenoidosa* No. 2 strain increased from 3.73 to 6.56 g/L, and the oil content increased by 19.75%, from 40.15 to 59.90%. In the present study, ultraviolet (UV) induced mutants were obtained, and the cultural condition was optimized, moreover, ISSR markers used to analyze the difference between the mutant and the wildtype were discussed.

MATERIALS AND METHODS

Microalgal strain, cultivation condition, and biomass measurement

Microalgae strain, *C. reinhardtii* CC124 (137C) kept in our laboratory was used in this study (Harris, 1998). Cells grown on agar plate were inoculated into a 2 mL centrifuge tube with 1 mL ddH_2O and were mixed well by pipetting gently. The mixtures were then inoculated into 100 mL Erlenmeyer flasks containing 50 mL of HSM (Harris, 1998) and N-deficient HSM (HSM-N) media, respectively. All cultures were maintained in an incubator shaker with 230 rpm at 24°C and were exposed to continuous illumination at a light intensity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The HSM medium is composed of NH_4Cl 0.500 $g.L^{-1}$, $MgSO_4 \cdot 7H_2O$ 0.020 $g.L^{-1}$, $CaCl_2 \cdot 2H_2O$ 0.010$g.L^{-1}$, K_2HPO_4 1.440 $g.L^{-1}$, KH_2PO_4 0.720 $g.L^{-1}$, C2H3NaO2 2.000 $g.L^{-1}$, H_3BO_3 0.001$g.L^{-1}$, $MnCl_2 \cdot 4H_2O$ 0.005 $g.L^{-1}$, $ZnSO_4 \cdot 7H_2O$ 0.022 $g.L^{-1}$, $FeSO_4 \cdot 7H_2O$ 0.005 $g.L^{-1}$, $CoCl_2 \cdot 6H_2O$ 0.002 $g.L^{-1}$, $Na_2Mo_7O_{24} \cdot 4H_2O$ 0.002 $g.L^{-1}$, and Na_2•EDTA 0.050 $g.L^{-1}$. The HSM-N (N deficiency) medium is composed of NaCl 0.547 $g.L^{-1}$, $MgSO_4.7H_2O$ 0.020 $g.L^{-1}$, $CaCl_2.2H_2O$ 0.011 $g.L^{-1}$, K_2HPO_4 1.440 $g.L^{-1}$, KH_2PO_4 0.720 $g.L^{-1}$, $CH_2COONa.3H_2O$ 2.000 $g.L^{-1}$, H_3BO_3 0.001 $g.L^{-1}$, $MnCl_2.4H_2O$ 0.005 $g.L^{-1}$, $ZnSO_4.7H2O$ 0.022 $g.L^{-1}$, $FeSO_4.7H_2O$ 0.005 $g.L^{-1}$, $CoCl_2.6H_2O$ 0.002$g.L^{-1}$, $Na_2MoO_4.2H_2O$ 0.002 $g.L^{-1}$, and Na_2.EDTA 0.050 $g.L^{-1}$.

Biomass concentration (g/L) was determined by measuring the optical density of samples at 490 nm (OD490). To generate the standard curve, a series of *C. reinhardtii* CC124 samples of different biomass concentrations were collected. Their OD490 value and cell dry weight were determined gravimetrically after drying the algal cells to plot the standard curve of OD490 versus biomass concentration (g/L). This experiment was repeated three times (Figures 1 and 2).

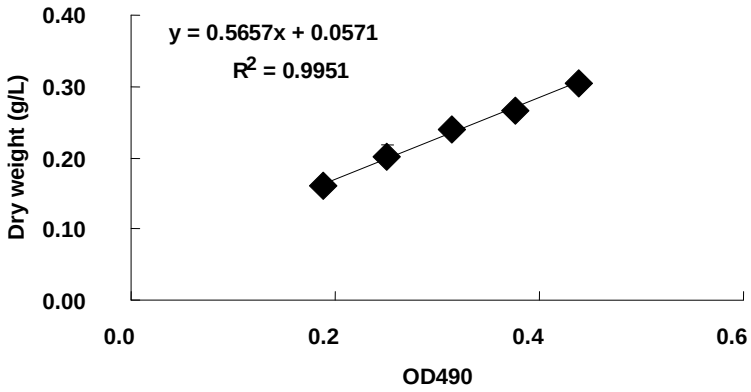

Figure 2. Linear relationship between weight of dry biomass and OD 490 from *C. reinhardtii* CC124-M25. Good line relationships exist between weight of dry biomass and OD 490 from both CC124 and the mutant CC124-M25.

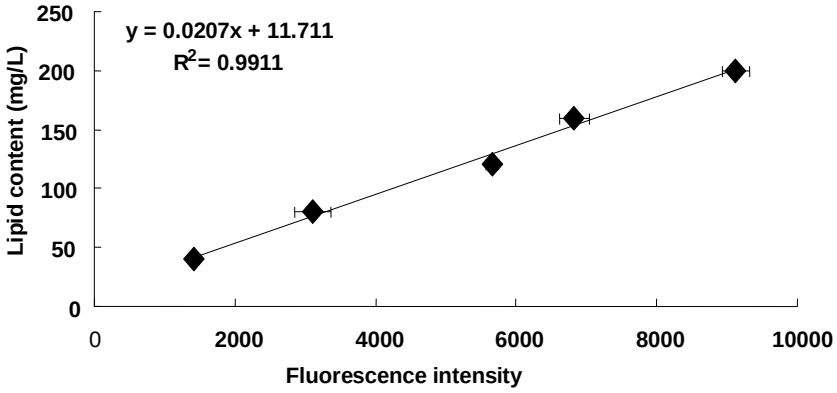

Figure 3. Linear relationship between lipid content and FD intensity.

Content of neutral lipid analysis

A Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) fluorescence method was applied in the determination of neutral lipids (Gao et al., 2008; Deng et al., 2011b). Algal cells of 200 μl were sampled at a 48 h interval during the cultivation period and were stained with 0.2 μl of 0.1 mg/mL Nile red for 10 min. 1.8 μl of DMSO was added before staining. Fluorescence (FD) was measured on a GloMax®-Multi Detection System (Promega, USA), whose excitation and emission wavelength were 470 and 570 nm, respectively. FD was calculated by the following equation: FD (470/570) = (A2-A1), where A2 is the FD value of algal cells after staining with Nile Red, and A1 is that of the algal cells before staining with Nile Red. The lipid content of the algal cell was calculated using a standard curve of FD value versus lipid content. For the standard curve generation, Triolein (Sigma, USA) was used as the lipid standard (Figure 3). In the microscopy assay, after staining the cells with Nile Red (10 μg/mL final concentration), images were acquired using a Nikon 80i Fluorescence Microscope. Nile Red signals were captured using an excitation wavelength of 480 nm; emission was collected between 560 and 600 nm (Huang et al., 2009; Chen et al., 2009).

Detection of total carbohydrate

Anthrone solution was prepared by dissolving 0.2 g of anthrone (9, 10-dihydro-9-oxoanthracene) in 100 ml sulfuric acid. The reagent was freshly prepared each day and used within 12 h (Yemm and Willis, 1954). The solution was allowed to stand for 45 min until it was perfectly clear. Solution of 10 ml was pipetted into thick-walled Pyrex tubes (150 x 25 mm) against 5 ml of Levoglucosan standard solutions (0.1, 0.2, 0.3, 0.4, 0.5, 1, 1.5, 2, 2.5, and 3 mg/l). The solutions were then transferred to a 100°C water bath for 6 min to produce the green dye, followed by incubation at 4°C for 5 min. All samples were read in a calorimeter at 620 nm against the deionized water. Then, the calibration curve (levoglucosan concentration versus absorbance) obtained from data for standard glucose solutions (0.1 to 3 mg/l) were drawn. This assay was carried out to determine the concentration of levoglucosan in particulate form 10 (PM) and residue samples using an improved technique. The order of addition of reagent proven to be essential for data reproducibility corrections was made for noncarbohydrate materials that could be present in the sample (Fartas et al., 2009).

