CHARACTERIZATION AND IDENTIFICATION OF FRESHWATER MICROALGAL STRAINS TOWARD BIOFUEL PRODUCTION

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Fifty-three algal cultures were isolated from freshwater lakes in Hainan, China. Four microalgal isolates were selected because they could be successfully cultivated at high density and demostrated a strong fluorescence after being stained with nile red. These cultures were identified as strains of *Chlorella sp. C11*, *Chlamydomonas reinhardtii C22*, *Monoraphidium dybowskii C29*, and *Chlorella sp. HK12* through microscopic and 18S rDNA analysis. Under similar conditions, the lipid productivity of *Chlorella* sp. *C11*, *Chla. reinhardtii C22*, *M. dybowskii C29* , and *Chlorella* sp. *HK12* were 1.88, 2.79, 2.00, and 3.25 g L^{-1}, respectively. *Chla. reinhardtii C22* yielded a higher lipid content (51%), with a lower biomass concentration (5.47 g dwt L^{-1}). *Chlorella* sp. *HK12* reached a growth rate of 0.88 day^{-1} at OD540nm and yielded a biomass concentration of 7.56 g dwt L^{-1}, with a high lipid content of 43%. Gas chromatography/ mass spectrometry analysis indicated that lipid fraction mainly comprises hydrocarbons including palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acids. Our results suggest that *Chlorella* sp. *HK12* is a promising species for biodiesel production, because of its high lipid productivity and a relatively high content of oleic acid.

Key words: Microalgae; Biodiesel; 18S rDNA; Fatty acids

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INTRODUCTION

Algae are the only primary producers in the oceans, which cover 71% of the Earth's surface (Andersen 2005). Algae are the original sources of fossil carbon found in crude oil and natural gas (Andersen 2005). Microalgae, which cover almost 75% algal species, contribute approximately 40% of the oxygen in the atmosphere. The first report on pure cultures of microalgae stems from Beijerinck (1890). He successfully isolated symbiotic green microalgae from *Hydra* and lichens (Beijerinck 1893). Microalgae have been attracting attention as a source of high-lipid material to produce biofuel because the biofuel they produce are biodegradable, renewable, non-toxic fuel and do not compete with food crops. In comparison to plant oils for biodiesel, microalgae oils are higher in lipid content, possess faster growth rates, and are capable of growing in saline waters that are unsuitable for agriculture (Alcaine 2010; Puppan 2002; Sheehan et al. 1998). Usage of biodiesel will allow for a balance to be achieved between environmental, agricultural, and economic development goals.

Hundreds of microalgal strains capable of producing large quantities of lipids have been screened, and their lipid production metabolisms characterized and reported. Some microalgal strains produce large quantities of lipid as a storage product, regularly achieving 50 to 80% of their dry weights as lipids. For instance *Nannochloropsis* sp. and *Neochloris* sp. can grow to a high cell density (50 and 27.5 g L^{-1}, respectively) under autotrophic growth conditions, and obtain a high lipid content (52% and 46%, respectively) (Moazami et al. 2011). Gouda et al. (2008) found that *Gordonia* sp. could accumulate lipids as high as 80% of dry weight under special culture conditions with a relatively low biomass (<2 g L^{-1}).

In the last decade, DNA sequencing and genomics have brought substantial change to microalgal taxonomy (Abou-Shanab et al. 2011). Both the subunit ribosomal DNA and ITS gene have been used in many studies for species identification because they include highly conserved regions at the species level (Rogers et al. 2006; Medlin et al. 1994; Bell and Grassle 1998).

Biomass productivity, total lipid content, and lipid productivity are among the main parameters that determine the economic feasibility of using microalgae as a source of biofuels. In order for microalgae to become an economically viable biofuel feedstock, the cost of producing biodiesel from microalgae needs to be reduced. Identifying a good strain for oil production, which should feature high lipid content, high biomass, and tolerance to extreme environments, remains a difficult prospect. Although microalgae lipids are not yet capable of bulk production, with the development of systems biology and genetic engineering, algae biofuel process can become economically feasible within 10 to 15 years (Wijffels and Barbosa 2010).

The aims of this work were to discover microalgae species with high biomass, high lipid content, suitable fatty acids, and tolerance to environments for biodiesel production. In this study, we selected microalgae with high lipid content from freshwater lakes at Haikou (110.35E, 20.02N) and Danzhou (109.57E, 19.52N), Hainan province, China. The growth rate, biomass, and lipid content of some microalgal isolates were determined. Furthermore, these microalgal species were subjected to fatty acid profile analysis. Moreover, 18S rDNA gene of the isolates was sequenced to confirm the identities of the microalgal species.

