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Screening and phylogenetic analysis of lipid-rich microalgae

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ABSTRACT

Microalgae are considered to be a potential alternative source for the production of biofuels, and isolation of microalgae capable of high lipid concentration and high biomass production is the foundation for microalgal biofuel development. To date, over 1200 microalgal clones have been isolated in our laboratory. Among them, 37 strains were identified and further compared based on total lipid content, growth rate and biomass production. From the ITS1-5.8S-ITS2 sequences and morphological characteristics, these strains were identified as belonging to the genera *Scenedesmus*, *Chlorella*, *Stichococcus*, *Nannochloropsis*, *Tetraselmis*, *Isochrysis*, *Phaeodactylum* and *Cylindrotheca*. The lipid content of these strains varied from 6% dry weight (dw) to 42% dw. All three *Isochrysis galbana* strains could accumulate lipid at more than 35% dw, and their average was the highest among the tested genera ($P < 0.05$), followed by genera *Nannochloropsis* and *Chlorella*. Based on the phylogenetic data, a taxonomic pattern of lipid accumulation was found in microalgae, which suggested that a genera strategy should be considered in screening for lipid-rich microalgal strains. A comprehensive consideration of growth rate, biomass production and lipid content indicated that marine species *Nannochloropsis maritima* strain IOAC710S, and *I. galbana* strains IOAC683S and IOAC724S could be promising candidates for biodiesel feedstock.

1. Introduction

Rapid depletion of fossil fuel reserves and concerns over global warming has received worldwide attention in recent years. According to many analysts, the world fossil oil reserves will be exhausted in less than 50 years at the present rate of consumption [1]. Fossil fuel combustion also contributes to global warming by releasing greenhouse gases such as CO_2. Thus, research on renewable, carbon neutral, economically viable, alternative sources of energy is needed.

Biodiesel from microalgae is one promising alternative, because it is generally comparable to diesel fuel in terms of its physical and fuel properties [2], but contributes neither net carbon dioxide nor sulfur to the atmosphere and emits less gaseous pollution than petrodiesel [3,4]. Compared with terrestrial bioenergy crops, microalgae as feedstock for biofuels have numerous advantages. Oil content of some microalgae is much higher than that of agricultural oil crops (exceeds 80% of the dry weight (dw) for *Botryococcus braunii* vs less than 5%, on a total biomass basis, for palm and soybean) [5]. On an area basis, microalgae can produce 300 times more oil than soybean [6]. Microalgae have greater photosynthetic efficiency, enjoy faster reproductive cycles, and require a limited nutrient supply for growth [7–9]. Microalgae can be cultivated in saline or brackish water along coasts, or on marginal lands (e.g. desert, arid and semi-arid lands), and do not compete for resources with conventional agriculture [1,8]. In addition, microalgae can assimilate CO_2 and utilize nitrogen and phosphorus from wastewater, providing the additional benefits of greenhouse gas emission reduction and wastewater bioremediation [8,10,11].

Based on the above advantages, biodiesel from microalgae is considered to be the only renewable biofuel having the potential to displace petroleum-derived transport fuels without affecting the food supply and other crop products [5]. However, the commercial production and large-scale use of biofuel from microalgae have been hindered by its high cost. Based on a biomass productivity of 20 g m^{-2} day^{-1} and an average lipid content of 24% dw, extracted at no further cost, biofuel from microalgae cost $209 US/bbl, whereas the petroleum oil price is only around $90 US/bbl [12]. To reduce the high cost of biofuel from microalgae, some strategies have been explored, such as selecting productive strains [13,14], optimizing cultivation conditions [15–17], developing efficient harvesting methods [18,19], combining microalgal cultivation with wastewater treatment [20,21], and so on. Among them, isolation of microalgae with a high lipid content and high biomass productivity is the first critical step towards the commercial production of microalgal biofuel [22,23]. As the total number of microalgal species is large (if the highest estimate is to be believed, the number of diatom species alone may reach 10 million [24]), collection and isolation of new, high-lipid-producing, microalgal strains from the natural environment presents a heavy workload challenge.

Since the 1970s, several countries have funded research programs to screen microalgae for renewable liquid fuel production [1]. Around

Abbreviations: dw, dry weight.