Extraction and detection of total protein

Algal proteins were extracted essentially as described by Allen et al. (2007). Cells were collected by centrifugation at 1000 xg for 5 min, washed in 10 mM sodium phosphate at pH 7.0, and resuspended in the same buffer to a concentration equivalent of 4 x 10^8 cells/ml. The cells were then broken by sonication (microtip; 30% intensity; two cycles of 30 s). Extracts were separated into soluble and

Table 1. Levels of factors.

Levels	N (mmol/L)	P (mmol/L)
1	0.00	10
2	0.50	20
3	1.00	30

Table 2. The concentration of N and P in orthogonal experiment.

Experiment code	NH_4Cl (g/L)	$K_2HPO_4.3H_2O$ (g/L)	KH_2PO_4 (g/L)	Kcl (g/L)
N0P10	0.00	1.39	0.53	2.40
N0P20	0.00	2.78	1.06	1.20
N0P30	0.00	4.17	1.59	0.00
N1/2P10	0.27	1.39	0.53	2.40
N1/2P20	0.27	2.78	1.06	1.20
N1/2P30	0.27	4.17	1.59	0.00
N3/2P10	0.53	1.39	0.53	2.40
N3/2P20	0.53	2.78	1.06	1.20
N3/2P30	0.53	4.17	1.59	0.00

insoluble protein fractions by centrifugation (10,000 xg, 10 min). The pellet was washed once and resuspended to the same volume as that of the soluble fraction. Protein concentration was determined using the Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, CHN).

Chlorophyll FD

Cells grown to the exponential phase were collected by centrifugation at 3,000 ×g for 5 min. Pellets were resuspended in 80% acetone for several hours until the cells turned gray in the dark. Chlorophyll FD was measured by absorbance OD_{663}, OD_{646}, and OD_{750} using a 752N UV-visible spectrophotometer (Shanghai Precision and Scientific Instrument Co., Ltd. CHN) and calculated with the following formula: Chla(mg/L)= 12.21*(OD663-OD750) – 2.81*(OD646-OD750), Chlb(mg/L) = 20.13*(OD646-OD750) – 5.03*(OD663-OD750) (Maxwell and Johnson, 2000; Lichtenthaler, 1987).

UV irradiation mutagenesis

According to the description of Harris (1998), microalgal cells of 1 mL in a logarithmic phase were placed in a 9 cm Petri dish, forming a thin layer covering the bottom. The dishes were exposed to a UV-A lamp for 0, 5, 10, 15, 20, 30 and 40 min, respectively. After UV irradiation, cells were placed in the dark for 12 h and then grown on HSM (Harris, 1998) agar plates for 15 days. Vigorous mutants were selected and applied into new HSM and HSM-N agar plates. After 15-day cultivation, oil contents of the mutants were detected. Finally, the mutants containing high oil content were inoculated into 100 ml flask with 50 mL HSM and HSM-N liquid media for further analysis. The cultivation condition was described as above.

Medium optimization of N and phosphorus (P) sources for enhanced oil content of the mutants

To enhance oil content and biomass weight of the mutant, medium optimization of N and P sources, including NH_4Cl, K_2HPO_4, and KH_2PO_4, was performed by orthogonal experiment with three levels of two factors (Wu et al., 1996). The concentration of N was 0.0, 0.5, or 1.0 mmol/L, and the concentration of P was 10.0, 20.0, or 30.0 mmol/L. The elements contained in the media are designed and shown in Tables 1 and 2. The algal cells were inoculated into 50 mL flasks with 10 mL medium. The cultivation condition was described as above.

Medium N0P20 optimization of carbon source

For the UV mutant CC124-M25, medium optimization of carbon sources, including sodium acetate and glucose, was performed after the most optimal concentration of N and P sources was found in N0P20 medium, which contains 0 g/L NH_4Cl and 2.78 g/L $K_2HPO_4.3H_2O$. The concentration gradient of sodium acetate was 0, 2, 4, 6, or 8 g/L and that of glucose was 0, 5, 10, and 15 g/L.

Effect of pH value on oil accumulation in the mutant

The N0P20 medium containing 0 g/L NH_4Cl and 2.78 g/L $K_2HPO_4.3H_2O$ was used as the basic medium. The pH of the medium was adjusted by NaOH or HCl, and the pH value was 4, 6, 8, and 10. All cells were maintained in an incubator shaker with 230 rpm at 24°C, and were exposed to continuous illumination at a light intensity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

ISSR analysis of genetic variation between the wild type and the mutants of *C. reinhardtii* CC124

Microalgal DNA was extracted by the glass beads method with some modification (Hikfors, 1986; Huang et al., 2009). Concentration of the DNA was determined by spectrophotometry, and the integrity was checked by 0.8% agarose gel electrophoresis. The DNA solutions were finally diluted to 50 ng/μL. In total, 100 ISSR primers designed by the University of British Columbia (UBC) were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). Polymerase

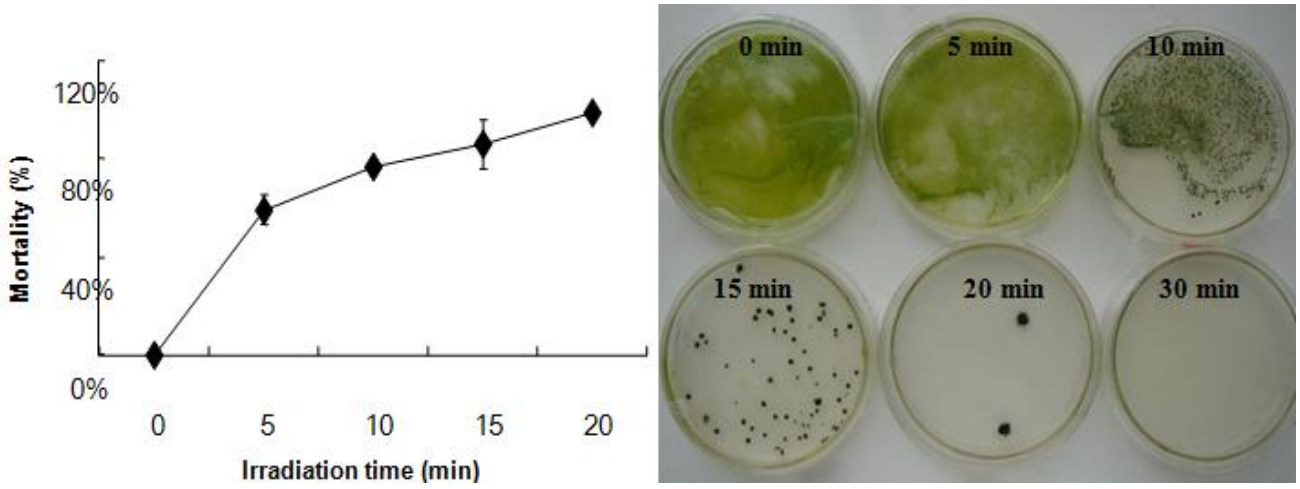

Figure 4. Lethality obtained from different dose of UV exposure. The Lethality of C. reinhardtii CC124 increased with rise of the exposure time.

