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The mutation of *Scenedesmus obliquus* grown in municipal wastewater by laser combined with ultraviolet

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Abstract−Mutagenetic breeding is an efficient technique for the enhancement of lipid productivity from microalgae. In this study, oil-rich microalga *Scenedesmus obliquus* were treated by Laser-UV composite mutagenesis. Among the 35 mutant strains, X5 was primely screened. Afterwards, a twice UV mutagenizing was operated on X5, and the optimal mutant strain X5-H13 was obtained. The growth rate, dry weight, lipid yield and lipid content of X5-H13 were 0.698×10^7 cells/mL·d, 0.99 g/L, 0.49 g/L and 48.8% while cultivated in municipal wastewater, respectively, which were increased by 45%, 58%, 109% and 32% than the original strain. The results of the subculture of repeated mutant showed that the biomass and lipid content of strain X5-H13 were up to 0.99 g/L and 48.8%. The growth of each generation was stable. Furthermore, the random amplified polymorphic DNA analysis indicated that the mutant strain X5-H13 was different from the starting strain, with their genetic similarity coefficient value of 0.815.

Key words: Microalgae, *Scenedesmus obliquus*, Biodiesel, Laser-UV Mutagenesis, Biomass

INTRODUCTION

The production of biodiesel from microalgae cultivated in municipal wastewater has attracted tremendous interest. Microalgae is considered to be one of the most promising raw materials for biodiesel production due to its easy cultivation, high photosynthetic efficiency, rapid cell growth and high biomass yield [1,2]. Microalgae can utilize the carbon, nitrogen, phosphorus and other nutrients from municipal wastewater for growth and lipid accumulation, promote the degradation of organic pollutants in wastewater, and reduce carbon emissions in wastewater [3,4]. The key issue for enhancing the lipid production from wastewater-cultivated microalgae is to obtain the optimum microalgal strains with high lipid content and growth rate, as well as the strong adaptability of wastewater. However, the general microalgae strains isolated from the natural environment cannot not fully meet the requirement. Other ways including co-cultivation with fungi/bacteria, selective stress for improving the lipid content could also improve lipid production of microalgae. However, the high cost and operational complexity limit its large-scale application. In contrast, the obtained microalgae species by mutagenesis have genetic stability and long-term advantages. Mutagenesis breeding could reduce the operating cost and operation complexity of microalgae biodiesel process. Therefore, it is necessary to apply mutagenesis treatment to obtain the proper microalgae species with higher quality.

Compared with the natural process, mutagenetic breeding could promote the variation of organisms by large scale and high frequency, which is beneficial to selecting organisms with excellent traits. Therefore, it is a highly efficient and practical breeding method for microorganisms. Mutagenetic breeding was generally performed through physical mutagenesis and chemical mutagenesis. Traditional physical mutagenesis methods included ultraviolet rays, X-rays and gamma-ray irradiation [5]. More recently, fast neutrons, laser and microwave stimulation have been also developed and applied [6]. Chemical mutagenesis is the use of chemical reagents on biological cell to cause base inversion, misinformation and information silencing of genes. The most efficient mutagenic agents are alkyl reagents, such as ethyl methanesulfonate (EMS) and nitroso arc (NTG) [7]. However, the chemical reagents are relatively toxic and cause many security concerns.

Microalgae mutation breeding with ultraviolet (UV) irradiation is a widely accepted method due to its safety, convenient operation, non-pollution, high efficiency and the ability to achieve sterile cultivation [8,9]. Thus, it is usually applied in the breeding of industrial microorganisms. Forjan et al. studied the effects of UV on cell growth and basic metabolic activities of *Nannochloropsis*. The results showed that lutein, carotenoids and polyunsaturated fatty acids were increased in *Nannochloropsis* cell after exposure to ultraviolet light