EXPERIMENTAL

Isolation, Purification of Microalgae

Water samples used to isolate microalgae were collected from freshwater lakes at Haikou (110.35E, 20.02N) and Danzhou (109.57E, 19.52N), Hainan province, China. Water samples (500 µL) were inoculated onto petri plates on BG-11 agar (1.5% w/v bacteriological agar). The petri plates were incubated at 26 °C under continuous illumination with white fluorescent light for fifteen days. A single colony was picked and inoculated into wells of a 96-well microtiter plate with 150 µl BG-11 medium in each well. The purities of the culture were ensured by repeated plating and regular observation under an optical microscope.

DNA Extraction, PCR Amplification, and Cloning of 18S rDNA PCR Products

DNA from microalgae was extracted using a fungus Genomic DNA extraction kit (BBI, Canada), following the manufacture′ s instructions. DNA extracts were stored at -4 °C before being used as template for PCR amplification. The 18S rDNA was amplified by PCR using primers: forward primer 5′-CCTGGTTGATCCTGCCAG-3′ and reverse primer 5′-TTGATCCTTCTGCAGGTTCA-3′ (Yuan et al. 2011; Xia et al. 2011). The 50 µL PCR amplification system contained 10 pM template, 1 µL (10 µM) forward primer, 1 µL (10 µM) reverse primer, 1 µL dNTPs, and 0.25 µL Taq DNA polymerase. The thermal cycling was programmed as follows: 5 min at 98 °C, 35 cycles of denaturation at 95 °C for 35 sec, annealing at 55 °C for 35 sec, and a 40 sec extension step at 72 °C with an additional 8 min cycle at 72 °C. PCR products were separated by electrophoresis on 1% agarose gel. Bands were extracted and purified from the gel with an SK1131 kit (Sangon, China).

The PCR products were ligated into the pUCm-T and then transformed into *E. coli* competent cells (SK2301, Sangon, China), following the manufacture′ s instructions. Positive clones were grown in LB medium, and the extraction of plasmids was performed with an SK1191 kit (Sangon, China) (Anderson et al. 2003; Berard et al. 2005). Sequence of 18S rDNA was determined by an ABI-3730 automatic capillary sequencer.

Phylogenetic Analysis

All sequences were compared with the GenBanK database using BLAST (Basic Local Alignment Search Tool) and were manually aligned with representative sequences from microalgae strains and related taxa, according to similarities in secondary structure identified by the ClustalX program (Altschul et al. 1997). A phylogenetic tree built with the neighbor-joining algorithm was inferred from the nucleic acid sequences of different microalgae using MEGA5 program.

Microalgae Cultivation and Biomass

The microalgal strains were cultivated in 2.3 L nutrient replete medium (2.1 L BG-11 medium and 0.2 L algal inoculums) in a 3 L photobioreactor (Infors 3, Germany) with continuous aeration at 26 °C, 300 rpm. The continuous illumination (200 µmol m^{-2} s^{-1}) was provided by plant growth lamps. Each strain was cultivated for about 16 days. Algal growth was monitored by measuring daily changes in optical density at 540 nm with a spectrophotometer. Microalgal cells were harvested by centrifugation and washed three times with deionized water. Microalga pellets were dried by freeze-drying for dry weight measurement.

Extraction of Total Lipids, Fatty Acid Analysis

Total lipid content was calculated with the modified Bligh-Dyer method as described by Luyen HQ (Bligh and Dyer 1959; Luyen et al. 2007). Dried cells (100 mg), 8 mL water, 10 mL chloroform, and 20 mL methanol were added to each bottle. After for 10 min sonication, 10 mL water and 10 mL chloroform were added, and the mixture was sonicated for another 10 min. The lower chloroform layer was collected and evaporated

in an oven at 60 °C. After cooled in a vacuum desiccator , the bottle with content was weighed, and the total lipid content was determined gravimetrically.

Form the extracts obtained in the total lipid determination, the transesterification of fatty acids was performed following the method by Christie (1982). Fatty acids were analyzed in an HP 6890 GC equipped with a mass spectrometer. 1.0 µL of each sample was injected into a fused silica capillary column (30 m x 0.25 mm. i.d., 0.25 µm film thickness), with helium as mobile phase at 1.0 mL min^{-1} under optimized conditions.