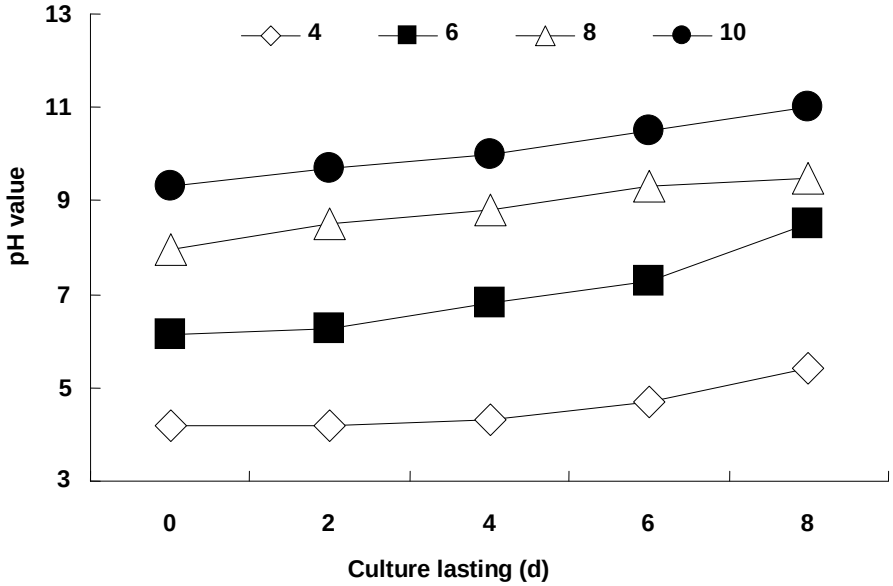

Figure 5. Detection of the pH value of the N0P20 medium when *C. reinhardtii* CC124-M25 was grown on. pH value slightly increased with the prolonging of culture time.

chain reaction was performed on Biometra T1 Thermocycle. The PCR program was as follows: 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. The PCR products were subsequently analyzed using 6% polyacrylamide gel electrophoresis. The silver-stained gels were prepared for further analysis.

RESULTS

Growth rate and lipid accumulation of the UV mutants from *C. reinhardtii* CC124

After UV irradiation, the number of viable cells decreased as UV exposure time increased from 0 to 20 min; lethality was counted after 3 days (Figure 4). The results show that a variety of UV doses has a lethal effect on *C. reinhardtii* CC124 cells. To obtain more viable mutants, 18 W UV lamp, an irradiation distance of 40 cm and irradiation of 8 min were the conditions considered for further studies. A total of 4500 viable algal colonies strains of *C. reinhardtii* CC124 were obtained after UV irradiation. These cells were inoculated into HSM and HSM-N media for mutant screening.

As shown in Figure 5, under the HSM culture condition, the mutant CC124-M5 grew better than the wildtype

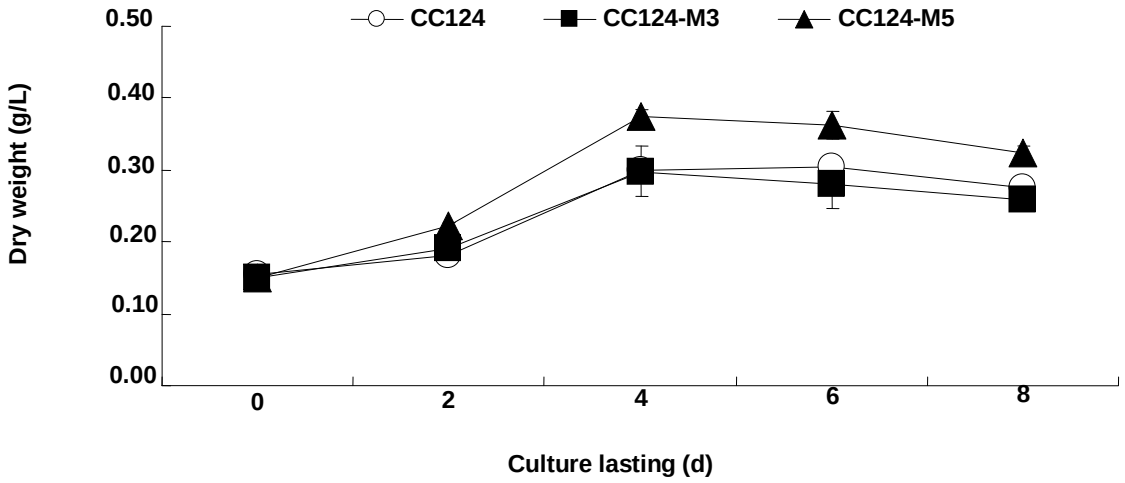

Figure 6. Growth curves of *C. reinhardtti* CC124 mutants grown in the HSM medium. The cultures were maintained in an incubator shaker with 230 rpm at 24℃ and were exposed to continuous illumination at a light intensity of 100 μmol•m^{-2}•s^{-1}.The mutant CC124-M5 grew better than the wildtype *C. reinhardtti* CC124, while the mutant CC124-M3 had a growth rate similar to that of the wildtype.

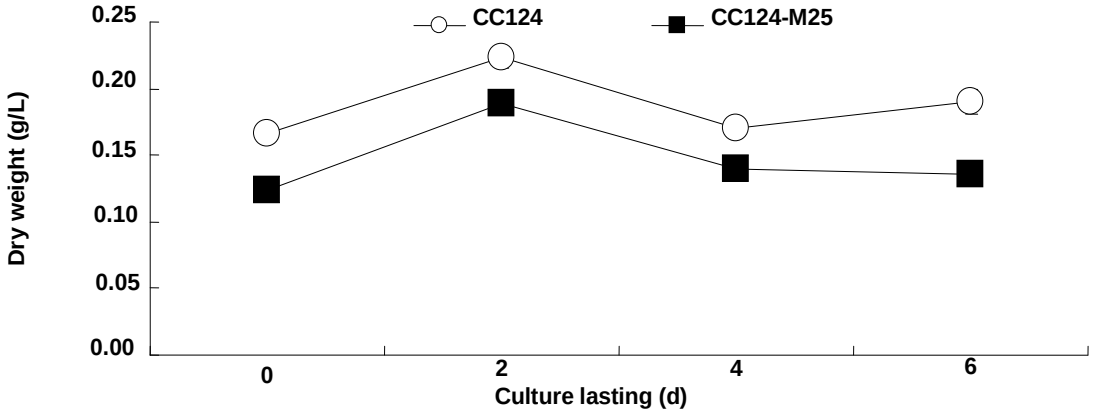

Figure 7. Growth curves of *C. reinhardtti* CC124 mutants grown in the HSM-N medium. The biomass of the mutant M25 was slightly lower than that of the wildtype.

C. reinhardtti CC124, while the mutant CC124-M3 had a growth rate similar to that of the wildtype (Figure 6). While the biomass of the mutant M25 was slightly lower than that of the wildtype under the HSM-N condition (Figure 7). Oil accumulation of the mutants and the wild type of *C. reinhardtii* are shown in Figure 8. Compared with the wild type with` 0.13 g/g oil content, the mutants CC124-M3 and CC124-M5 grown in HSM media for 8 days had lower oil content, only containing 0.11 and 0.09 g/g, respectively. In contrast, after 4 days of culture in HSM-N media, the mutants CC124-M25 accumulated 10% more oil than the wild type, reaching up to 0.42 g/g (Figure 9). As shown in Figure 10, brighter yellow FD indicates that neutral lipids accumulated in CC124-M25 in the main form of triglyceride, which can be transesterificated to biodiesel (Yusuf, 2007). These results show that, compared with the wild type, the mutant CC124-M25 has a similar growth rate but higher oil content, which is considered an ideal feedstock for biodiesel production.

Genetic stability analysis of the mutant *C. reinhardtii* CC124-M25

The mutant *C. reinhardtii* CC124-M25 was inoculated into HSM-N for five subsequent generations. The growth curve and oil content of the first generation and the fifth generation of the mutant are shown in Figures 11 and 12. Either growth rate or oil content had no significant difference between the two generations, indicating that

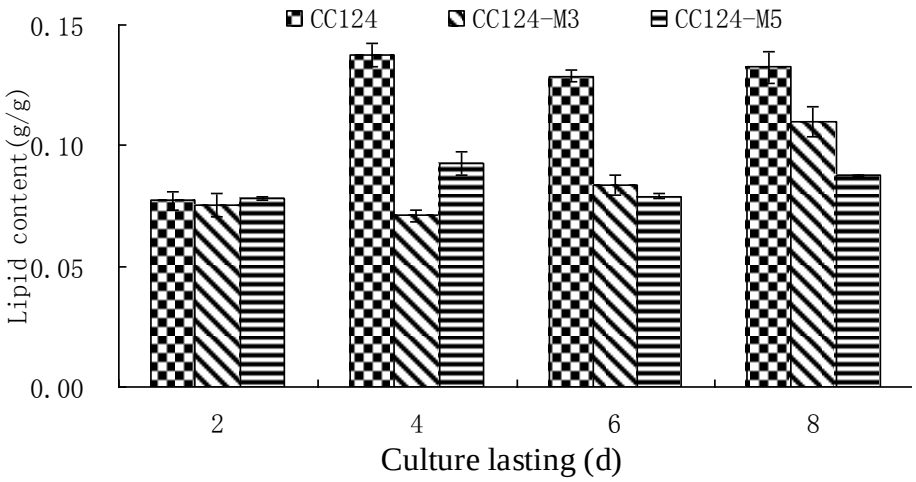

Figure 8. Lipid content of the mutants CC124-M3 and CC124-M5 grown in the HSM medium. Compared with that of the wildtype *C. reinhardtti* CC124, the oil content of the CC124-M3 and CC124-M5 significantly decreased after 4, 6, and 8 days of cultivation.

