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Purification of a Diatom and Its Identification to *Cylindrotheca closterium*

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Abstract A diatom was purified with colony selection and continuous dilution methods. It was identified to *Cylindrotheca closterium* according to its morphological characteristics and *rbcL* and 18s rRNA gene sequences. The alga was not sensitive to ampicillin and neomycin, but sensitive to chloramphenicol which inhibited its growth at concentrations ranging from 50 to 150 $\mu g\,mL^{-1}$. The purified alga was easy to culture and its specific growth rate was 0.207 ± 0.002 (d^{-1}). It was resistant to pollution and could be harvested in an easy way. It was relatively high in lipid content (20.08% ± 0.67% of dry weight) and the combined amount of its 16:0 and 16:1 (n-7), the most suitable resource of biodiesel, was as high as 64% of the total fatty acids, while the amount of polyunsaturated fatty acids reached 19.96%–20% of the total fatty acids. Thus the purified *C. closterium* can be cultured as a biodiesel producer or a nutrition supplement producer.

Key words *Cylindrotheca closterium*; antibiotics sensitivity; fatty acid composition; total lipid content

1 Introduction

Diatoms are a main group of microalgae, and are highly diverse. More than 200 genera and 100000 species have been recorded in this group (Mann, 1989). Diatoms are widely distributed in seawater, fresh water, soil and moist surface. Although diatoms often aggregate into communities, they are unicellular and diverse in shapes with sizes varying from a few to hundreds of micrometers. These algae are the main primary producers in seawater and play a crucial role in global carbon cycle (Tréguer *et al*., 1995; Field *et al*., 2003). The most prominent feature of diatoms is their silicic shells which enable them to sink to the bottom under some conditions. This performance makes their harvest easy, which are highly appreciated by algal culturing community. Diatoms have been cultured for a long time as the feed of aquaculture animals (Lebeau and Robert, 2003; Xu *et al*., 2012), the tester of estuary sediment toxicity (Ignacio *et al.*, 2007) and PUFA producers (Wang and Zeng, 2007). In recent years, diatoms have drawn more and more attentions as they may serve as biodiesel producers. Aquatic Species Program has chosen 50 diatom species from 3000 species as the candidates of biodiesel producers in 1998, considering the characteristics like fast growth, rich in lipid content, tolerance to stress condition and well performance in large-scale cultivation. The wholegenome sequence of *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* are available at present, and several more will be sequenced soon (Armbrust *et al*., 2004; Bowler *et al*., 2008; Courchesne *et al*., 2009).

Eurythermal and euryhaline *Cylindrotheca closterium* (Cai *et al*., 2007) belongs to family Bacillariaceae, order Bacillariales, class Bacillariophyceae and phylum Bacillariophyta. It mainly distributes in the intertidal flats, can be cultured and harvested easily and resists to pollution (Liang *et al*., 2000), and is rich in PUFA (Liang *et al*., 1999; Lan *et al*., 2012). It is worthy to purify this diatom (Lin, 2000) and culture it axenically (Lin *et al*., 2000). In this study, we purified a strain of this alga and cultured it axenically, aiming to offer an applicable strain for future large scale culture.

2 Methods

2.1 Algal Purification and Identification

A mixture of diatoms was obtained from The Microalgae Library of Ocean University of China. The mixture was cultured in *f/2* seawater medium at (22 ± 1)℃, salinity 30 and 70 $\mu mol\,photons\,s^{-1}\,m^{-2}$ with a rhythm of 12 h light and 12 h dark. The diatom was isolated with algal colony selection and continuous dilution methods as described early (Mcmanus and Katz, 2009). Morphological images were created under an optical microscope (Nikon E 50i)

