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Characterization of the Growth, Quality and Antibiotics Inhibition in *Scenedesmus obliquus*

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The freshwater eukaryotic algae has potential applications in sewage treatment, biofuel and food production. Here, we characterized a freshwater microalgae *Scenedesmus obliquus (S. obliquus)*, which could grow normally under low light intensity and deposit at the bottom of the medium, facilitating the harvesting. We found that the dry mass of *S. obliquus* contained 45.90% of proteins and 9.53% of lipids that were mainly composed of 42.59% of palmitoleic acid (C16:1), 24.58% of palmitic acid (C16:0), and 19.14% of eicosapentaenoic acid (EPA). Besides,our results suggests that both pumping air into the culture medium and moderate darkness stress could increase the growth rate of *S. obliquus,* whereas shaking had no obvious effects.Moreover, the effects of eight antibiotics on the growth of *S. obliquus* were investigated. Among them, chloramphenicol (Cm) and streptomycin (Str) had the strongest growth inhibitory effects, as well as higher concentration of geneticin (G418) and gentamycin (Gen), suggesting their potentiality to be used as selection markers for transformation experiments. Together, our data indicated that the *S. obliquus* is a promising resource for biofuel and food production. The identification of several candidate selection markers will also facilitate genetic manipulations to further improve the lipid yield in *S. obliquus.*

Key words: *Scenedesmus obliquus*, Growth, Lipid composition, Antibiotics, Selection marker.

The energy shortage is one of key factors influencing world economy. While environmental protection has become the focus of attention among the world.The fossil energy is a limitedÿnon-renewable resource, generating greenhouse gases[1]. Thus, there is a urgent need to search for substitute resources. Currently, eukaryotic microalgae are at the forefront of research for biofuel production[2-4].

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Compared to oil plants, microalgae are more efficient in converting sunlight into chemical energy. Most kinds of the microalgae grow easily in diverse conditions, and do not take over agricultural land. Microalgae contains many kinds of essential substances, such as C_{16} and C_{18} fatty acids, which are useful for biodiesel production. It also contains multiple vitamins and the single cell protein (SCP). It can be harvested batch-wise nearly all-year-round, providing a reliable and continuous supply of oil and other substances[5]. However, the potential application for most of the microalgaes is limited because they are difficult to harvest and contain relatively lower level of lipids to be used for biofuel production[6-7].

Genetic manipulations have been used to identify the gene function. *Chlamydomonas*

reinhardtii is a powerful model system for microalgae research[8-10], and the chloroplasts, mitochondria and nuclear genome of it could be targeted for transformation using different methods[11-12]. *Chlorella,* a freshwater microalgae, is also used to express heterogonous proteins[13] and is developed as a bioreactor utilizing efficient transformation technique like electric shock[14]. In addition, *Gonium pectorale*, a freshwater microalgae with intermediate complexity between unicellular *Chlamydomonas* and multi-cellular relatives with differentiated cell types, can also be transformed stably[15]. The critical step of transformation of microalgae.is to choose the suitable selection markers, Therefore, the selectable maker research of the host microalgae in genetic transformation should be investigated first.

Scenedesmus, like *Chlamydomonas*, is a freshwater microalgae[16]. It plays an important role in sewage treatment[17], and is also a promising resource for biofuel, feed and food[18-20]. However, there are few reports about the growth and nutrients of *S. obliquus*, and no selection markers have been identified for transformation in *S. obliquus*. In this study, we firstly characterized the growth of *S. obliquus*, determined its lipid contents and composition and protein contents, and measured its sensitivity to antibiotics. The results showed that *S. obliquus* can grow and be harvested easily, It contains high amount of proteins and fatty acids useful for health and biofuel production. We also found *S. obliquus* is very sensitive to Cm and Str, as well as higher concentration of G418 or Gen, suggesting their usefulness for genetic engineering to further improve the yield of lipids or other important substance.

MATERIALS AND METHODS

S. obliquus strains and culture conditions

S. obliquus was grown in BG-11 medium[21]. To examine the growth of *S.obliquus* under different cultural conditions including static, shake and aerobic cultivations. The S. obliquus was cultured with a volume of 500 ml in a 1000 ml flask with 16 hours of continuous cool fluorescent light of 60 μmol m^{-2} s^{-1} and 8 hours of darkness per day at 25°C. To determine the antibiotics sensitivity, the *S. obliquus* was cultured with a volume of 5 ml in a 12-well plate in the incubator with a similar cycle of light and darkness

Determining the growth curve of *S. obliquus*

Algae samples were taken from the solid culture medium, and then transferred into a 500 ml lipid culture medium in 1000 ml flask. The OD (680nm) of *S. obliquus* per 48 hours was measured and the growth curve was plotted.