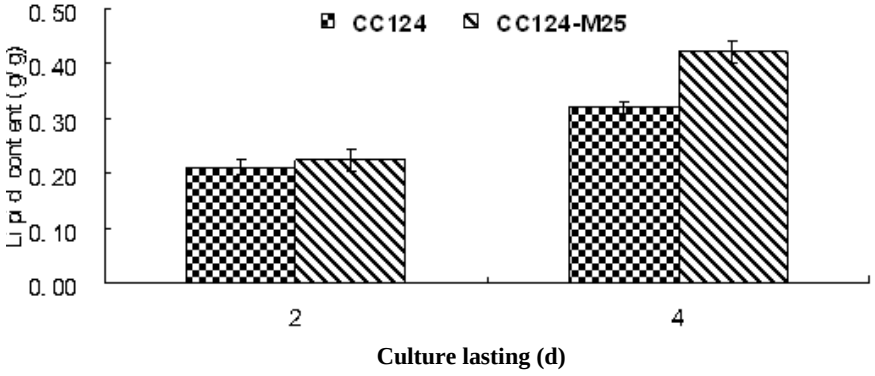

Figure 9. Lipid content of the mutant CC124-M25 grown in the HSM-N medium. At 4 days, the lipid content of the mutant M25 significantly increased compared with that of the wildtype.

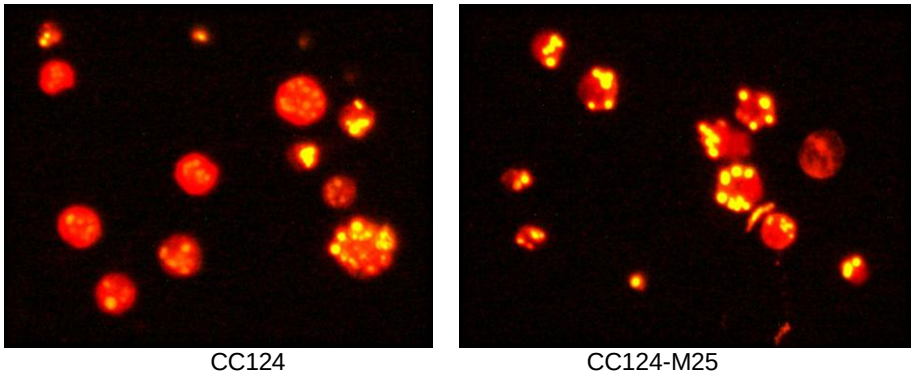

Figure 10. Microscopic observation of the mutant *C. reinhardtii* CC124-M25 and the wildtype *C. reinhardtii* CC124 after 4 days of cultivation (10X40). Bright yellow FD represents the neutral lipid, which can be used to produce biodiesel through transesterification. Red fluorescence represents the polar lipids, such as phospholipids, which are mainly involved in membrane composition and metabolic regulation.

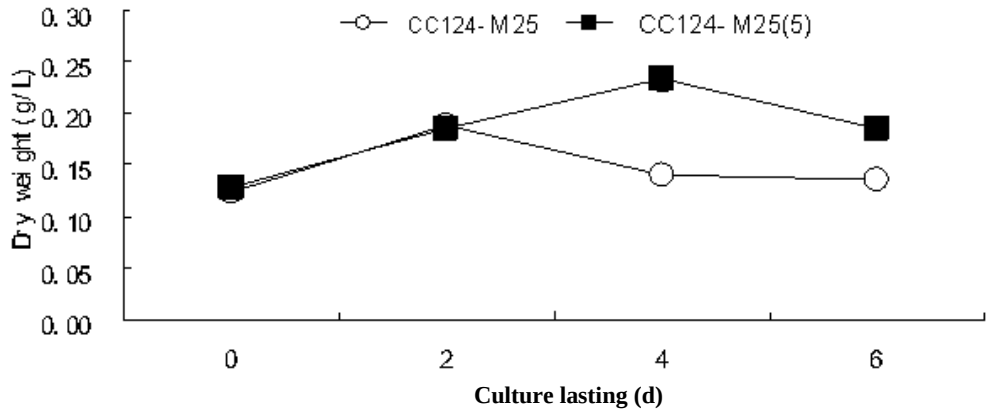

Figure 11. The growth curve of the first generation and the fifth generation of the mutant *C. reinhardtii* CC124-M25 maintained in the HSM-N medium.

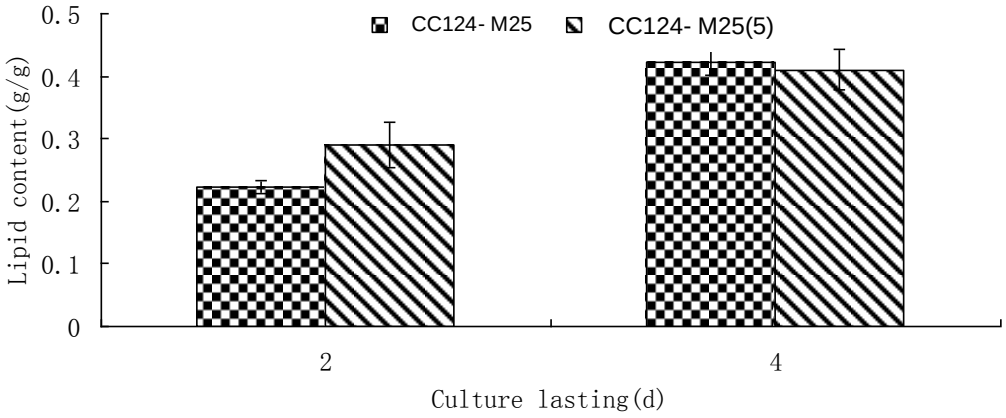

Figure 12. The oil content of the first generation and the fifth generation of the mutant *C. reinhardtii* CC124-M25 grown in the HSM-N medium. No significant changes were observed on the weight of dry biomass and oil content between the first generation of the mutant CC124-M25 and the fifth generation, suggesting the genetic stability of the mutant.

the mutant CC124-M25 has stable genetic traits.

Optimization media for lipid accumulation of *C. reinhardtii* CC124-M25

Oil content of the mutant, CC124-M25, significantly increased to 0.5 or 0.45 g/g after 6 days of cultivation in HSM-N media with 20 or 30 mmol/L P element, respectivly, which is more than that of the mutant grown in HSM-N medium with 10 mmol/L P (0.34 g/g oil content). Howerer, under these culture conditons, their biomass accumulated very slowly (Figures 13, 14, 15, 16, 17, and 18).

When the medium was supplied with a 0.5 mmol/L N source, the dry weight biomass of the mutant reached a maximum of 0.49 g/L (Figure 14) which is more than that of the mutant grown in the medium with free N source. However, the mutant accumulated less than 0.2 g/g oil after N supplement, significantly lower than that under the HSM-N medium condition. Considering the combination of biomass and oil content, oil content of the mutant CC124-M25 reached up to 0.5 g/g in the medium with 20 mmol/L P source and free N source called N0P20. Thus, the medium N0P20 is considered the most suitable medium of oil accumulation for the mutant.