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[10]. Eva et al. studied the effects of UV irradiation on diatoms and reported that UV mutagenesis played an important role in the changes of C/N/P ratio, photosynthetic pigment composition and cell numbers [11]. Liang et al. applied UV irradiation on *Phaeodactylum tricornutum*; the results showed that the content of fatty acids (especially EPA) was significantly increased [12]. Beacham et al. reported that the lipid content of the mutant *Nannochloropsis salina* was more than double after UV mutagenesis [13]. On the other hand, laser irradiation had the advantages of high energy density, easy accessibility, non-toxicity and pollution [14]. However, till now, most of the studies on laser mutagenesis microbial breeding focused on industrial microorganisms, and few studies on microalgae had been conducted [15,16]. More recently, the combination of laser and UV mutagenesis was also used to enhance the mutagenic effects. Zhuang et al., reported that the astaxanthin content of *Haematococcus pluvialis* was increased by 52.2% after laser-UV compound mutagenesis [17]. Up to now, no studies have been conducted to investigate the effects of laser-UV composite mutagenesis on lipid yield of microalgae cultivated in wastewater. In this study, oil-rich microalga *S. obliquus* was primarily treated by laser-UV composite mutagenesis. Thereafter, the mutant strains of *S. obliquus* were screened for obtaining optimum strains with higher lipid yield and better adaptability in wastewater, which will shed light on the simultaneous biodiesel production and wastewater treatment.

MATERIALS AND METHODS

1. Cultivation of Microalgae

The microalgae *S. obliquus* FACHB 276 used in the tests were obtained from Freshwater Algae Culture Collection at Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The pre-cultured microalgae at logarithmic phase were diluted 10, 100, 1,000, 10,000 times before spreading on the plate, then cultivated with light intensity of 10,000 lux, illumination period was 12L/12D, temperature was kept as 23.2±2 °C. After four days, ten strains of microalgae with optimal growth conditions were first screened for repeated inoculation. After 12 days of culture, 1 strain of *S. obliquus* was finally selected as starting strain, then subcultured and preserved by SE medium as described by Tan et al. [18]. During the tests, artificial wastewater [19] and real wastewater [20] were used as the medium. The main characteristics of the wastewater were: COD_{Cr} 360-430 mg/L, TP 4-7 mg/L, TN 70-90 mg/L, NH_3-N, 8-16 mg/L, SS 112 mg/L.

2. Laser-UV Compound Mutagenesis

A continuous wave semiconductor laser with a wavelength of 473 nm was used as the mutagen. A solid state low-power blue laser generator (Guoke Century Laser Technology Co., Ltd. Beijing, China) with a divergence angle of ≤4 mrad and a power input of 220 V/50 Hz/AC. The stability was characterized by continuous output for four hours, and suitable working ambient temperature was 10-35 °C. To enhance the penetration of the laser, a quartz cuvette was used as the container for algae solution. 3 mL of fresh microalgae liquid at logarithmic growth phase was pipetted into the cuvette, which was a sample for mutagenesis. The cuvette was placed 4 cm away from the laser for 5 min, and the microalgae solution was continuously stirred during the irradiation to ensure uniform irradiation.

After laser irradiation, microalgae strains were used for UV irradiation mutagenesis. UV with a wavelength of 254 nm was used as the mutagen. To avoid spattering of the microalgal liquid during stirring and to maintain uniform irradiation, the microalgal liquid was diluted and spread on the solid medium, which was composed of 1 L SE medium with 15 g agar. Then the spread plate was exposed to a 20 W UV lamp at 10 cm away for 25 min. The whole process was operated in the dark, and the irradiated plate was placed in the dark for 24 hours, then cultured in a constant temperature culture chamber. After laser and first round of UV mutagenesis, 45 mutant strains of *S. obliquus* were screened and divided into three groups (L, X, Y), 15 strains for each group and their growth rates, biomass and lipid production were investigated to select the optimum strain.

To enhance the mutagenic effect and obtain more beneficial mutant strains, the select strain was subjected to secondary UV irradiation mutagenesis treatment, and the UV mutagenic treatment process was performed as the same as above.

3. Analytical Methods

3-1. Cell Counting and Determination of Growth Rate

The cell density was measured by the standard method with blood counting chamber [21]. The growth rate was calculated as follows:

$$\mu=\ln(N_T-N_0)/T \quad (1)$$

where μ is the growth rate, N_T is the cell density of the microalgal solution after time T, and N_0 is the initial cell density of the microalgal solution at the time of inoculation.

3-2. Determination of Dry Weight of Microalgae

The dry weight of microalgae was determined by gravimetric method. 10 mL of microalgae liquid was filtered, washed by pre-weighed (W_1) microporous membrane (Φ0.45 μm), and dried at 105 °C to constant weight (W_2). The dry weight of the blank control group without microalgae was W_0, and the dry weight of microalgae DW (g/L) was calculated as follows:

$$DW=(W_2-W_1-W_0)/0.01 \quad (2)$$

3-3. Lipid Production of Microalgae

Lipid was extracted from microalgae out using chloroform-methanol extraction method [22]. After the extraction, the chloroform phase was collected and transferred to a pre-weighed tin foil tray. The organic solvent was evaporated under a stream of nitrogen, then the residues were baked in an oven at 80 °C to a constant weight to obtain the lipid production. The lipid content of microalgae was the percentage of lipid production and dry weight of microalgae.