Confocal microscopy observation

S. obliquus cells were observed with scanning confocal microscope of Leica TCS SP5 II. Excitation and emission filters Ex 488 nm/BA520-560 nm were used for chloroplast observation. The white light was used for whole cell observation. The final picture was the merge of the two observations.

Cell number calculation of *S. obliquus*

The cell number of the *S. obliquus* was counted at the time points when OD (680nm) reached 0.1, 0.3, 0.5, 0.8, 1.0 utilizing blood count plate. The optical density value and cell number is of an linear relationship and the 0.1 OD corresponds to $2.1x10^6$ cells.

The relative growth rate of *S. obliquus*

$K=(\lg N_t-\lg N_0)/T$ [22]; *K,* relative growth rate; N_0, the initial concentration of the microalgae; N_t, the concentration of the microalgae after cultivating phase of *T* time; *T,* culture time.

Measurement of total lipid content

Approximately 2 g to 5 g dried powder of *S. obliquus* grown to logarithmic phase was harvested and weighed, then was packaged in filter paper and placed into the sample cup of Soxhlet extractor. 150 ml petroleum ether was added to the flask of Soxhlet extractor and incubated for 8 hours. The flask was dried and weighed after the petroleum ether was evaporated completely. The formula to calculate the percentage of lipid content was $x=(m_2-m_1)/m$; x, the percentage of lipid content; m, the weight of microalgae sample; m_1, the weight of the flask of the Soxhlet extractor; m_2, the total weight of the flask and the lipid after extraction[23].

Methyl ester derivation and GC-MS analysis of fatty acid (FA)

The *S. obliquus* biomass was harvested on the 36th day by centrifugation at 4000 g and then lyophilized. 100 mg Freeze-dried sample of biomass was treated with 1ml extraction buffer of methanol: sulfuric acid (1:2.5%, v/v) in a glass tube

with screw cap for 2 hours in a water-bath at 80 ! after 20 µg standard fatty acid C15:0 was added. This mixture was homogenized every 20 min to hydrolyze ester bonds of membrane lipids and triglycerides. 1 ml 0.9% NaCl (v/v) and 1 ml hexane were added to reduce the solubility in the extraction buffer after the mixture was cooled to the room temperature, then centrifuged at 4000 g for 10 min. The upper phase collected was dried under vacuum and kept at -20°C. Three replicates for each extraction were performed.

The GC-MS analysis for fatty acids methyl esters (FAME) was performed on Trace GC Ultra gas chromatography (Thermo, USA) instrument coupled with an ITQ1100 mass selective detector (Thermo, USA) and an injector. A capillary column TR-5MS with dimension of 30 m × 0.25 mm × 0.25 µm thickness (Thermo Hypersil-Keystone, USA) was used for separation of fatty acid methyl esters utilizing ethyl acetate. The sample was maintained for 1 min at 120°C, then the temperature was raised to 150 °C at the rate of 10°C/min, continued to 250 °C at the rate of 4 !/min, and kept at 250°C for 10 min. The split ratio was 1:20, and the helium was used as a carrier gas with the flow rate of 1ml/min. The injector and detector temperature were 260°C and 250°C, respectively. The mass spectrometer was operated in the electron impact (EI) and the temperature of the ion source is 230°C in the scan range of 29-450 m/z [24].

Measurement of total protein content

The microalgae was harvested from samples of logarithmic growth phase, and approximately 2 g dried microalgae was used to measure the protein content with Kjeldahl nitrogen analysis. The formula was $x=(v_1-v_2)$ c×0.014×F×100%/0.1m [25]; x, the protein content of sample (g/100g or g/100ml); v_1, sample consumption volume of HCl standard solution (ml); v_2, blank reagent consumption volume of HCl standard solution (ml); c, the concentration of HCl standard solution (mol/L); 0.014, the nitrogen content corresponding to 1.000 mol/L HCl standard solution; m, the sample weight (g) .