Effects of carbon source

The growth curves of the mutant CC124-M25 grown in the N0P20 medium with different concentrations of glucose are shown in Figure 19. No significant changes in biomass weight were observed in these samples cultivated at various glucose levels from 0 to 6 d. After the glucose concentration increased to 15 g/L, oil content reached a maximum of 0.43 g/g (Figure 20).

When sodium acetate was used as the external carbon

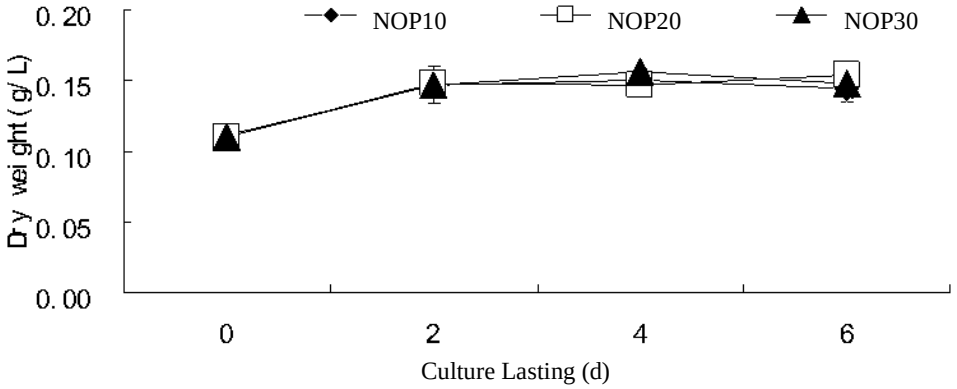

Figure 13. Growth curve of the mutant *C. reinhardtii* CC124-M25 grown in the HSM medium with various N and P concentrations. The mutant CC124-M25 had a similar growth rate in the HSM medium with three different concentrations of P.

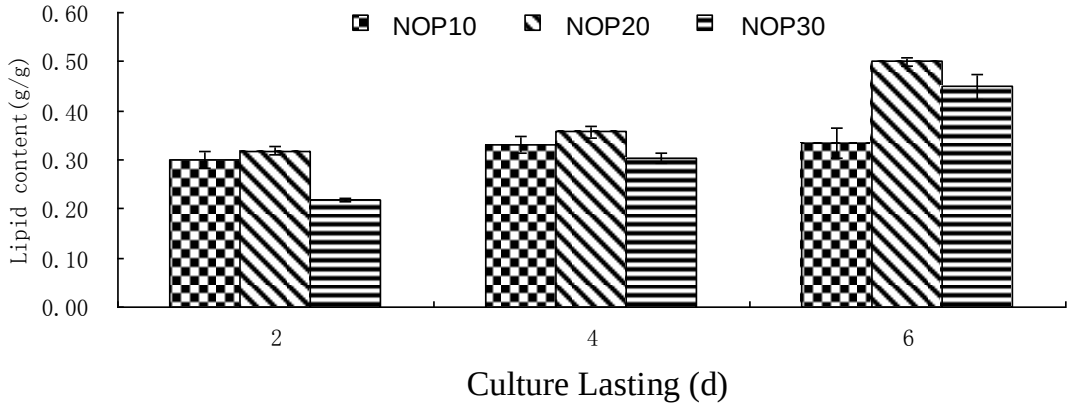

Figure 14. Oil content of the mutant *C. reinhardtii* CC124-M25 grown in the HSM medium with N-free and various P concentrations. The oil content of the mutant increased with the increase in the P content of the medium. When P concentration was 20 mmol/L, the oil content reached a maximum of 0.5 by weight of dry biomass. The oil content then decreased.

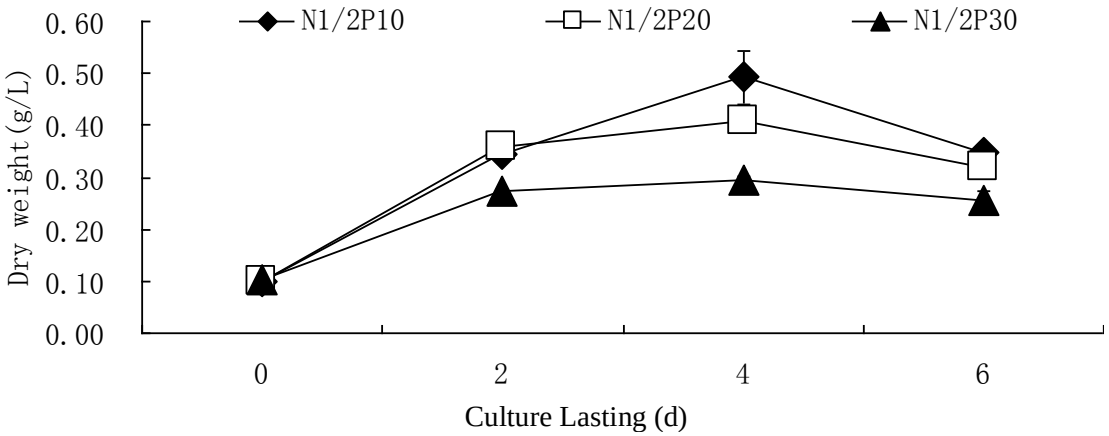

Figure 15. Growth curve of the mutant *C. reinhardtii* CC124-M25 grown in the HSM medium with 0.5 mmol/L N and various concentrations of P element. The weight of dry biomass of the mutant CC124-M25 decreased with the increase in P concentration. It reached the maximum when the concentration of P was 10 mmol/L.

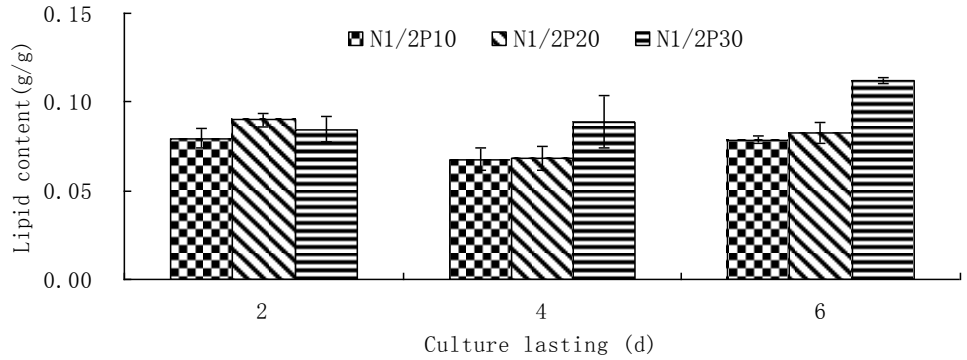

Figure 16. Oil contents of the mutant *C. reinhardtii* CC124-M25 grown in the HSM medium with 0.5 mmol/L N and various P concentrations. The oil content of the mutant CC124-M25 maintained in the medium with 30 mmol/L P source reached a maximum of 0.15 by weight of dry biomass at 6 days.

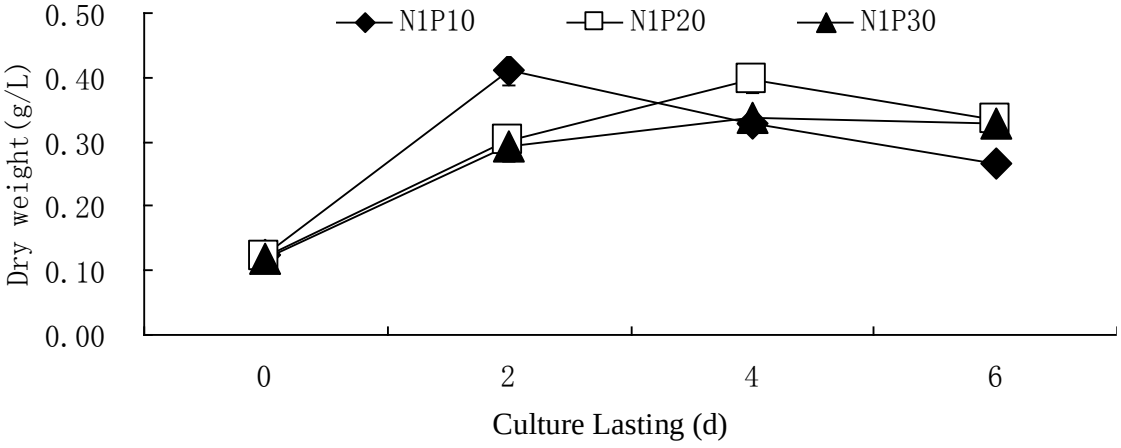

Figure 17. Growth curve of the mutant *C. reinhardtii* CC124-M25 grown in the HSM medium with 1.0 mmol/L N and various concentrations of P element. When 10 mmol/L P source was added to the medium, the weight of dry biomass reached a maximum.