RESULTS AND DISCUSSION

Isolation and Identification of Microalgae

Two freshwater samples obtained from Haikou (110.35 E, 20.02 N) and Danzhou (109.57 E, 19.52 N) lakes were screened for high-lipid content microalgae. A total of 53 algal cultures were isolated, and four green microalgal isolates (C11, C22, C29, HK12) were selected because they could be cultivated at high cell density. Also, the selected microalgal strains indicated high neutral lipid content according to nile red staining (data not shown) (Matsunaga et al. 2009; Chen et al. 2009; Elsey et al. 2007). So far, little information about the oil-producing microalgal strains discovered and isolated from Danzhou has been obtained, especially regarding freshwater microalgae. Light microscopic images of the species isolated in this study are shown in Fig. 1. Microscopic analysis of the samples allowed preliminary identification of isolates C11, C22, C29, and HK12 as genus *Chlorella*, *Chlamydomonas*, *Monoraphidium*, and *Chlorella*, respectively.

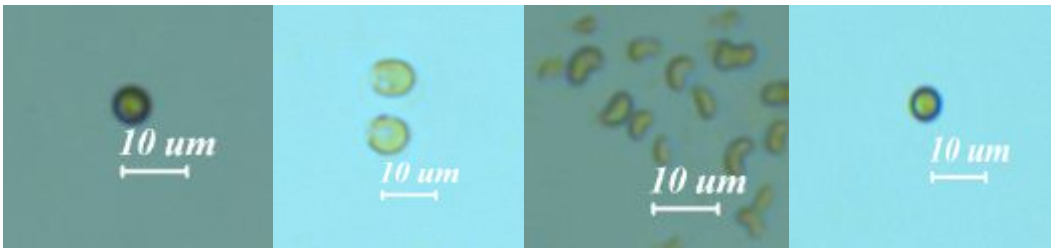

Fig 1. Light microscope (40X) pictures of the tested microalgal isolates

PCR Amplification and Sequence Analysis

In order to further confirm the identity of the isolates, DNA from microalgae was extracted (Stach and Turbeville 2002). The expected amplicons of microalgal DNA were amplified by the universal forward and reverse primers. In each case, PCR products of the expected sizes appeared as intense bands on agarose gels. The approximate sizes of the amplified PCR products were 1.6 to 1.8 kb. According to the 18S rDNA sequences, we concluded that microalgal isolates C11, C22, C29, and HK12 were closely related to *Chlorella sorokiniana*, *Chlamydomonas reinhardtii*, *Monoraphidium dybowskii*, and *Chlorella zofingiensis*, based on 96%, 99%, 97%, and 95% sequence similarities, respectively. Identification of the four microalgal strains was also supported by the results from the phylogenetic analysis of the 18S rDNA sequence. In the phylogram (Fig.

2), the 18S rDNA sequences of isolates C11, C22, C29, and HK12 confirmed their identification as *Chlorella* sp., *Chlamydomonas reinhardtii*, *Monoraphidium dybowskii*, and *Chlorella* sp.. They had sequence similarities of 96%, 99%, 97%, and 95% to *Chlorella sorokiniana X62441.2*, *Chlamydomonas reinhardtii M32703.1*, *Monoraphidium dybowskii Y16939.1*, and *Chlorella zofingiensis X74004.1*.