Sensitivity of *S. obliquus* to the eight antibiotics

About 10% of *S. obliquus* in logarithmic phase was vaccinated and cultivated initially in the BG-11 medium containing different concentration of Amp, Kan, Cm, Gen, Str, G418, Hyg, and PPT respectively (Table1), and the responses to these compounds were measured in terms of optical density value (OD 680 nm) using Synergy *HT* Multi-Mode Micro-plate Reader per 48 hours. Triplicate experiments were performed.

RESULTS

The phenotype and growth of *S. obliquus*

S. obliquus cells were observed with confocal microscopy. As shown in Figure 1A, the *S. obliquus* exists with 2 cells living together. The diameter of the single cell is about 8.9 um. The circled part in the cell center is nucleus, and the red parts are chloroplasts. It is interesting to note that the growth pattern of *S. obliquus* is unique. It is deposited to the flask bottom (Figure 1B left), while the *Chlamydomonas reihardtii* is suspended in the medium (Figure 1B right). This feature greatly facilitates the harvesting of *S. obliquus*.

As shown in Figure 1C, the growth rate of *S. obliquus* from shake culture is very similar to

Table 1. Different concentrations of antibiotics to *S. obliquus*

Antibiotics	Concentrations (µg/ml)			
Amp	0	100	300	500
Kan	0	100	300	500
Cm	0	100	300	500
Gen	0	100	300	500
Str	0	100	300	500
G418	0	100	300	500
Hyg	0	100	300	500
PPT	0	20	50	80

Table 2. Composition and content of fatty acids in *S. obliquus*

Fatty acid	Retention time (min)	Content (%)
Myristic acid (C14:0)	11.15	5.06
Palmitoleic acid (C16:1)	15.02	42.59
Palmitic acid (C16:0)	15.46	24.58
Oleic acid (C18:1)	19.39	6.75
Stearic acid (C18:0)	19.82	1.88
EPA(C20:5)	22.78	19.14

The retention time represents the peak time of various fatty acids, and the peak areas indicate the content of these fatty acids. Data reported were analyzed by GC-MS, and were mean values of three independent experiments.

that from static culture (in shelf). Interestingly, the *S. obliquus* from aerobic culture (pumping air) grew much faster and reached to stable growth phase four days earlier than the control (Fig 1D), and the final cell concentration was also higher than the control. In addition, the average accumulation rate of *S. obliquus* dry mass was 0.148 g L^{-1} d^{-1} , which is also higher than the control (about 0.115 g L^{-1} d^{-1}).

Lipid content, lipid composition and protein content of *S.obliquus*

The total lipid content of *S. obliquus* was 9.53% of dry mass as measured by Soxhlet extractor. The total lipid was applied for gas chromatogram, and the results were shown in Figure 2 and Table 2. The majority of fatty acids of *S .obliquus* is unsaturated (68.48%), including Palmitoleic acid (42.69%) , Oleic acid (6.75%) and Eicosapentaenoic acid (19.14%), which are beneficial to human's health. Other fatty acids are saturated (accounted for 29.64%), including myristic acid (5.06%) and palmitic acid (24.58%), which are useful for bio-diesel production.

The protein content of *S. obliquus* was 45.90% of dry mass, as were measured utilizing Kjeldahl nitrogen analysis, indicating a good supply source for proteins.

The sensitivity of *S. obliquus* to antibiotics

To identify useful markers for genetic manipulations, we transferred about 10% of vaccinated *S. obliquus* of logarithmic phase to the medium of BG-11 containing different concentrations of 8 antibiotics and incubated for two weeks (Table1). The growth of *S. obliquus* was not sensitive to Amp, Kan and PPT a plant growth inhibitor (Figure 3,A, B,I). Remarkably, the growth of *S. obliquus* was almost completely inhibited two days after inoculation when the concentration of Cm reached 100 µg/ml (Figure 3C), whereas the ethanol used to dissolve Cm had no inhibitory effect (Figure 3D). Higher concentration of Gen ranged from 100 – 500 µg/ml also had an inhibitory effect on the growth of *S. obliquus* (Figure 3E). Str showed strong inhibitory effect on the growth of *S. obliquus*, the biomass of which did not increase much after two days of inoculation of very low concentration of Str (Figure 3F). G418 at a concentration of 100 µg/ml had no significant effect on the growth of *S. obliquus*, but it showed significant inhibitory effect at a concentration of 300 µg/ml (Figure 3G). High concentration of Hyg (500 µg/ml) had only a moderate inhibitory effects (Figure 3H), suggesting *S. obliquus* was not sensitive to Hyg. The inhibitory effect of the Hyg was gradually becoming obvious after six days incubation, and almost 50% cells were still alive in the medium containing 500 µg/ml of Hyg on the fourteenth growth day compared to the control group (Figure 3H). The relative growth rates of the tested on various concentrations of inhibitors *S. obliquus* was listed in Table 3. After two weeks, compared to the control, the relative growth rate of *S. obliquus* showed a significantly decrease in the medium with different tested concentrations