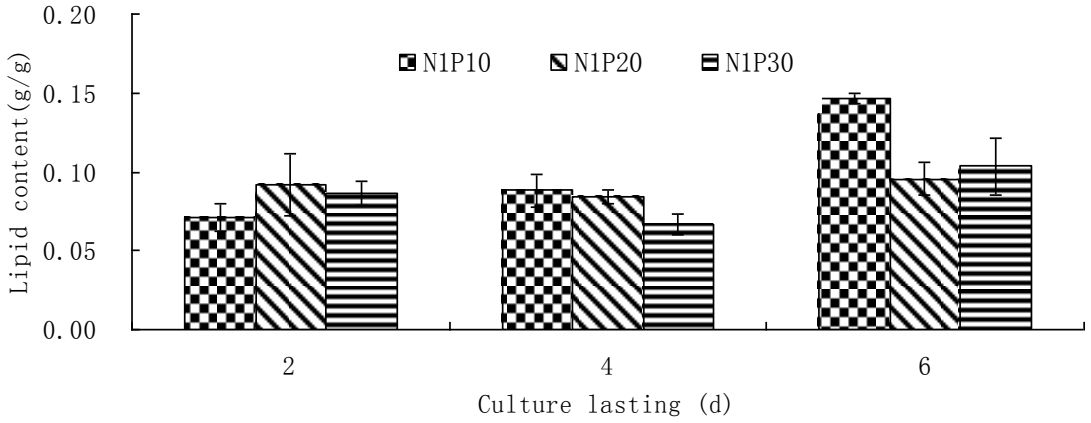

Figure 18. Oil contents of the mutant *C. reinhardtii* CC124-M25 grown in the HSM medium with 1.0 mmol/L N and various P concentrations. The oil content of the mutant CC124-M25 maintained in the medium with 10 mmol/L P source reached a maximum of 0.15 by weight of dry biomass at 6 days.

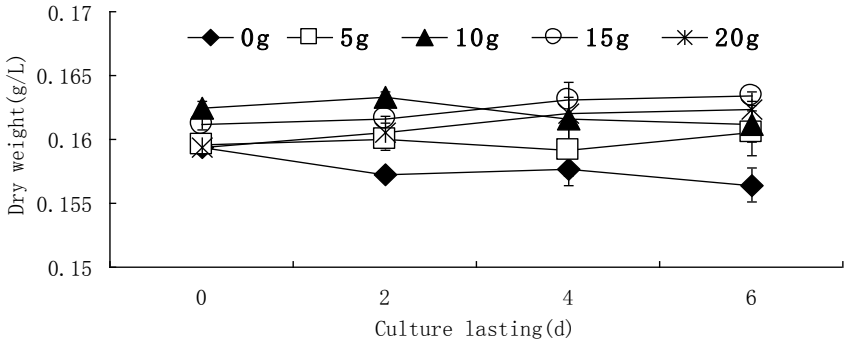

Figure 19. Growth curves of the mutant *C. reinhardtii* CC124-M25 grown in the N0P20 medium with various concentrations of glucose. There was no significant difference in the weight of dry biomass among these samples maintained in the media with different glucose concentrations.

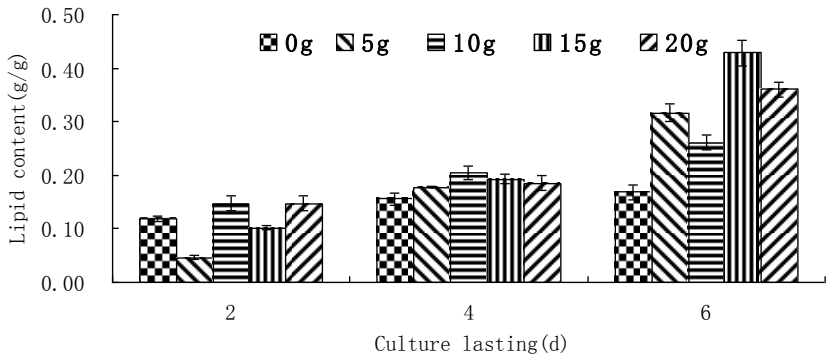

Figure 20. Oil contents of the mutant *C. reinhardtii* CC124-M25 grown in the N0P20 medium with various concentrations of glucose. The oil content of the mutant CC124-M25 reached the maximum at 6 day. When the concentration of glucose was 15 g/L, the maximum of the oil content was 0.43 g/g by weight of dry biomass, much higher than that of the cells grown in the medium with no glucose.

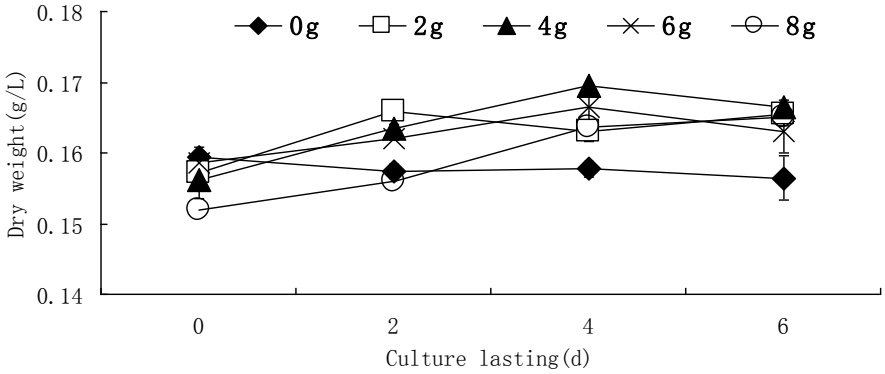

Figure 21. Growth curves of the mutant *C. reinhardtii* CC124-M25 grown in the N0P20 medium with various concentrations of sodium acetate. The weight of dry biomass reached the maximum.

source, the biomass weight of the mutant CC124-M25 slightly increased with the prolonging of culture time (Figure 21). The oil content of the mutant grown in the N0P20 medium with 2 g/L sodium acetate reached up to 49.84%. Subsequently, as the concentration of sodium acetate increased, the oil content of the mutant gradually

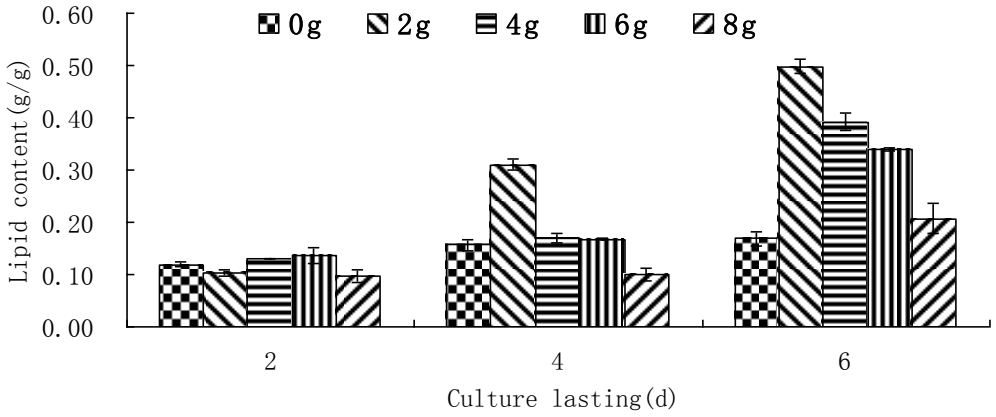

Figure 22. Oil contents of the mutant *C. reinhardtii* CC124-M25 grown in the N0P20 medium with various concentrations of sodium acetate. The oil content of the mutant CC124-M25 reached the maximum at 6 days. When the concentration of sodium acetate was 2 g/L, the maximum of the oil content was 0.50 g/g, much higher than that of the cells grown in the medium with no sodium acetate. Oil content decreased with the increase in the concentration of sodium acetate.

