African Journal of Agricultural Research Vol. 6(16), pp. 3768-3774, 18 August, 2011
Available online at http://www.academicjournals.org/AJAR
DOI: 10.5897/AJAR10.810
ISSN 1991-637X ©2011 Academic Journals

Full Length Research Paper

Effects of selective medium on lipid accumulation of chlorellas and screening of high lipid mutants through ultraviolet mutagenesis

Xiaodong Deng[1], Yajun Li[1] and Xiaowen Fei[1,2]*

[1]Key Laboratory of Tropical Crop Biotechnology, Ministry of Agriculture, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agriculture Science, Haikou, 571101, China.
[2]Department of basic medicine, Hainan medical college, Haikou, 57110, China.

Accepted 31 March, 2011

Two micro-algae Y019 and Y041 were isolated from tropical fresh water in Hainan, China. Through phylogenetic analysis of 18S rDNA sequences, they had been identified as *Chlorella vulgaris* and *Chlorella pyrenoidosa.* Subsequently, both of them and C. *vulgaris* FACHB-31 were cultured respectively in four selective medium (SE, BG11, HSM1, DS) at 25℃, 230 r/min, 24 h full light, 50-110 μE/m2/s. Effects of these media on the cell growth and lipid content were detected. The results showed that, the HSM1 medium is more suitable for the growth of Chlorella, while the DS medium is more conducive to the lipid accumulation. In the DS medium, the lipid content of *C. vulgaris* Y019 was 1.84 ~ 2.64-fold than others at 12 days. So the local separated algae *C. vulgaris* Y019 is thought of an ideal raw material for biodiesel production. After that, we conducted Ultraviolet mutagenesis for *C. vulgaris* Y019. A total of 1086 algae strains had been screened for lipid production in HSM and HSM-N media.. Results showed: in HSM medium, mutants Y019-M57 and Y019-M66 were identified as high lipid producing mutants, while in HSM-N medium Y019-M67 was detected as high lipid content mutants. These mutants laid the foundation for further research and commercial application.

Key words: Chlorella, culture medium, ultraviolet mutants, lipid accumulation.

INTRODUCTION

With global shortages of fossil fuels, especially oil and natural gas, renewable biofuel production has become a major focus worldwide. Algae, with a much higher unit area oil yield than terrestrial oilseed crops, are a promising biofuel feedstock (Scragg et al., 2003; Spolaore et al., 2006; Gouveia et al., 2009). Another issue from burning of fossil fuels is the ever-increasing carbon dioxide (CO_2) emission, whose trend will continue with the fast pace of modern industry development if a feasible energy source replacement could not be found. Algae, which can assimilate CO_2 photoautotrophically or mixtrophically, is a perfect candidate for CO_2 sequestration and greenhouse gas reduction. Now more and more scientists and companies focus on this field though biochemisty and molecular biology to get ideal algae strains for commercial use (Deng et al., 2009). Chlorellas, an easy culture micro-alga, normally contain high oil in its cell, is thought of a suitable candidate for biofuel feedstock (Hsieh et al., 2009; Liang et al., 2009). In this paper, we isolated two micro-algae strains from Hainan province, China, and through phylogenetic analysis of 18S rDNA, classified them as *Chlorella vulgaris* and *Chlorella pyrenoidosa*. Then, the growth rate and lipid content of these Chlorellas strains in four selective medium had been detected. After that, we conducted ultraviolet mutagenesis for *C. vulgaris* Y019. Several high lipid content mutants had been identified and discussed.

*Corresponding author. E-mail: feixw2000@hotmail.com.

MATERIALS AND METHODS

Algal strains

Alga strain *C. vugaris* FACHB-31 was purchased from freshwater alga culture collection center at the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). *C. vulgaris* Y-019

and *C. pyrenoidosa* Y-041 were isolated from the freshwater lakes in haikou, Hainan province, China.

Preparation of genomic DNA

Chlorellas cells in mid-log time were collected for extraction of genomic DNA according to the method of Deng and Erikson (2007) with modification. About 1.5 ml of algal cells was centrifuged for 30 s at 12000 rpm. The supernatant was aspirated off and the cells were resuspended in 150 μl H_2O. 300 μl of SDS-Buffer (2% SDS; 400 mM NaCl; 40 mM Na_2EDTA; 100 mM Tris-HCl, pH 8.0), 5 μl of RNase (20 μg/μl) and 0.1 g of acid-washed glass beads (sigma, St. Louis, MO) were added, mixed by vortex and kept at room temperature for 15 min. After a series of phenol-chloroform extraction, DNA was precipitated with two volumes of absolute ethanol, and then washed with 70% ethanol. Finally, the air-dried pellet was dissolved in 40 μl of TE buffer (10 mM Tris HCl, pH 8.0; 1 mM EDTA). Concentration of genomic DNA was determined by spectrophotometer and the DNA integrity was checked by agarose gel electrophoresis.