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and a scanning electron microscope (JEOL JSM-840). Universal primers, 18S rRNA (5'-AAC CCC TGG TTG ATC CTG CCA GT-3' and 5'-GAT CCT TCC GCA GGT TCA CCT AC-3', and *rbcL* gene, 5'-TCA GAA CGG ACT CGA ATA AA-3' and 5'-CCA ATA GTA CCA CCA AAT-3', were used to amplifying the genomic DNA of the diatom with expected products of about 1200 bp and 1070 bp in length, respectively. PCR was carried out in a volume of 50 μL containing 2.0 U *Taq* DNA polymerase, 10 nmol dNTP (each), 1nmol primer each, 1×buffer and 2.5 ng template DNA. The nested PCR was performed by denaturing at 94℃ for 3 min, followed by 30 cycles of denaturing at 94℃ for 30 s, annealing at 55℃ for 30 s, and extending at 72℃ for 2 min. The PCR product was sequenced commercially with ABI3730 sequencer. The sequences were also deposited into NCBI databank with accession number KC899347 and KC899348. The sequences were aligned with those retrieved from GenBank with ClustalX 1.81 (http://www.ncbi.nlm.nih.gov/genbank/), and the phylogenetic trees were constructed with MEGA4 (http://www.megasoftware.net/).

2.2 Determination of Antibiotics Sensitivity

Ampicillin, neomycin and chloramphenicol were purchased from Beijing Solarbio Co., Ltd. with their stock solutions prepared following standard protocol (Sambrook *et al*., 1987). Fifty milliliters of *f/2* medium containing different concentrations of ampicillin (0, 300, 500, 800, 1000 μg mL^{-1} each) or neomycin (0, 50, 100, 300, 500 μg mL^{-1} each) or chloramphenicol (0, 10, 50, 100, 150 μg mL^{-1} each) were used to culture the diatom (initial cell density 10^6 cells mL^{-1}) to either plateau growth phase (ampicillin and neomycin) or exponential growth phase (chloramphenicol) with OD_{680} value read every 2 days (starting from day 1).

2.3 Determination of Growth, Total Lipid Content and Fatty Acid Composition

The algal cells at the end of exponential growth phase were collected with centrifugation at 6000 g and 20℃, dried with a vacuum freeze drier at 0.03 atm and −50℃ for 12 h, and then were accurately weighed to determine the biomass. The specific growth rate was calculated to measure the growth of algal cells at exponential growth phase (Jiao *et al*., 2011). About 50 mg of algal biomass was used to determine the total lipid content with gravimetric method (Bligh and Dyer, 1959). Then lipid productivity was calculated from the biomass and total lipid content (Su and Yu, 2013). About 30 mg of dry algae were used to extract fatty acids with the method described early (Volkman *et al*., 1989) and the fatty acids content was analyzed with gas chromatography (HP5890E).

3 Results

3.1 Algal Purification and Identification

Algal colonies appeared on solid *f/2* medium in about 25 days. A colony was inoculated into a well of a 96-well plate with cells in this well diluted step by step by removing 1 μL medium from one well to the other. In about two weeks, pure diatom cultures were obtained from the last dilution with growing alga. Among colonies purified, one was further morphologically characterized and phylogenetically analyzed.

The purified alga was about 50 μm in length, needle-like and thin in shape. Two ends of the cell extended far from the center of the cell (Fig.1A). The chromatophore of the purified alga was in the middle of the cell. Under scanning electric microscope, the cell was found to be wrapped by the raphe canal of valves (Figs.1B and C), which is a typical characteristic of *Cylindrotheca closterium*. The algal cell moved around when water streamed through the raphe canal, and divided longitudinally along the raphe canal (Figs.1D, E). Thus, the purified alga may be identified to *Cylindrotheca* sp. morphologically.

The phylogenetic tree of 18S rRNA and *rbcL* gene amplified from the purified alga showed that it was closer to *C. closterium* than to other species; it was 99% and 100%

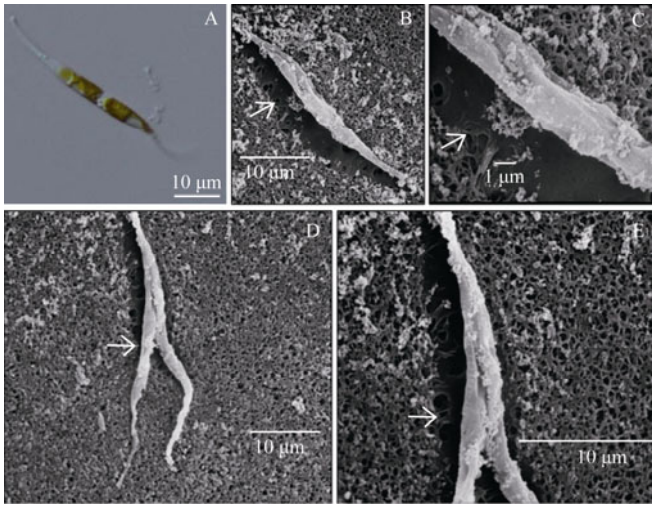

Fig.1 Morphological characteristics of the diatom purified in this study. A, under optical microscope; B and C, under scanning electric microscope, white arrow points to raphe canal running through the whole cell; D and E, under scanning electric microscope, white arrow points to the dividing position.