3-4. DNA Isolation and PCR Analysis

Genomic DNA and PCR analysis was based on the methods of our previous study [23]. Genomic DNA was isolated using plant DNA kits (E.Z.N.ATM HP, Omega Bio-tek, USA). The DNA were first electrophoresed on 1.2% agarose gels at 100 V for 45 min, with 5 μL DNA samples and 1 μL bromophenol blue were added. Then photgraphs were taken for the gels under ultraviolet light. The PCR program was first desaturated for 1 min at 98 °C, followed by 10 s at 98 °C, 30 s at 56 °C, and 1 min at 68 °C for 35 cycles, and 15 min at 68 °C. The samples were stored at 4 °C. Finally, 25 primers were screened. Genetic similarity among the treated and untreated

cells was calculated by the following formula to estimate the level of genetic polymorphism [24]:

$$I=2Nij/(Ni+Nj)$$

where, Nij is the number of bands in common between line i and j, and Ni and Nj are the total number of bands in the lines, respectively.

3-5. Data Analysis

Data were analyzed by one-way ANOVA using statistical software SPSS 21.0, and statistical tests were performed by LSD method ($P<0.05$).

RESULTS AND DISCUSSION

1. Characteristics of Mutant Strains

1-1. Screening of Mutant Strains

After laser-UV composite mutagenesis, 45 mutant strains of *S. obliquus* were selected and separated into L, X and Y groups (15 strains each). All of the selected strains were dark-green and the cells were evenly dispersed in the culture. The biomass (dry weight) after six days and growth rates of the mutant strains in artificial wastewater are shown in Fig. 1(a)-(c). According to Fig. 1(a), the dry weights of the mutant strains L2, L4, L7, L9, L12, L14 and L15 from L group were higher than that of the starting strain. Among them, mutant strain L12 had the highest biomass and the optimum growth rate. Compared with the starting strain, the biomass and growth rate of L12 were increased by 8.9% and 26%, respectively. Therefore, mutant strain L12 was selected for subsequent experiments. As shown in Fig. 1(b), the biomass of X5, X6, X7, X8 and X12 from X group was 42%, 32%, 43%,35%, 34% higher than the starting strain, while the growth rates of X3, X6, X7, X12 were improved by 24%, 42%, 44%, 37%. Thus, considering the growth rate and biomass, the mutant strains X5, X6, X7, X8 and X12 were chosen for subsequent experiments. In group Y, the biomass of Y3, Y4, and Y10 strains was significantly increased, which was 33%, 34%, and 27% higher than the original strain, as shown in Fig. 1(c). Similarly, the growth rate of Y3, Y4, Y5 and Y10 was 21%, 39%, 42%, and 43% higher than the original strain, respectively. Therefore, four mutant strains Y3, Y4, Y5 and Y10 were selected from Y group.

In summary, based on the screening results of the L, X and Y groups, 11 mutant strains with better growth conditions were screened for subsequent experiments, i.e., re-screening by lipid yield and content measurement.

1-2. Lipid Yield and Content of Mutant Strains

The high growth rate and lipid accumulation ability were the main characteristics of high-yield strains of *S. obliquus*. The lipid yield was an important indicator for judging the microalgae as raw materials for biodiesel production, which was determined by the dry weights and lipid content of the microalgae. The lipid yields in artificial wastewater of the 11 screened mutant strains of *S. obliquus* are shown in Fig. 2. After laser-UV composite mutagenesis, the lipid yield of mutant strains was varied. Among them, mutants X5 and X7 had the most significant lipid yields and contents. Compared with the starting strain, the lipid yields were increased by 95% and 84%, while the lipid content was increased from 36.9% to 46.8% and 44.1%, respectively. However, the lipid yields of mutant strains X6, X8, X12, Y4, Y5 and Y10 were lower than that of the