PCR amplification of 18S rDNA sequence and phylogenetic analysis

18S rDNA gene sequence was amplified using the primers 5'-CAGCMGCCGCGGTAATWC-3' and 5'-ACGGGCGGTGTGTRC-3' (Morgan-Kiss et al., 2008). Polymerase chain reaction (PCR) amplification was performed in a final volume of 25 μl containing 1 x PCR reaction buffer, 2 mM $MgCl_2$, 0.4 μM of each primer, 0.25 mM dNTPs, and 0. 5 U Taq DNA polymerase (Promega, USA), and subjected to the following program: 4 min at 95°C; 30 cycles of denaturation for 40 s at 95°C, annealing for 40 s at 68 to 58°C (decreasing 1°C per cycle in the first 10 cycles), and elongation for 20 s at 72°C; 10 min at 72°C. PCR products were purified using EZ Spin Column DNA Gel Extraction Kit (BBI, Canada) and cloned into pMD18-T Vector following the manufacturer's instructions (TaKaRa, Japan). Sequencing was performed by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, China). The sequences were aligned and compared with a dataset of algal sequence selected from GenBank using ClustalX 1.83 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Phylogenetic analysis were conducted using MEGA version 4.1 (Tamura et al., 2007), based on the neighbor joining method and 1000 bootstrap replications.

Cultivation conditions and biomass measurement

The chlorellas grown on plate were inoculated into 100 ml Erlenmeyer flasks with 50 ml of SE , BG-11, HSM1 and DS culture media, respectively. SE medium: $NaNO_3$ 0.25 g.L^{-1} , $K_2HPO_4.3H_2O$ 0.075 g.L^{-1} , $MgSO_4.7H_2O$ 0.075 g.L^{-1}, $CaCl_2.2H_2O$ 0.025 g.L^{-1}, KH_2PO_4 0.175 g.L^{-1}, NaCl 0.025 g.L^{-1}, soil extract 40 ml, $FeCl_3$•$6H_2O$ 0.005 g.L^{-1}, Fe-EDTA 1 ml, A5 solution 1 ml, BG11 medium: $NaNO_3$ 1.5 g.L^{-1}, $K_2HPO_4.3H_2O$ 0.04 g.L^{-1}, $MgSO_4.7H_2O$ 0.075 g.L^{-1}, $CaCl_2.2H_2O$ 0.036 g.L^{-1}, citric acid 0. 006 g.L^{-1}, Ferric ammonium citrate 0.006 g.L^{-1}, EDTA (dinatrium-salt) 0.001 g.L^{-1}, Na_2CO_3 0.02 g.L^{-1}, A5 + Co solution 1 ml, HSM1 medium:NH_4Cl 0.05 g.L^{-1}, K_2HPO_4 1.44g.L^{-1}, KH_2PO_4 0.74g.L^{-1}, $MgSO_4$•$7H_2O$ 0.02 g.L^{-1}, $CaCl_2$•$2H_2O$ 0.01 g.L$_{-1}$, Trace 1 ml; DS medium : $NaNO_3$ 0.30 g.L^{-1}, $CO(NH_2)_2$ 0.15 g.L^{-1}, $K_2HPO_4.3H_2O$ 0.05 g.L^{-1}, $FeC_6H_5O_7$ 0.006 g.L^{-1}, $CaCl_2.2H_2O$ 0.025 g.L^{-1}, soil extract 10 ml, Vb1 200 μg.L^{-1}, Vb12 400 ng.L^{-1}, Trace 1 ml). All cultures were maintained in an incubator shaker with 230 rpm at 25°C, and exposed to a

Biomass was determined by measuring the optical density of samples at 490 nm (OD490). Then the dry weight of the diluted samples were detected and made for standard curve. The average relative growth rate (R) was calculated using the following formula: R = (lnQ2 - lnQ1) / (t2 - t1), where Q1 is the dry weight of the algal cells at the first sampling time point (t1) and Q2 is that of second sampling time point (t2). The dates were collected at 24 h intervals with three replications per sample.