similar to *C. closterium* in 18S rRNA and *rbcL* gene sequence (Figs.2A and B), respectively. Therefore, the strain was identified to *C. closterium*.

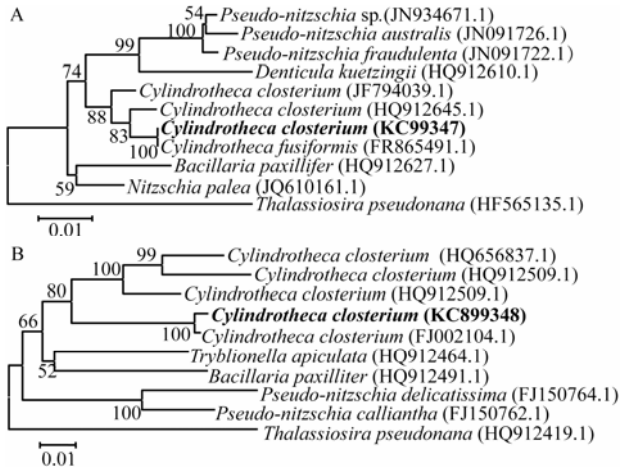

Fig.2 Phylogenetic tree of 18S rRNA (A) and *rbcL* (B) gene of diatom constructed with neighbor-joining method.

3.2 Antibiotics Sensitivity of *C. closterium*

As showed in Fig.3, ampicillin at a concentration range from 300 to 800 μg mL^{-1} enhanced the growth of *C. closterium*. Ampicillin at 1000 μg mL^{-1} significantly affected the growth of *C. closterium*. Little effect of neomycin on the growth of *C. closterium* was observed. Chloramphenicol at concentrations higher than 10 μg mL^{-1} significantly inhibited the growth of *C. closterium*. When the concentration of chloramphenicol was higher than 50 μg mL^{-1}, the growth of *C. closterium* was completely inhibited. So the purified alga was resistant to ampicillin and neomycin but sensitive to chloramphenicol.

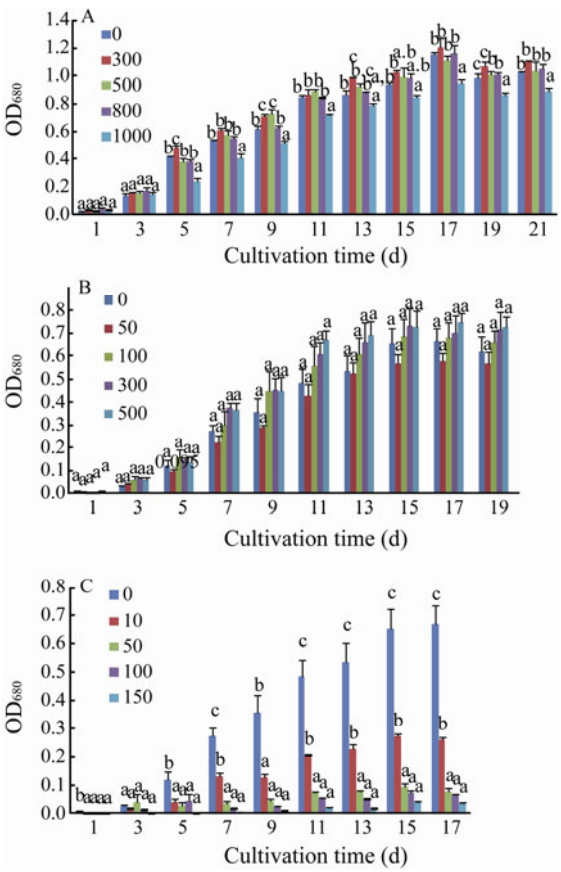

Fig.3 Effect of ampicillin (A), neomycin (B) and chloramphenicol (C) on the growth of *C. closterium*.