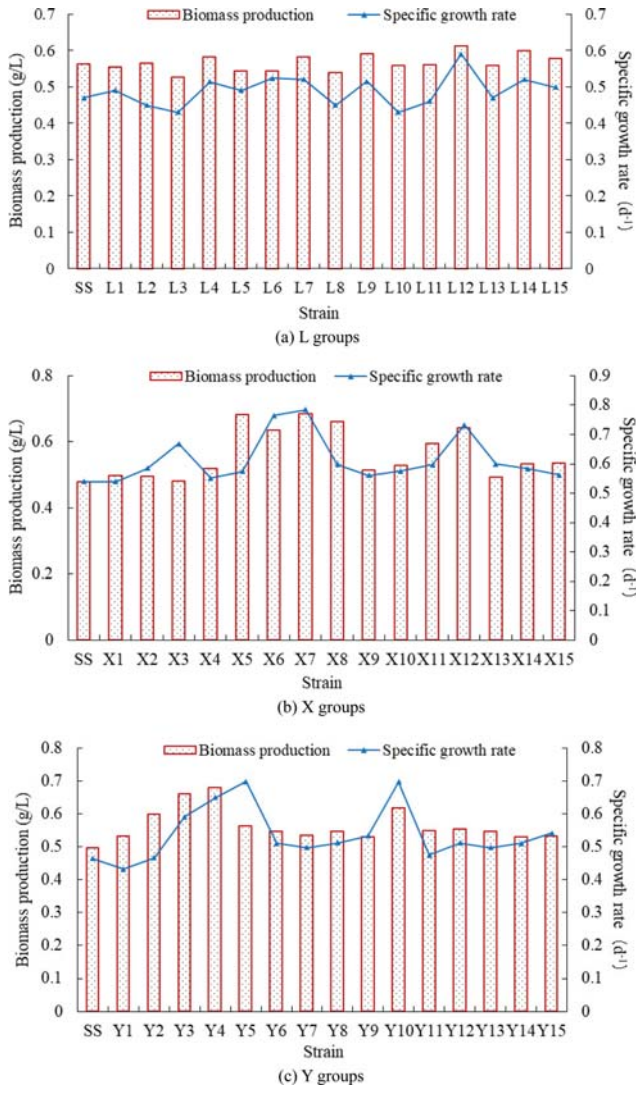

Fig. 1. The biomass (dry weight) after six days and growth rates of the mutant strains. (a) L groups; (b) X groups; (c) Y groups (SS: starting strain).

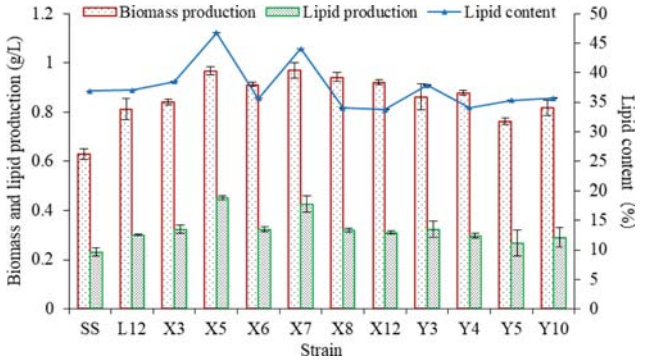

Fig. 2. The lipid yields of the 11 screened mutant strains of *S. obliquus* (SS: starting strain).

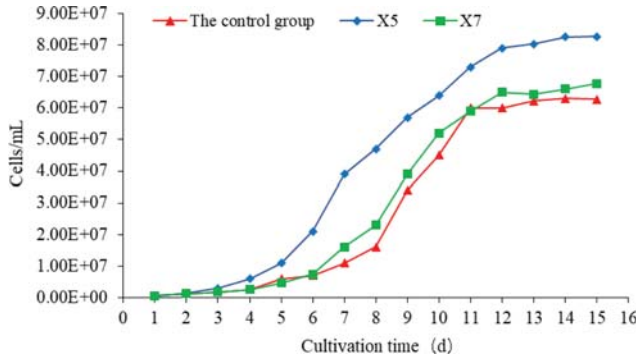

Fig 3. The microalgal cell density of the control group and mutant strains.

starting strain. Therefore, by further investigating the growth of the mutant strains in wastewater, the mutant strains X5 and X7 were screened for subsequent experiments.