Neutral lipid analysis

A Nile Red fluorescence method was applied to the determination of neutral lipids (Gao and Xiong, 2008). The algal cells were directly stained with 0.1μg/ml Nile Red (final concentration) for 10 min, and then fluorescence was measured on a GloMax®-Multi Detection System (Promega, USA), which excitation and emission wavelengths were 470 and 570 nm, respectively. The fluorescence value was calculated by the equation:

FD(470/570)= (A2-A1)

Where A2 is the fluorescence value of algal cells after staining with Nile Red, A1 is that of the algal cells before staining with Nile Red. Triolein (Sigma) was used for standard sample to generate standard curve of lipid. For microscopy, after stained with Nile Red (10 μg/ml final concentration), cells images were acquired using Nikon 80i Fluorescence Microscopes. Nile Red signal was captured using an excitation line at 480 nm, and emission was collected between 560 and 600 nm (Huang et al., 2009; Chen et al., 2009).

Ultraviolet mutagenesis of *C. vulgaris* Y-019 cells

According to the method described by Zhang (2007) with modification. *C. vulgaris* Y-019 cells were grown to a density of 5 × 10^6 cell/ml. One milliliter of the cells was placed in a 9 cm diameter glass Petri dish bottom, which exposed to 18W UV light at a distance of 15.0 cm for 13 min. The mutagenized cells were kept in the dark for 24 h to prevent photoreactivation, then plated onto TAP medium and grown under constant light.

Screening *C. vulgaris* Y-019 mutants

After UV mutagenesis, single algal colonies were subcultured onto agar plates containing high salt medium (HSM), HSM-N (N deficiency) and HSM-Fe (Fe deficiency) for 10 days, respectively. Neutral lipids were detected using Nile Red fluorescence method as described previously. The mutants with high lipid content were selected and inoculated into 50 ml centrifuge tubes with 30 ml of HSM, HSM-N and HSM-Fe, respectively. Neutral lipids were determined at 48 h interval. Images were also acquired using Nikon 80i Fluorescence Microscopes. The experiment was repeated three times.

RESULTS

Phylogenetic analysis of the isolated algal strains

Molecular phylogenies of two isolated algae strains Y-019 and Y-041 were performed using partial sequences of the18S rDNA fragment. Based on the 18S rDNA

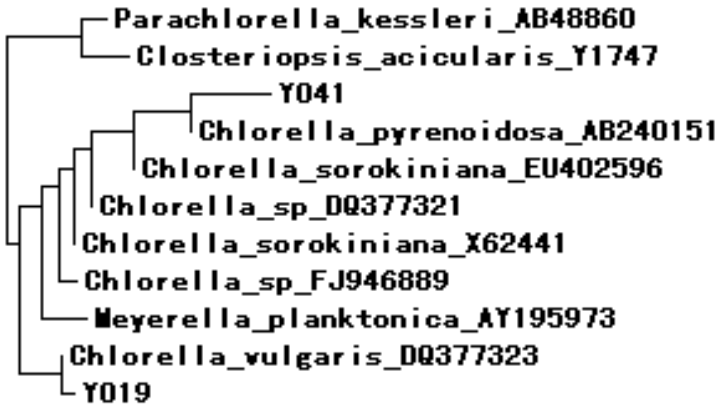

Figure 1. 18S rDNA phylogenetic tree of algae strains Y-019, Y-041. Phylogenetic analysis were done using MEGA version 4.1. Result Showed Y-019 was closely related to Chlorella vulgaris DQ377323, and Y-041 was to Chlorella pyrenoidosa AB240151.

organisms of the *Chlorella* spp. (Figure 1). The closest match to the strain Y-019 is *C. vulgaris* DQ377323, and the strain Y-041 is most closely related to *C. pyrenoidosa* AB240151.

The effects of selected media on cell growth of the algal strains

C. vugaris FACHB-31, *C. vulgaris* Y-019 and *C. pyrenoidosa* Y-041 were grown in SE, BG11, HSM1 and DS media for 12 days, respectively. As shown in Figure 2. The relative growth rates of all algal strains in HSM1 were between 1.19 to 1.57, which were three times more than those in the other three media, however no significant difference was observed among the biomass of the Chlorellas cultured in HSM1 and those in SE medium (Table 1). Similar growth curves were generated for the three algal strains growing in SE and BG11 media (Figure 2a-d). The difference was that biomass at plateau phase in SE medium were approximately 0.53 to 0.73 g/L, more than doubling that obtained with BG11 medium, which were 0.29 to 0.34 g/L. Cell biomass at plateau phase in DS medium were much lower than those in SE, HSM1 and BG11 media, which only were 0.08 to 0.09 g/L (Table 1).