Table 3. Relative growth rate of *S. obliquus* treated with various antibiotics

Antibiotics	Concentrations			
	0	100 µg/ml (PPT 20)	300 µg/ml (PPT 50)	500 µg/ml (PPT 80)
Ampicillin Amp	0.0762±0.0057	0.0707±0.0074	0.0693±0.0063	0.0689±0.0022
Kanamycin Kan	0.0637±0.0042	0.0576±0.0035	0.0553±0.0037	0.0538±0.0039
Chloramphenicol Cm	0.0636±0.0044	0.0018±0.0003	0.0016±0.0007	-0.015±0.0002
Gentamycin Gen	0.0574±0.0030	0.0456±0.0042	0.0448±0.0045	0.0397±0.0027
Streptomycin Str	0.0831±0.0083	0.0147±0.0016	0.0077±0.0017	0.0108±0.0019
Geneticin G418	0.0778±0.0074	0.0551±0.0056	-0.0084±0.0009	-0.0106±0.0003
Hygromycin Hyg	0.0605±0.0046	0.0565±0.0056	0.0513±0.0024	-0.0114±0.0008
Phosphinothricin PPT	0.0617±0.0042	0.0525±0.0047	0.0526±0.0029	0.0478±0.0023

The samples were taken from the logarithmic growth phase of *S. obliquus* treated with different antibiotics, and the growth rate of each tested sample was measured after two weeks exposure to Amp, Kan, Cm, Ethanol, Gen, Str, G418, Hyg and PPT. The results was corresponding to Figure 3. Data reported were mean values of three independent experiments .

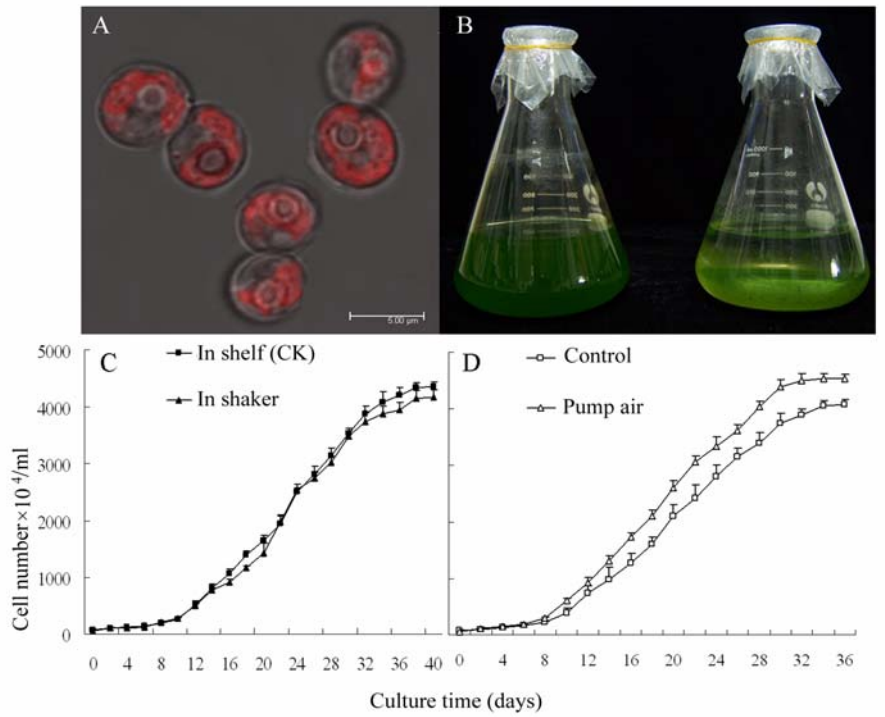

Fig. 1. The growth characteristics of *S. obliquus*. (A) Confocal microscopy. The cells of *S. obliquus* at stable growth phase were observed.. The red colored parts are chloroplasts and the circled part in the center is nucleus. Bar=5μm, (B) The growth pattern of *Chlamydomonas reihardtii* (left) and *S. obliquus* (right). Both microalgaes were in the stable growth phase. (C) The growth curves of *S. obliquus* with the treatment of shaking or without shaking (In shelf). (D) The growth curves of *S. obliquus* with the treatment of pumping air or without pumping air (Control). Data reported are mean values of three independent experiments and presented as means ± SE