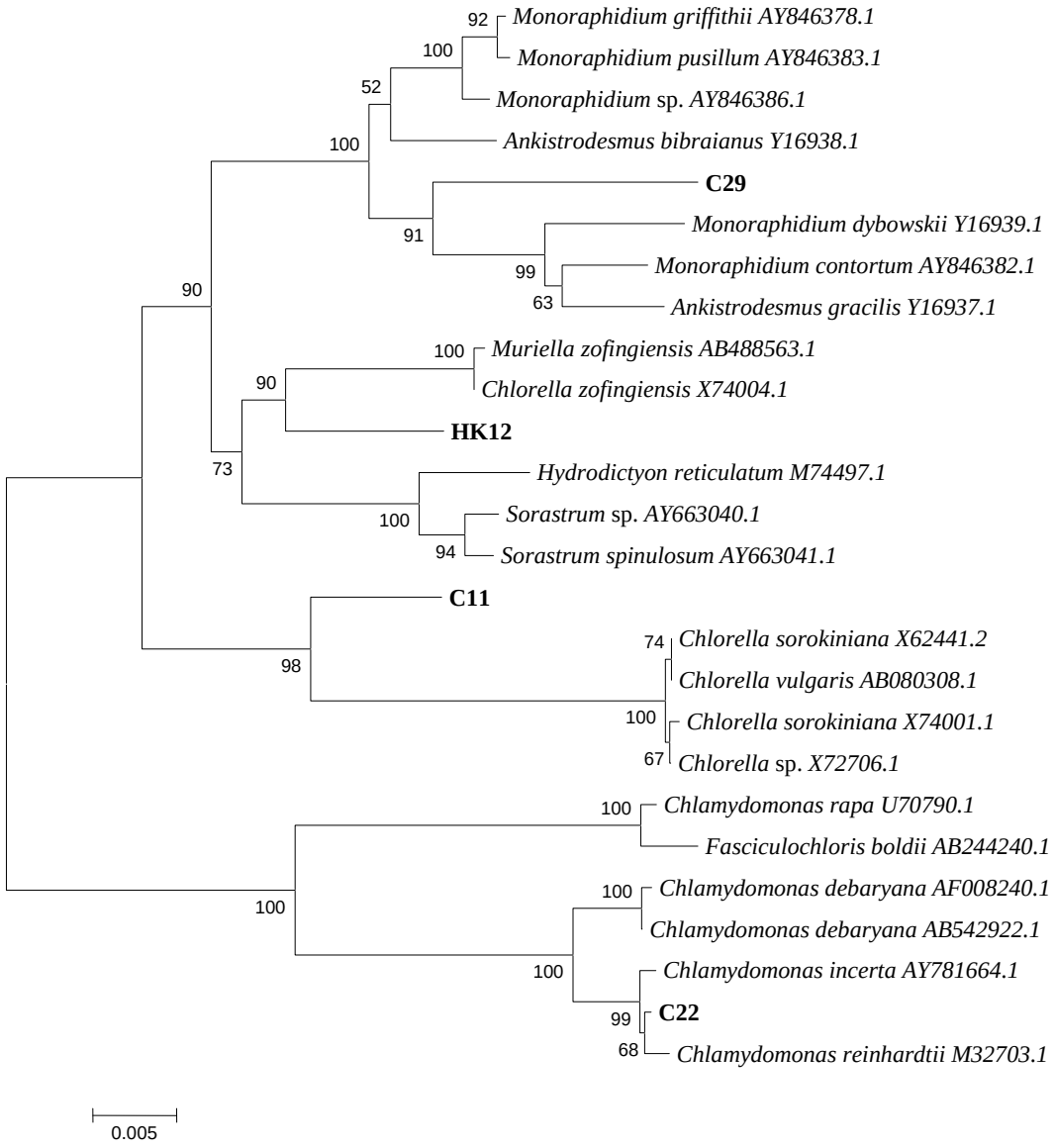

Fig. 2. Phylogenetic analysis realized on nucleotide sequences of PCR products from 18S rDNA extract and purified from the tested microalgal isolates (C11, C22, C29, HK12). Comparison was with the most similar sequences retrieved from the NCBI nucleotide database.

Growth Rate and Biomass Production

Microalgae can grow profusely when supplied with sufficient nutrients and suitable conditions. Algal growth is directly affected by the availability of nutrients, temperature, light, and pH. Under similar environmental conditions the net growth rate differed among the four microalgal species (Fig. 3). The average specific growth rates of *Chlorella* sp. *C11*, *Chla. reinhardtii C22*, *M. dybowskii C29*, and *Chlorella* sp. *HK12*, were 0.56, 0.21, 0.67, and 0.88 day^{-1} at OD 540 nm, respectively. The growth rate of *Chlorella* sp. *HK12* after 16 days of incubation was 14.12, compared with an initial reading of 0.09. Biomass productivities of 6.70, 5.47, 5.13, and 7.56 (g dwt L^{-1}) were obtained for *Chlorella* sp. *C11*, *Chla. reinhardtii C22*, *M. dybowskii C29*, and *Chlorella* sp. *HK12*, respectively (Table 1). The biomass of those microalgal strains was much higher than the same genus′ reported by Abou-Shanab et al. (2011). *Chlorella* sp. *HK12* showed the highest biomass productivity at 7.56 (g dwt L^{-1}), while *M. dybowskii C29* had the lowest biomass productivity at 5.13 (g dwt L^{-1}). Therefore, *Chlorella* sp. *HK12* is suitable for high-density culture.