1-3. Growth of Mutant Microalgae Strains in Real Wastewater

The mutant strains X5 and X7 as well as the starting strain were inoculated into real wastewater and cultivated for 16 days, and the microalgal cell density was measured daily to monitor the growth of the microalgae strains. As shown in Fig. 3, the microalgal cell density of the mutant strains and the starting strain at the initial stage of cultivation (the first three days) was low, and the growth conditions were similar. However, from the 6th day, the mutant strain X5 showed a significant increase than the original strain and mutant strain X7, and microalgal cells entered the high-speed proliferative phase on the 7th to 8th day of cultivation, while the growth conditions of mutant strain X7 and the original strain remained. On the 14th day of the cultivation, the mutant strain X5 reached the highest cell density of 8.3×10^7 cells/mL, while the cell density of original strain was 6.3×10^7 cells/mL. On day 15, the highest cell density of mutant strain X7 reached 6.8×10^7 cells/mL, which was not obviously higher than the original strain. Comparing the microalgal density curves of the starting strain and the mutant strains, the biomass of the mutant strain X5 was significantly higher than both of the original strain and mutant strain X7. Therefore, X5 was selected as the target for further UV mutagenesis.

2. Effect of Secondary UV Mutagenesis on Mutant Strain X5

2-1. Growth of the Mutant Strains After Secondary UV Mutagenesis

The mutant strain X5, which had optimum lipid yield and growth rate, was further mutagenized by UV mutagenesis (irradiation for 25 min). After screening, 18 mutant strains X5-H1~X5-H18 were obtained. After inoculation in artificial wastewater medium for two weeks, the growth rates were measured as shown in Fig. 4. After second UV mutagenesis, the growth rates of mutant strains X5-H4 and X5-H13 increased significantly, as 0.680×10^7/mL/d and 0.698×10^7/mL/d, respectively. The results demonstrated that secondary UV mutagenesis could further enhance the growth rate of microalgae.

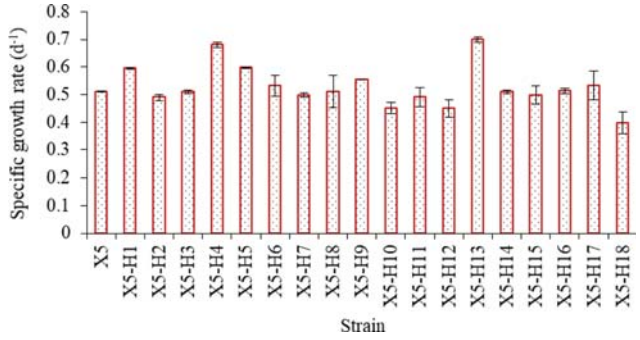

Fig. 4. The growth rates of repeated mutant strain.

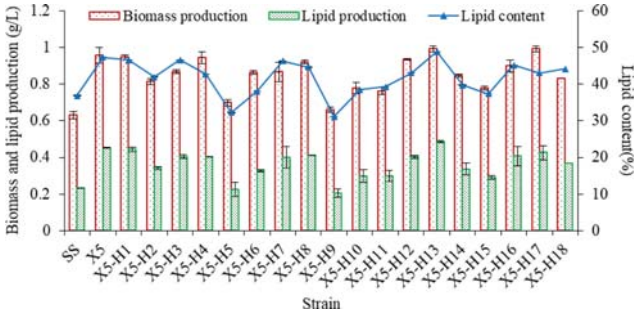

Fig. 5. Lipid production of repeated mutant strain (SS: starting strain).

2-2. Lipid Yields of the Mutant Strains After Secondary UV Mutagenesis

The lipid yields of the mutant strains after secondary UV mutagenesis were investigated; the results are shown in Fig. 5. Compared with X5, only mutant strain X5-H13 had better lipid yields, which was increased from 0.453 g/L to 0.485 g/L. Therefore, the final mutant strain was identified as *S. obliquus* X5-H13.

3. Genetic Stability Analysis of the Repeated Mutant

We continuously sub-cultured the repeated mutant strain X5-H13 and detected their traits stability. Consequently, the genetic stability of the mutants was measured at molecular level by random amplified polymorphic DNA (RAPD) analysis.