Neutral lipid accumulation in chlorella strains

To monitor neutral lipid accumulation in the algal strains, the Nile Red fluorescence assay was applied to detect the lipid content. The standard curve of the lipid content was made according to the results of Nile Red fluorescence value of Triolein. The lipid content of the algal cell was calculated as:

Lipid content (g/g) = [8.276* FD(470/570) +13.624]/Cell dry wet.

Figure 3 showed the lipid content (g/g) of *C. vugaris* FACHB-31*, C. vulgaris* Y-019 and *C. pyrenoidosa* Y-041 growing in SE, BG11, HSM1 and DS media, respectively. It revealed that DS medium was most favorable for the lipid accumulation under the investigating conditions. *C. vulgaris* Y-019 showed highest lipid accumulation in all detected media, especially in DS medium with the highest lipid content of 0.55 g/g after 10 days (Figure 3d). In contrast, all algal strains grown in BG11, HSM1 and SE media gave very low lipid production (Figure 3a-c). Subsequently, *C. vulgaris* Y-019 was selected for further analysis.

Identification of high lipid-producing ultraviolet mutants from *C. vulgaris* Y-019

Three *C. vulgaris* Y-019 mutants were selected following UV mutagenesis from 1028 algal colonies. During 8 day cultivation in HSM and HSM-N (N deficiency), algal cell growth and neutral lipid accumulation were determined and the data are shown in Figure 4 and Figure 5. Compared with wild-type *C. vulgaris* Y-019, Mutants Y019-M37, Y019-M56, and Y019-M67 accumulated more neutral lipids. The lipid content of Y019-M37 dramatically increased from the initial value of 0.11 g/g at day 2 to 0.26 g/g at day 8 in HSM medium, which was approximately 2.5 times of that obtained with parental strain Y-019 (Figure 4A). A significant increase of lipid content (0.258 g/g) was also observed in Mutant Y019-M56 after 8 days cultivation in HSM (Figure 4A). In HSM-N medium mutant Y019-M67 started to accumulate neutral lipids at day 4, and reached the highest lipid content (0.39 g/g) at day 8 (Figure 4B). However, no significant difference was detected in cell growth between these mutants and the wild type (Figure 4C-D), revealing