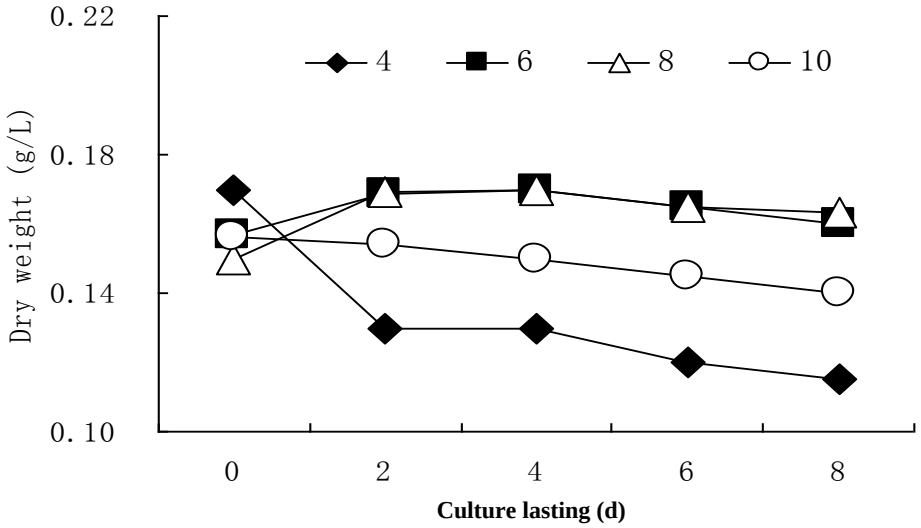

Figure 23. Growth curves of the mutant *C. reinhardtii* CC124-M25 grown in the N0P20 with different pH values. When the pH value was 4.0, the weight of dry biomass of the mutant M67 decreased with the prolonging of culture time. When pH was 6 and 8, the biomass did not change compared with the control.

decreased (Figure 22). In general, sodium acetate is more suitable than glucose for oil accumulation in the mutant CC124-M25, the optimal concentration of which is 2 g/L.

Effects of pH value on oil accumulation

As shown in Figure 5, the pH value of the N0P20 medium increased with culture time when the mutant CC124-M25 was cultivated. The biomass weight of CC124-M25 with initial pH 6-8 slightly increased. However, it decreased at an initial pH of 4 or 10 (Figure 23). The oil content of CC124-M25 reached a maximum of 0.55 g/g when the cells were maintained in the N0P20 medium with initial pH 8.0 for 8 days, suggesting that the N0P20 medium with initial pH 8 is the most suitable for oil accumulation in CC124-M25 (Figure 24).

Content of lipid, sugar, protein, chlorophyll, and carotenoid in *C. reinhardtii* CC124-M25

As shown in Figure 25, oil is most abundantly produced in CC124-M25 grown in HSM-N and in the modified N0P20 medium (with 2 g/L sodium acetate and medium

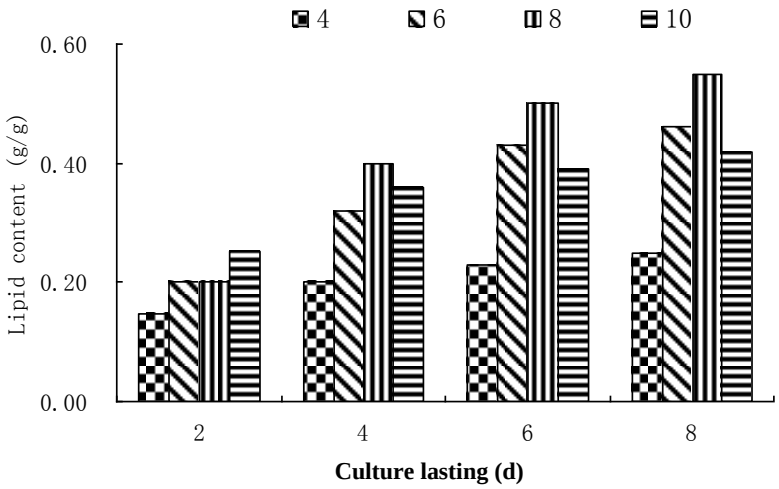

Figure 24. Oil contents of the mutant *C. reinhardtii* CC124-M25 grown in the N0P20 with different pH values. The oil content of the mutant CC124-M25 reached the maximum of 0.55 g/g after 8 days of culture.

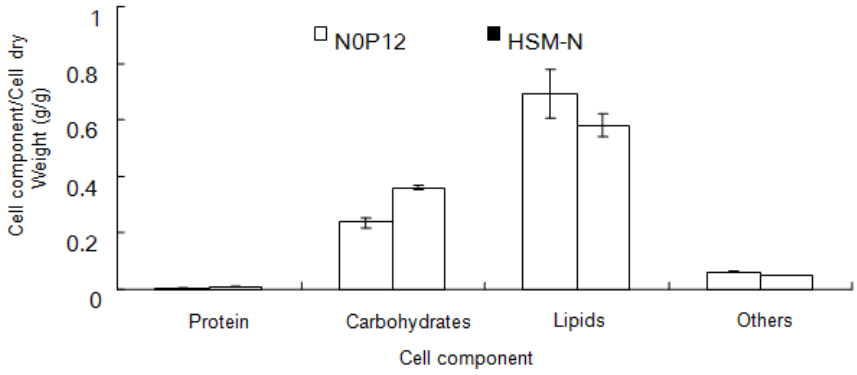

Figure 25. Cell component content of the mutant *C. reinhardtii* CC124-M25 grown in the N0P20 medium and HSM-N medium. Sugar content of the mutant under the HSM-N medium was 8% higher than that of cells grown in the N0P20 medium. The oil content decreased by 8%, and no significant changes were observed in the protein content from the cells grown in the two media.

with initial pH 8), followed by sugar. Protein content accounted for a small proportion. Compared with those in the mutant CC124-M25 grown in the HSM-N medium, sugar levels dramatically decreased and oil content significantly increased in CC124-M25 grown in the N0P20 medium with 2 g/L sodium acetate. This result implies that the carbon source used for oil synthesis was mainly derived from sugar in CC124-M25 after the external carbon source was added.

No significant changes were observed in chlorophyll and carotenoid content of the mutant CC124-M25 grown in the HSM-N medium and N0P20 medium with 2 g/L sodium acetate (Figure 26). The content of chlorophyll slightly increased in CC124-M25 under the N0P20 medium condition, affecting the photosynthetic efficiency of the algal strains.

ISSR analysis of *C. reinhardtii* CC124-M25

DNA was extracted for the mutant CC124-M25 and the wildtype CC124 as described above. These DNA samples were used for ISSR analysis. The results of the ISSR analysis showed that PCR products can be obtained by 24 out of the 100 primers, of which 4 primers (that is, UBC808, 809, 816, and 835) can amplify distinct bands with good polymorphism. The PAGE gel picture is shown in Figure 27. The polymorphism information content (PIC) for CC124 was 78% and that for the mutant CC124-M25 was 50% (Tables 3 and 4).