3.3 Growth, Total Lipid Content and Fatty Acid Composition

The specific growth rate of *C. closterium* was 0.207±0.002 (d^{-1}), reaching a biomass density of 291.12±14.6 mg L^{-1}. Its total lipid accounted for 20.08%±0.67% of dry weight (Table 1). Saturated fatty acids accounted for 40.9%–42.3% of the total fatty acid. The amount of 16:0 was the most abundant, reaching 29.90%±0.33% of the total fatty acids. Monounsaturated fatty acids accounted for 37.17%–38.37% of the total. The most abundant fatty acid was 16:1 (n-7), which accounted for 34.28%±0.48% of the total. Polyunsaturated fatty acids consisted of 19.96%–20% of the total fatty acids and the most abundant two were EPA and arachidonic acid. The combined amount of 16:0 and 16:1 (n-7) was as high as 64% of the total, indicating that the alga was suitable for biodiesel production (Table 2). EPA and arachidonic acid are excellent antioxidants and nutrition additives. The purified alga may serve as EPA and arachidonic acid producer as well.

Table 1 Growth, lipid content and lipid productivity of *C. closterium*

Specific growth rate (d^{-1})	Biomass (mg L^{-1})	Total lipid content (%)	Lipid productivity (mg L^{-1} d^{-1})
0.207±0.002	291.12±14.6	20.08±0.67	3.90±0.33

Notes: Total lipid content was expressed as the percentage of dry weight.

Table 2 Fatty acid composition of *C. closterium*

Lipid and fatty acid		Content (%)
Saturated fatty acid	14:0	4.18±0.06
	15:0	0.99±0.03
	16:0	29.90±0.33
	17:0	0.87±0.08
	18:0	5.32±0.15
Monounsaturated fatty acid	16:1 (n-7)	34.28±0.48
	17:1 (n-7)	3.49±0.12
Polyunsaturated fatty acid	18:2 (n-6)	3.27±0.17
	18:3 (n-6)	1.41±0.14
	20:4 (n-6)	8.08±0.38
	20:5 (n-3)	8.22±0.33

Note: Fatty acid content was expressed as the percentage of total lipid.

4 Discussion

The dominating fatty acids of diatoms are 14:0, 16:0, 16:1 (n-7) and EPA, which all together account for 68.7% of the total fatty acid (Dunstan *et al.*, 1993). In this study, we found that the combined amount of 16:0 and 16:1 (n-7)

of *C. closterium* was as high as 64% of the total. *C. closterium* was obviously different from *C. fusiformis* in morphology; however, it was only a single base different from *C. fusiformis* in 18s rRNA gene. Under a similar condition, the specific growth rate of the alga was much higher than that of *Phaeodactylum tricornutum* (Liu *et al*., 2008); however, its lipid content and lipid productivity were lower than most of the biodiesel producing candidates (Kong *et al*., 2010; Yang *et al*., 2011). Fortunately, chemical and physical mutation (Nečas, 1975; Niwa *et al*., 2009) and genetic modification may modify this alga to a highly efficient lipid producer. The *C. closterium* purified and characterized in this study was insensitive to ampicillin and neomycin but sensitive to chloramphenicol; thus ampicillin and neomycin may be applied to its axenical culture while chloramphenicol may serve as the selection marker in its genetic modification. We found that *C. closterium* sinks to the bottom of flask in only a few minutes without aeration. All these characteristics make this alga a biodiesel-producing candidate.

Growth rate, lipid content and fatty acid composition can be greatly influenced by culturing conditions. For example, nitrogen starvation has been widely applied in increasing lipid content and TAG production in a wide range of microalgal species (Widjaja *et al*., 2009). In addition, temperature and salinity may influence algal fatty acid composition. Eurythermal and euryhaline *C. closterium* mainly inhabits intertidal zone and is often directly exposed to sunlight, implying its high tolerance to high irradiation, temperature and salinity. It has been found that *C. closterium* remains metabolic activity with no statistically significant change in sensitivity to copper when illumination and temperature drastically change (Araujo *et al*., 2008). Therefore, *C. closterium* may be genetically improved to become a desirable oil producer.

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