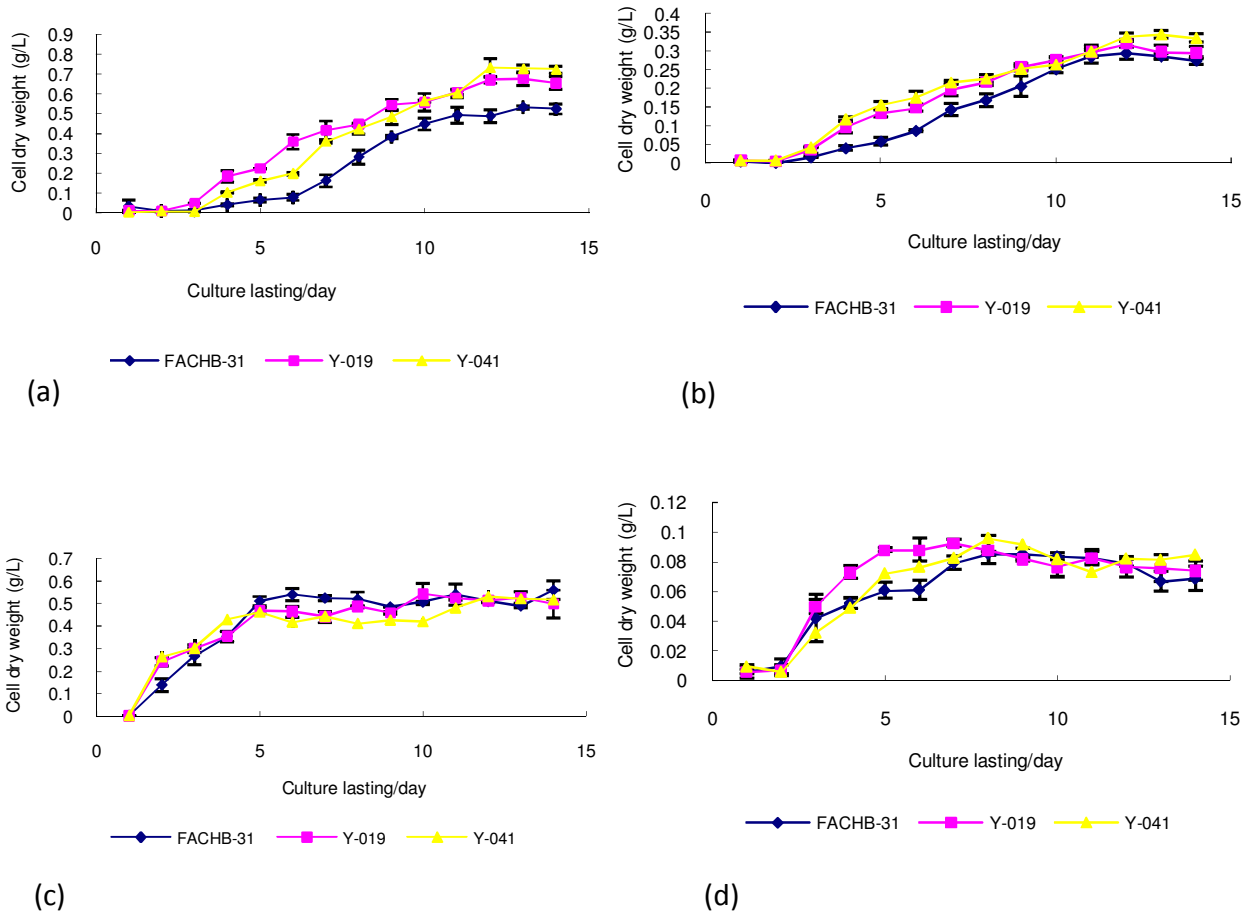

Figure 2a-d. Growth curve of chlorellas in SE, BG11, HSM1 and DS medium.

Table 1. Relative growth rate and maximum biomass of 3 Chlorella in four kinds of culture mediums.

Algae species	SE		BG11		HSM1		DS	
	Growth rate	Biomass g/L	Growth rate	Biomass g/L	Growth rate	Biomass g/L	Growth rate	Biomass g/L
FACHB-31	0.39±0.03	0.53±0.08	0.56±0.04	0.29±0.02	1.26±0.05	0.56±0.04	0.37±0.07	0.09±0.01
Y-019	0.45±0.02	0.67±0.03	0.44±0.01	0.31±0.02	1.57±0.12	0.52±0.03	0.47±0.07	0.08±0.01
Y-041	0.49±0.04	0.73±0.04	0.40±0.01	0.34±0.01	1.19±0.03	0.53±0.03	0.52±0.01	0.08±0.01

From the image of Figure 5, brilliant yellow fluorescence in neutral lipid environment was detected by fluorescence microscopy in mutants Y019-M37, Y019-M56, and Y019-M67 8 days after cultivation.

DISCUSSION

Microalgae as a promising feedstock for biodiesel production have attracted growing attention in recent years (Chisti, 2007). Many efforts have been made to realize the commercialization of algae biodiesel by governments, researchers and entrepreneurs, such as seeking optimal microalgal species, large-scale cultivating the algal strain and identifying low cost oil extraction and harvesting methods (Mata et al., 2010; Basova et al., 2005). Among of these, identifying optimal microalgal strains is the key challenge. In general, algae with high oil content such as *Botryococcus braunii* grow slowly, whereas algae with lower oil content such as *Dunaliella salina* will grow more quickly. Therefore,