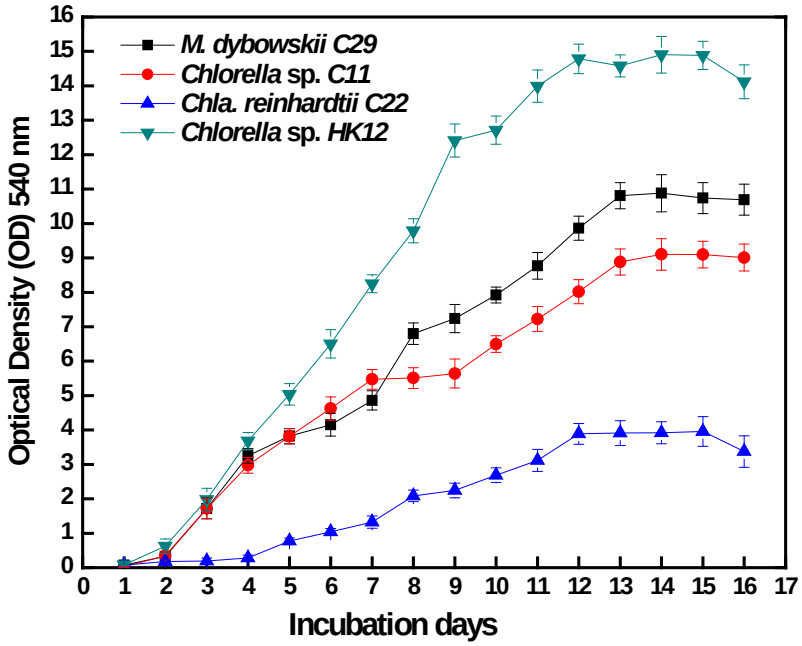

Fig. 3. Growth curves of four microalgal species cultivated in BG-11 medium in 3-L photobioreactor and flushed with air at a temperature of 26 °C with stirring at 300 rpm, under continuous illumination for 16 days

Lipid Content and Fatty Acid Composition

The four microalgal species were tested for their lipid production by evaluating lipid content in a 3 L photobioreactor for 16 days of incubation under similar conditions (Table 1). Under our experimental growth conditions, the total lipid contents of the microalgae cultured in this study ranged from 28% to 51% of their dry weight. The lipid contents of *Chlorella* sp. *C11*, *Chla. reinhardtii C22*, *M. dybowskii C29*, and *Chlorella* sp. *HK12* were 28%, 51%, 39%, and 43%, respectively. The lipid productivity of *Chlorella* sp. *HK12* was the highest at 3.25 g L^{-1}, compared with the other tested microalgal species

(Table 1). In previous studies, total lipid contents representing 20 to 60% of the dry biomass weight were found to be quite common, and some microalgae even exceeded 80% as a response to different culture conditions (Mata et al. 2010). Some *Chlorella* and *Chlamydomonas* species can produce more lipids under certain conditions. *Chla. Reinhardtii*, which is considered as a good biomass producer and lipid producer, has been isolated mainly from ponds, lakes, and oceans, and it is also used as a source of hydrogen production (Melis and Happe 2004).

Table 1. Biomass Productivity, Lipid Content and Lipid Productivity of the Microalgal Strains

Microalgal strain	Biomass productivity (g dwt L^{-1})	Lipid content (% biomass)	Lipid productivity (g L^{-1})
Chlorella sp. *C11*	6.70	28	1.88
Chla. Reinhardtii C22	5.47	51	2.79
M. dybowskii C29	5.13	39	2.00
Chlorella sp. *HK12*	7.56	43	3.25