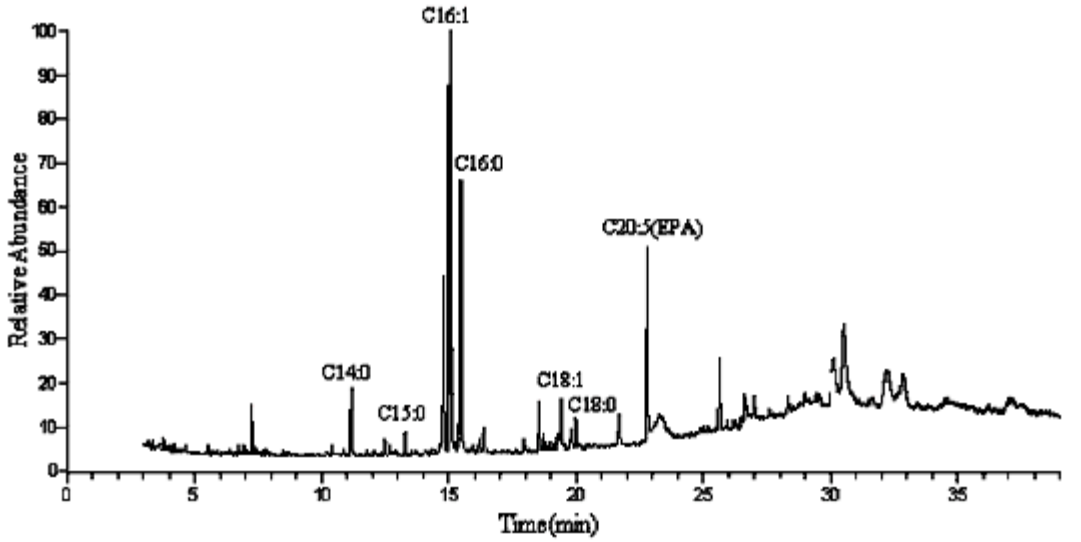

Fig. 2. The chromatographic of fatty acid methyl esters in *S. obliquus*. The sample extracted by methanol was harvested from the dried microalgae grown to the logarithmic phase. The peaks represent different types of fatty acids