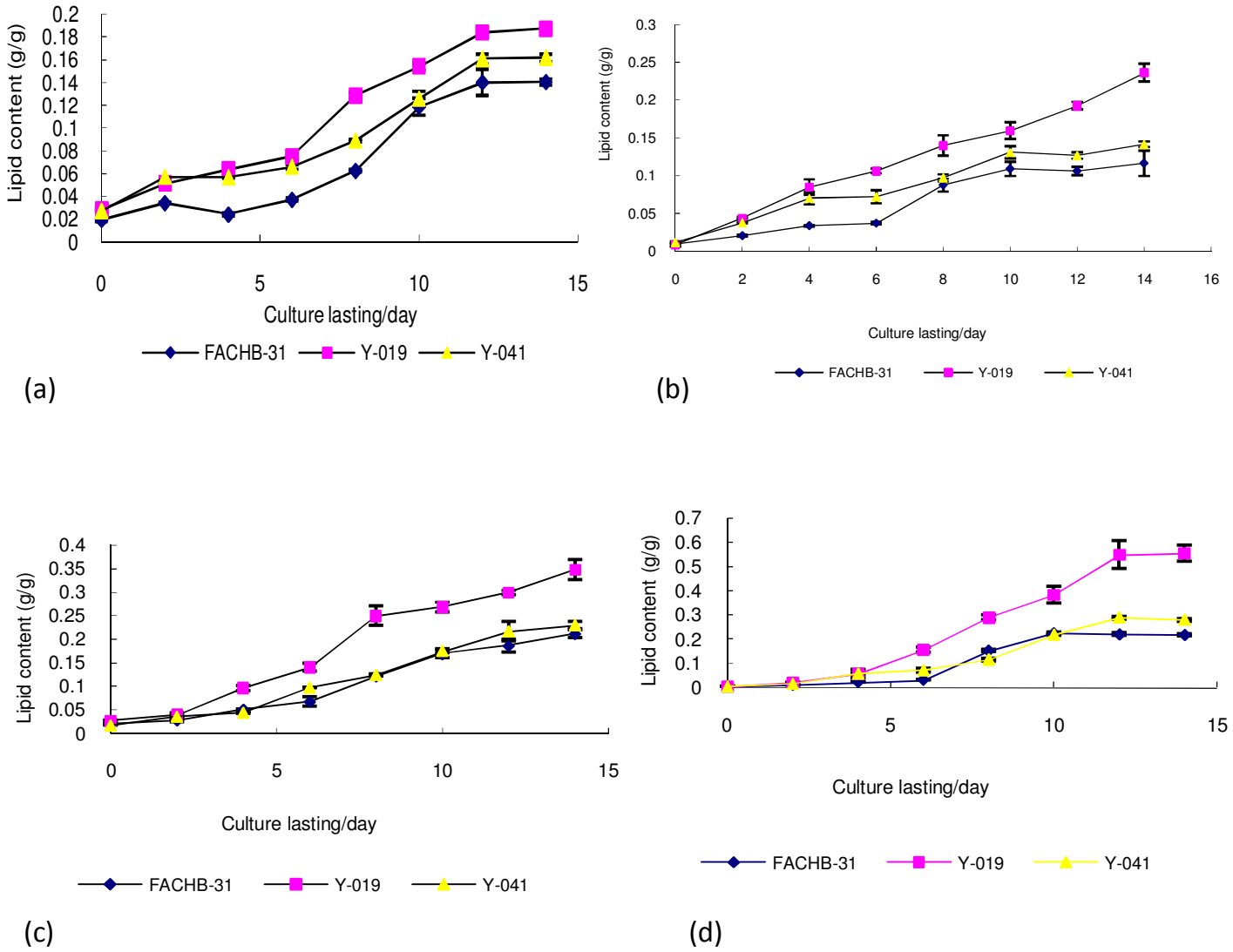

Figure 3a-d. Lipid accumulation of chlorellas in SE, BG11, HSM1 and DS medium.

production. However, sometimes we can hardly isolate an ideal algae with high lipid content and rapid growth rate. We have to use physical or chemical mutagenesis to reach our destination.

In this article we analyzed the growth rate and lipid content of three algae strains: *C. vulgaris* FACHB-31, *C. vulgaris* Y-019 and *C. pyrenoidosa* Y-041 in SE, BG11, HMS1 and the DS media. The results showed that, the HSM1 medium is more suitable for the growth of *Chlorella*, while the DS medium is more conducive to the lipid accumulation. In the DS medium, the lipid content of *C. vulgaris* Y019 was 1.84 ~ 2.64-fold than others at 12 days. So the local separated algae *C. vulgaris* Y019 is thought of an ideal raw material for biodiesel. *C. vulgaris* has gained considerable attention for biodiesel production due to its faster growth rate and easier cultivation than other strains. Various *Chlorella* strains have been proved to be suitable for biodiesel production (Feng et al., 2011; Liu et al., 2010). To data, many researchers focus on further improving the lipid content of these strains through strategies. In this study, using ultraviolet mutagenesis analysis, a total of 1086 algae strains had been selected, and then transferred to HSM and HSM-N medium. Lipid contents of these strains had been detected when they grew up. Results showed: in HSM medium, Y019-M57 and Y019-M66 were identified as high lipid content mutants, while in HSM-N medium Y019-M67 was detected as high lipid content mutants. These mutants will be studied on their genetic stability through long term cultivation and detection of their lipid

content. Furthermore, the molecular markers teleology such as single nucleotide polymorphism (SNP) or