3-1. Continuous Subculture of the Repeated Mutants

Strain X5-H13 was subcultured five times with real wastewater before the genetic stability analysis was conducted at the condition of light intensity of 10,000 lx, light cycle of 12L/12D with temperature maintained at 23.2±2 °C in the incubator. The growth

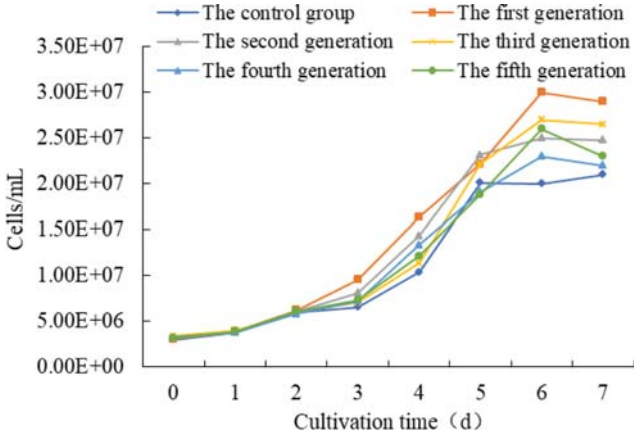

Fig. 6. Microalgal growth of each generations of X5-H13.

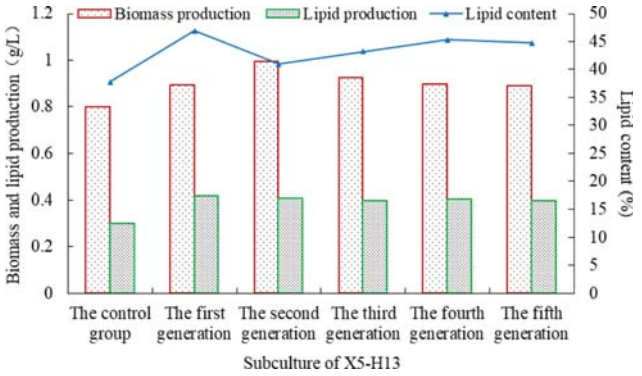

Fig. 7. Lipid production of each generations of X5-H13.

and lipid production of X5-H13 at each generation are shown in Fig. 6 and Fig. 7. The final density of the algae cells of X5-H13 at each generation was higher than that of the starting strain, implying the increase of the X5-H13 biomass after repeated ultraviolet mutagenesis (Fig. 6). In terms of the whole growth curve, we observed the stable growth of all generations for the mutant strain, which evidenced the stable performance of the mutant strain X5-H13. The performance of the lipid production for the mutant was steady for each generation. The microalgal dry weight, lipid production and the lipid content of mutant at each generation were higher than those of the starting strain, in which the first generation achieved the highest lipid production and lipid content, with the value of 0.419 g/L and 46.9%, respectively (Fig. 7). The microalgae production was improved compared with 0.185 g/L reported by Salama et al. [25] in which the microalgal lipid production was achieved by batch culturing of the *Chlamydomonas mexicana* for ten days with municipal wastewater. Therefore, X5-H13 was a stable mutant strain.

3-2. RAPD Analysis

The total DNA was extracted from starting *S. obliquus* strain and the repeated mutant strain with the DNA extraction kit; their gel electrophoresis was thus obtained (Table 1). The total size of the extracted DNA was 20 kb, which was the same as that of the starting strain, demonstrating the total DNA extracted by this method was intact and could be used for the following PCR amplification.

Eight of 25 RAPD primers generated scorable bands with 81 total bands. 33 bands showed polymorphism among them (Table 1). The genetic similarity coefficient I between the repeated mutant strain and starting *S. obliquus* was 0.815, which could be confirmed as different strains.

Table 1. A list of primers, their sequences and the amplification results

Primer names	Amplification sequences	Total amplification bands	Polymorphic amplification bands
L-06	TTGCTGGCGT	8	4
L-11	TCCAGCCGTT	11	6
L-12	GAGGACGATG	13	3
L-13	AGCGCCTACG	8	2
L-14	CCAGCAACGA	9	3
L-20	ACAACGCGAG	10	4
L-21	ACATGCCGTG	12	6
L-22	AGAGGGCACA	10	5

CONCLUSION

A highly efficient laser-UV composite mutagenesis was primarily applied to oil-rich microalga *S. obliquus* cultivated in artificial wastewater and real wastewater. After twice mutagenesis, the mutation rates of *S. obliquus* were thus enhanced. X5-H13 was chosen as the optimal mutant strain from the mutant library, while the dry weight and lipid content were up to 0.99 g/L and 48.8%. Also, after continuous subculture of the repeated mutant, the biomass and the lipid production of X5-H13 strain increased compared with the starting strain, and the growth for each generation was stable. Moreover, the mutant strain X5-H13 and starting strain can be confirmed as different strains based on their genetic similarity coefficient value of 0.815 from RAPD analysis.

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