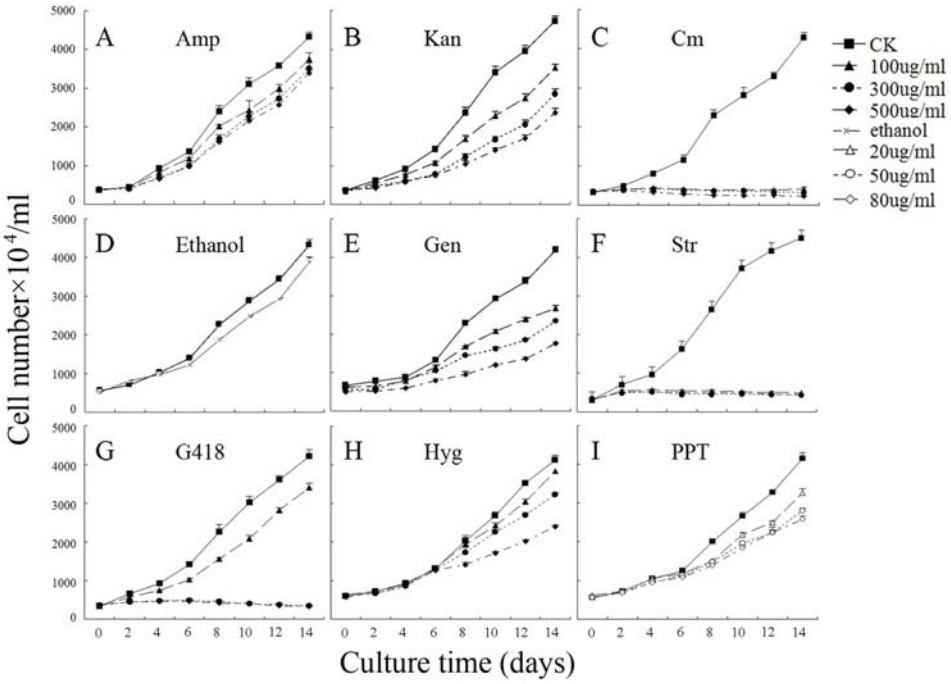

Fig. 3. Effect of antibiotics on the growth of *S. obliquus*. Inhibition of growth (Cell number) was measured in the *S. obliquus* test strains after two weeks of exposure to eight antibiotics. (A) Amp, (B) Kan, (C) Cm, (D) Ethanol, (E) Gen, (F) Str, (G) G418, (H) Hyg, (I) PPT. Data reported were mean values of three independent experiments and presented as means±SE

of Cm, Str and the relative high tested concentrations of G418 ($P<0.05$). Whereas, the relative growth of *S. obliquus* didn't decrease significantly in the medium with different tested concentrations of other antibiotics ($P>0.05$).

DISSCUSION

In recent years, microalgae has attracted a lot attentions in the field of biofuel research. *S. obliquus* is considered to have the potentiality to be developed as a new generation of bio-energy producer. In this study, we characterized the growth, lipid content and composition, protein content, and antibiotic sensitivity of *S. obliquus.*

We found that the *S. obliquus* can be deposited to the flask bottom, while the majority of microalgae belonging to the Chlorophyta grow in the floating medium. It greatly facilitates the harvesting and is one of its advantages for production of biofuel and other materials. We also found that the growth rate and biomass accumulation of *S. obliquus* can be significantly increased by simply pumping air into the culture, whereas static or shake cultivation does not have this effect. It is reported that carbon dioxide can improve the capacity of the photosynthesis to increase the microalgae biomass[26].Thus, it is possible that pumping air may help provide a continuous supply of carbon dioxide to enable the fast growth of *S. obliquus*.

Although *S. obliquus* contains low level of total lipid as commonly found from members of the Chlorophyceae[27], it contains a lot short-chain fatty acids, which is an important advantage for biofuel production. In addition, a large percentage of the unsaturated fatty acids is C20:5 (Table 2), which is considered to be able to reduce low-density lipoprotein cholesterol[28] to improve human's health. It also contains a lot proteins (45.90% of dry mass), indicating its potentiality to be used as the feed and food.

Genetic engineering could be used to increase the content of the fatty acid in microalgae. However, there have no established transformation techniques for *S. obliquus*. As the first step to

implement genetic manipulation in *S. obliquus*, we started to search for useful selection markers. Our data showed that the growth of *S. obliquus* was very sensitive to Cm, Str and G418, and also to high concentration of Gen and Hyg. Cm can disturb the protein synthesis. The resistance gene corresponding to Cm is called *cat* coding the chloramphenicol acetyltransferase, which could acetylate and inactivate the Cm. The *cat* gene has been widely used as a selection maker for the terrestrial higher plants and marine plants[29]. G418 and Str belong to the amino glycoside antibiotics and can inhibit the protein synthesis[30]. The phosphoric acid transferase gene obtained from Tn5 transposon is the resistance gene for Str, which is also used as selection marker in some higher plants like tobacco and some marine organism like Cyanobacteria[31]. The coding product of *npt* (Neomycin phosphotransferase) may deactivate the amino glycoside antibiotics G418, which is commonly used as a selection marker in plant transformation. The previous reports have already suggested that both the *Chlamydomonas reinhardtii* and the *Chlorella* were sensitive to the G418 [32]. And our study also indicated that G418 may be used in *S. obliquus* as a selection marker. It is interesting to find that both Kan and Amp have no effects on the growth of *S. obliquus*. Thus, they may be used to inhibit bacterium growth in the *S. obliquus* culture without degrading the performance of this microalgae.

In all, our data suggest that *S. obliquus* is a good resource for production of biofuel- and healthy-related materials, We also found several useful selection markers, which may be used for subsequent genetic engineering to further improve the yield of important substances in *S. obliquus*.

Abbreviations

S. obliquus, Scenedesmus obliquus; Amp, ampicilin; Kan, kanamycin; Cm, chloramphenicol; Gen, gentamycin; Str, streptomycin; Hyg, hygromycin; G418, geneticin; PPT, phosphinothricin; OD, optical density; GC-MS, gas chromatographymass spectrometry.

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