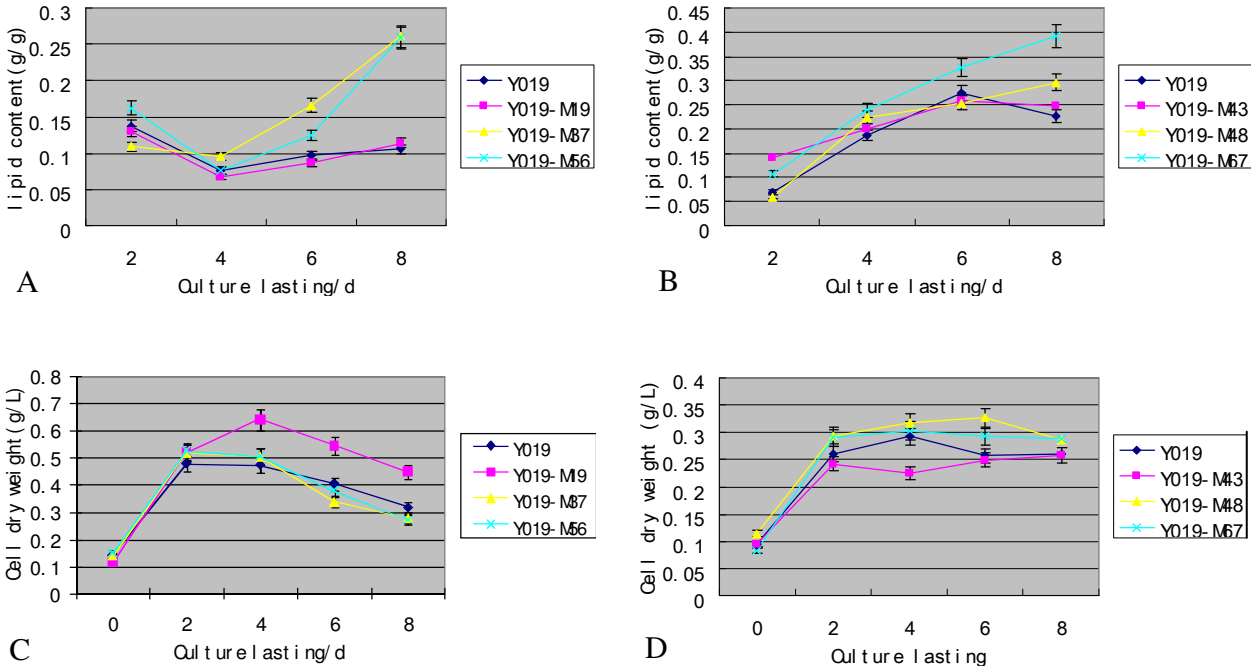

Figure 4. The growth curve and lipid content of *C. vulgaris* Y-019 and its ultraviolet mutants. lipid content comparison of ultraviolet mutant Y019-M19, Y019-M37, Y019-M56 and control Y019 in HSM medium: (A) lipid content of UV mutant Y019-M45, Y019M-48, Y019-M67 and Y019 in HSM-N medium; (B) the growth curve of UV mutants Y019-M19, Y019-M37, Y019-M56 and Y019 in HSM medium; (C) the growth curve of UV mutant Y019-M45, Y019-M48, Y019-M67 and Y019 in HSM-N mediums (D).