The fatty acid compositions of four microalgae species (*Chlorella* sp. *C11*, *Chla. reinhardtii C22*, *M. dybowskii C29*, and *Chlorella* sp. *HK12*) at the stationary growth phase were determined with gas chromatography/ mass spectrometry. The fatty acids profiles of the four isolates (Table 2), indicated the presences of C12:0, C14:0, C16:0, C16:1n-7, C16:2n-6, C18:0, C18:1n-9, C18:2n-6, C18:3n-3, and C26:0. The major fatty acids of the four isolates were C16:0, C18:0, C18:1n-9, and C18:2n-6 comprising 8-33%, 23-38%, 4-30%, and 6-26% of the total fatty acids, respectively, whereas C12:0 and C14:0 existed as minor fatty acids. C16:0 and C18:0 comprised 33% and 38% of total fatty acids in *Chla. reinhardtii C22* and *Chlorella* sp. *HK12*, respectively. Oleic acid(C18:1n-9), which is an ideal component of biodiesel, occupied up to 30% and 29% of the total fatty acids in *M. dybowskii C29* and *Chlorella* sp. *HK12*. For biodiesel, oleic acid was a strong candidate for improving fuel properties. Higher oleic acid content increases the oxidative stability of fuel, decreases the cold filter plugging point (GFPP) of the fuel, and allows it to be used in cold regions (Knothe 2008). All the oil of the species contained a certain amount of saturated fatty acids; this component would negatively impact cold flow properties, but most of the fatty acid produced by microalgal strains have this problem (Pratoomyot et al. 2005; Abou-Shanab et al. 2011). Solutions to this problem often entail increasing the problematic behavior of another property and have included the use of additives or mixing biodiesel with petrodiesel, either through physical processes, such as winterization (Knothe 2008). *M. dybowskii C29* showed the highest oleic acid content, making it suitable for the production of good quality biodiesel. Although the genus of *Monoraphidium* was described a long time ago, little information about the biofuel production in *Monoraphidium* has been obtained. Therefore, among the tested microalgal species, *Chlorella* sp. *HK12* was the most appropriate for biodiesel production based on its high biomass concentration (7.56 g dwt L^{-1}), lipid content (43%), and a relatively high content of oleic acid (29%).

Table 2. Fatty Acids Composition (% of total fatty acids) of 4 Microalgal Species

Fatty acid	Fatty acid composition (wt%)			
	Chlorella sp. *C11*	*Chla. reinhardtii C22*	*M. dybowskii C29*	*Chlorella* sp. *HK12*
C12:0	6	4		6
C14:0	1	2		4
C16:0	28	33	23	8
C16:1n-7			2	
C16:2n-6			4	
C18:0	25	23		38
C18:1n-9	18	4	30	29
C18:2n-6	6	13	26	15
C18:3n-3	16	21	10	
C26:0			5	
Total	100	100	100	100

CONCLUSIONS

1. In the present study, four lipid-producing microalgae species were isolated and selected from freshwater samples. Using microscopic analysis, the strains were tentatively identified as genus *Chlorella*, *Chlamydomonas*, *Monoraphidium*, and *Chlorella*, respectively. 18S rDNA sequences recovered from the four microalgal strains showed high similarities to *Chlorella sorokiniana X62441.2*, *Chlamydomonas reinhardtii M32703.1*, *Monoraphidium dybowskii Y16939.1*, and *Chlorella zofingiensis X74004.1*. Phylofenetic analysis of sequences also supported identification of the four microalgal strains.
2. The average biomass productivity of *Chlorella* sp. *C11*, *Chlamydomonas reinhardtii C22*, *Monoraphidium dybowskii C29*, and *Chlorella* sp. *HK12* were 6.70, 5.47, 5.13, and 7.56 g dwt L^{-1}, respectively. The total lipid contents of the algae were 28%, 51%, 39%, and 43% for *Chlorella* sp. *C11*, *Chlamydomonas reinhardtii C22*, *Monoraphidium dybowskii C29*, and *Chlorella* sp. *HK12*, respectively. The composition of fatty acids in the studied species was mainly C12:0, C14:0, C16:0, C16:1n-7, C16:2n-6, C18:0, C18:1n-9, C18:2n-6, C18:3n-3, and C26:0. These results indicated that the *Chlorella* sp. *HK12* was the most suitable strain among the four strains for biodiesel production based on its high biomass concentration, lipid content, and content of suitable fatty acids.
3. Economics of producing microalgal biodiesel need to improve substantially to make it competitive with petrodiesel. Producing low-cost microalgal biodiesel requires primarily improvements to algal biology through metabolic engineering and genetics. Our research provided favorable microalgae candidates for biodiesel production, especially the strain of *Chlorella* sp. *HK12*, which showed high biomass productivity and lipid content under normal growing conditions of BG-11 medium. Further research should study the effect of elements such as nitrogen, phosphorus, and iron on biomass and lipid yield, investigate how the biomass productivity and lipid content of this strain be improved, and also research tolerance to extreme environments.

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