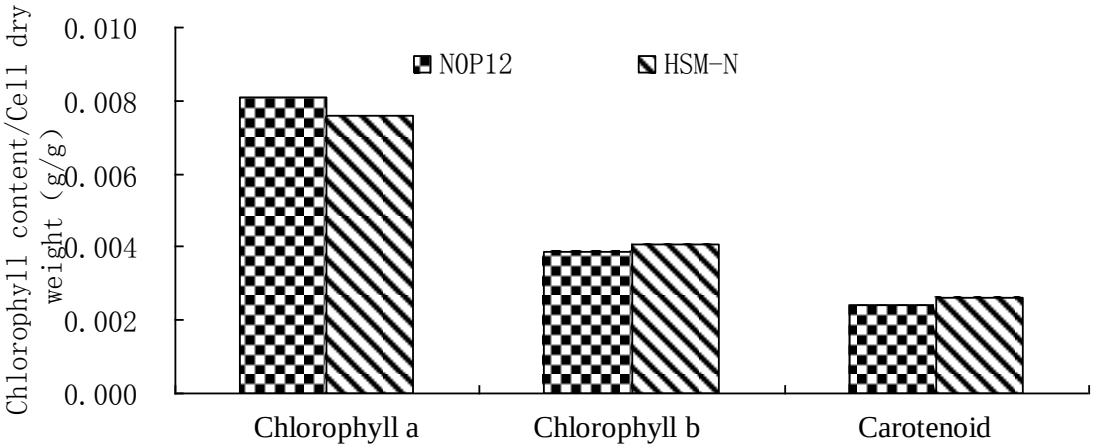

Figure 26. Chlorophyll content of the mutant *C. reinhardtii* CC124-M25 grown in the N0P20 medium and HSM-N medium. The contents of chlorophyll a, b, and carotenoid from the cells grown in N0P20 medium slightly increased compared with those from the cells grown in the HSM-N medium.

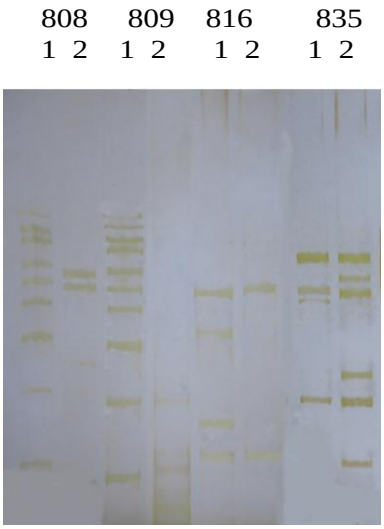

Figure 27. ISSR gel picture of *C. reinhardtii* CC124 (lane 1) and *C. reinhardtii* CC124-M25 (lane 2).

DISCUSSION

Ultraviolet (UV) irradiation has strong mutagenic biological effects on organisms, and UV mutagenesis is an effective breeding method (Zhang et al., 2009). Compared with chemical mutagenesis, UV mutagenesis offers many advantages such as less pollution, simple operation, and sterile cultivation condition (Huang et al., 1993). Several successful cases on microalgae strains for UV mutagenesis have been documented. For example, Zhang et al. (2007) reported that three high-yield mutants, that is, M51, M59, and M73, were selected from *C. vulgaris* using UV mutagenesis breeding technology, through which the relative growth rate increased by 6.23, 3.8, and 5.92%. In the current study, the mutant CC124-M25 was obtained from *C. reinhardtii* CC124 strains through UV mutagenesis. Its oil content reached up to 0.42 g/g, increasing by 10% compared with the wildtype CC124 in HSM-N medium. As the mutant achieved rapid growth rate and high oil content, it has been considered a prospect for biodiesel development.

Many researches are focused on the effects of N and P elements on the growth rate and oil content of microalgae. The results show that N source and concentration are the key factors required for microalgal growth and oil accumulation (Laing and Utting, 1980; Mutsumi et al., 2006). Under an N deficiency condition, cell growth and biosynthesis of many cell components will be suppressed, but the cells maintain a high level of lipids. Microalgae grown in an N deficiency medium have been proved to produce 2 to 3 times more lipids than those under normal growth condition (Ratledge, 2002). In the current study, the results of the medium optimization of N and P sources showed that, the HSM-N medium with 20 mmol/L P element is the most suitable for oil accumulation in the mutant CC124-M25, in which the maximum oil content of the mutant can increase by 8% compared with the basic HSM-N medium, which can reach up to 0.5 g/g.

Culture methods can affect the composition and content of fatty acid in microalgae. Several studies have found that some algal species can utilize organic carbon source to grow heterotrophically (Tatsuzawa et al., 1996) and mixotrophically (Lalucat et al., 1984). Under these culture conditions, light suppression can be eliminated, and cell density can be increased. Moreover, an abundant carbon source is beneficial to the accumulation of more oil for microalgae (Marguez et al., 1995). Under a heterotrophic culture condition, the oil content of *C. protothecoides* can reach 0.55 g/g by weight of dry biomass, which is four times more than that from

Table 3. Primer with bands.

UBC	808	809	816	825	827	828	829	830	835	849	855	856
UBC	858	873	875	877	878	880	886	887	888	889	890	891

Table 4. Total amount of the belt in the ISSR graph and the amount of the polymorphic belt.

Species	Amount of primers	Total amount of belt	Amount of polymorphic belt	Proportion of polymorphic belt (%)
CC124	4	27	21	78
CC124-M25	4	12	6	50

autotrophically grown cells (Roessler, 1990). Wei and Liu (2008) reported that the oil content of *Chlorella vulgaris* reached a maximum of 0.3 g/g when 10 g/L glucose was added to the culture medium. As the concentration of glucose increased, the oil content decreased. The oil content decreased to 0.18 g/g after 50 g/L glucose was used. In the present study, glucose and sodium acetate were used as carbon sources for *C. reinhardtii* growth. The results showed that the oil content of the mutant CC124-M25 was maximum, 0.43 g/g, when the concentration of glucose was 15 g/L. In contrast, the oil content reached up to 0.5 g/g, after 2 g/L sodium acetate was used as the carbon source. Thus, 2 g/L sodium acetate is more suitable for the oil accumulation of CC124-M25.

pH value affects many biological processes, such as enzyme activity and ion absorption, in microalgae. Qi (2008) showed that, when pH value varied from 4.0 to 6.0, the biomass and oil level of C*. pyrenoidosa* No.2 increased with the increase in pH value. The oil content reached a maximum of 0.43 g/g when the pH was 6.0. Afterwards, the oil content significantly decreased with the increase in pH value. In the current study, the oil content of CC124-M25 reached a maximum of 0.55 g/g when pH was 8.0.

Medium composition also affects the component of microalgae cells. To date, studies have found that fatty acid composition of microalgae varies with the composition of the culture medium (Liang et al., 2000). In the present study, sugar content of the mutant M25 grown in the HSM-N medium was 8% higher than that grown in the N0P20 medium. Oil content decreased by 8%, and the protein content was similar when the algae were maintained in the two different media. The results imply that carbon source from sugar flowed into the oil biosynthesis in the mutant M25 grown in the N0P20 medium.

ISSR is a recent molecular marker technology based on a microsatellite (SSR) developed by Zietkeiwitcz et al. (1994). It has been widely used in many aspects, such as plant species identification, genetic mapping, genetic diversity, and evolution and gene location (Rakoezy et al., 2004). The genetic diversity of *Dendrobium tassels* in Xishuangbanna region was analyzed using ISSR markers (Ma and Yin, 2009; Chen et al., 2008), and located a parthenocarpic gene in cucumber using ISSR markers. In the current study, ISSR markers were used to analyze the difference between the mutant CC124-M25 and the wildtype CC124. In total, 24 out of the 100 primers designed by the UBC could amplify clear bands, among which four primers (that is, UBC808, 809, 816, and 835) amplified distinct bands with good polymorphsim. The PIC for *C. reinhardtti* CC124 was 78% and that for the mutant CC124-M25 was 50%. These distinct bands showed the difference between the mutant and wild type in DNA level, and this difference may connect with algae lipid metabolism. Further studies of these bands may give us more details about the mutant.

ACKNOWLEDGMENT

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