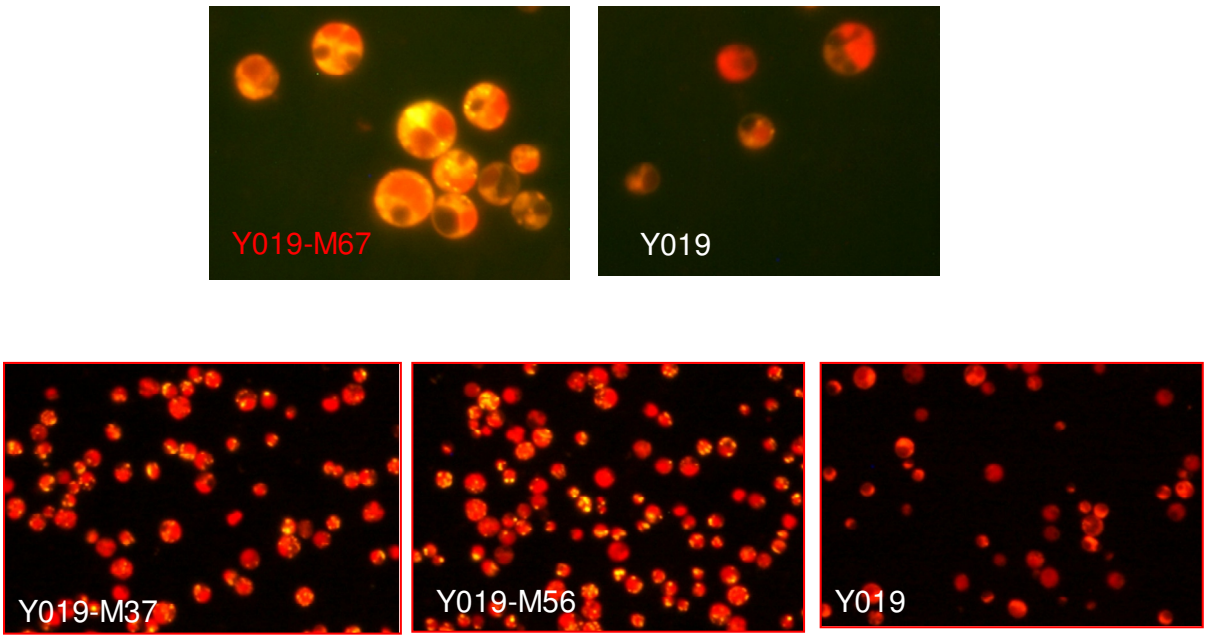

Figure 5. Fluorescence analysis of lipid content of *C. vulgaris* Y-019 and its ultraviolet mutants.

simple sequence repeat (SSR) will be used to find the possible DNA sequences that caused lipid accumulation in chlorellas.

ACKNOWLEDGEMENT

This work was supported by grants from the National Natural Science Foundation of China (30860028, 30960032), National Nonprofit Institute Research Grants (CATAS-ITBBZX0841) and National Natural Science Foundation of Hainan province (061012).

REFERENCES

Basova MM (2005). Fatty acid composition of lipids in microalgae. Int. J. Algae, 7: 33-57.

Chen W, Zhang CW, Song LR (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J. Microbiol. Methods, 77: 41-47.

Chisti Y (2007). Biodiesel from microalgae. Biotechnol. Adv., 25: 294-306.

Deng XD, Eriksson M (2007) Two Iron-Responsive Promoter Elements Control Expression of *FOX1* in *Chlamydomonas reinhardtii*. Eukaryotic Cell, 6: 2163-2167

Deng XD, Li YJ, Fei XW (2009). Microalgae: a promising feedstock for biodiesel. Afri. J. Microbiol. Res., 3: 1008-1014.

Feng Y, Li C, Zhang D (2011). Lipid production of *Chlorella vulgaris* cultured in artificial wastewater medium. Bioresour Technol., 102: 101-105.

Gao CF, Xiong W, Rapid (2008). quantitation of lipid in microalgae by time-domain nuclear magnetic resonace. J. Microbilol. Methods, 75: 437-440

Gouveia L, Oliveira AC (2009). Microalgae as a raw material for biofuels production. Ind. Microbiol. Biotechnol., 36: 269-274.

Hsieh CH, Wu W (T100). Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresour. Technol., pp. 3921-3926.

Huang GH, Gu C, Chen F (2009). Rapid screening method for lipid production in alga based on Nile red fluorescence. Biomass and Bioenergy, 5: 1-7.

Liang Y, Sarkany N, Cui Y (2009). Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol. Lett,, 31: 1043-1049.

Liu J, Huang J, Fan KW, Jiang Y, Zhong Y, Sun Z, Chen F (2010). Production potential of Chlorella zofingienesis as a feedstock for biodiesel. Bioresour. Technol., 101: 8658-63.

Mata TM, Martins AA, Caetano NS (2010). Microalgae for biodiesel production and other applications. Rev. Renew. Sustain. Energy Rev., 14(2010): 217-232.

Morgan-Kiss RM, Ivanov AG, Modla S (2008). Identity and physiology of a new psychrophilic eukaryotic green alga, *Chlorella* sp., strain BI, isolated from a transitory pond near Bratina Island, Antarctica. Extremophiles, 12: 701-711.

Scragg AH, Morrison J, Shales SW (2003). The use of a fuel containing *Chlorella vulgaris* in a diesel engine. Enzym. Microb. Technol., 33: 884-889.

Spolaore P, Joannis-Cassan C, Duran E (2006), Commercial applications of microalgae. J. Biosci. Bioeng., 101: 87-96.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.

Zhang XC, Shi YX, Meng Z (2007). Screening hig-yield strains Chlorella by UV mutagenesis, J. Ocean Univ. China, 37: 749-753.