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30,000 species have already been identified [25], and many microalgal species belonging to *Botryococcus* [26], *Chaetoceros* [27], *Chlorella* [28], *Dunaliella* [29], *Haematococcus* [30], *Isochrysis* [31], *Nannochloropsis* [32], *Scenedesmus* [33] etc. have been investigated for their lipid content. Significant differences between various species have been found; for example, lipid content in *Chlorella zofingiensis* can reach 65.1%, by weight, of dry biomass [34], while that of *Chaetoceros socialis* is only 2.2% dw [23]. How the lipid-rich strains distribute among clades of microalgae, or whether the lipid content of strains with a closer phylogenic relationship is similar is unknown. Clarification of this situation would benefit further isolation of new microalgae having a high lipid content. Besides lipid content, growth rate and biomass production should also be considered when isolating candidate strains for biofuel production. In this study, 37 strains were isolated and tested for their lipid content, and a phylogenetic tree was constructed to determine if there were taxonomic patterns of lipid production. In addition, the growth rate and biomass of some lipid-rich strains were also investigated and compared, so as to select potential strains for biofuel production.

2. Materials and methods

2.1. Collection of water samples

Marine and fresh water samples were collected in the four seasons for isolation of lipid-rich microalgae. The marine water samples were collected from the China Seas and the Western Pacific Ocean. Fresh water samples were obtained from lakes in terrestrial areas including mountainous regions.

The water samples were filtered soon after collection. About 300 ml of each sample was first passed through a 60-μm plankton net to remove protozoa, and then algae were collected using a 0.45 μm filter membrane. The microalgae on the filter membrane were cultivated on plates with solid medium, which consisted of 1.3% agar plus L1 medium [35] for marine samples, or MCM medium [36] for freshwater samples. The solid plates were incubated at 25 ± 1 °C using a 14:10 light:dark cycle under 20 μmol photon m^{-2} s^{-1} illumination.

2.2. Isolation of microalgal clones

After 4–12 weeks, micro-colonies grown on the solid medium were streaked onto new plates with corresponding medium, which were cultivated under the same conditions as above for a further 4–8 weeks. This process was repeated many times until axenic microalgal colonies were obtained. Then the clonal colonies were kept as pure cultures in the algal collection (IOAC) of the Institute of Oceanology, Chinese Academy of Sciences (IOCAS).

2.3. Microalgal strains and culture conditions

The purified freshwater microalgal strains were transferred to MCM liquid medium, and marine strains were transferred to L_1 liquid medium for further experimentation. The strains were cultured in conical flasks (500 ml) containing 300 ml fresh medium, with three replicates of each strain. The culture conditions were 25 ± 1 °C, and a 14 h:10 h light:dark rhythm at a light intensity of 20 μmol photon m^{-2} s^{-1}, with illumination provided from the top by cool-white fluorescent lamps. During the growth periods, the microalgal cultures were manually shaken 2–3 times daily to avoid sticking.

For each strain, the cells were observed using a 37XB inverted microscope (Shanghai, China) to determine their morphological characteristics.

2.4. DNA isolation and sequencing

About 40 ml of each algal culture at the logarithmic phase was centrifuged (4000×*g*, 6 min), and DNA was extracted using the Universal Genomic DNA Extraction Kit Ver.3.0 (TaKaRa, Dalian). The primers used for DNA amplification of the ITS1-5.8S-ITS2 region of the ribosome were ITS-F1 (forward): 5′ GAAGTCGTAACAAGGTTTCC 3′, and ITS-R1 (reverse): 5′ TCCTGGTTAGTTTCTTTTCC 3′ [37]. The following thermal profile was used: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s; and a further 10 min elongation at 72 °C. The PCR products were purified using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver. 3.0 (TaKaRa, Dalian), and then sequenced by Invitrogen Biotechnology Co., Ltd. (Shanghai) with an ABI 377 sequencer. The overlapping fragments were assembled using the Contig Express program in the Vector NTI software package (Version 11).

2.5. Sequence analysis

The amplified sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) algorithm at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) to identify the species of microalgae. The ribosomal ITS1-5.8S-ITS2 regions were aligned using the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). A neighbor-joining (NJ) algorithm-based, [38] unrooted, phylogenetic tree was constructed using MEGA 5.05 software [39]. Bootstrap analysis [40] was used with 1000 replications to test the relative support for the branches produced by NJ analysis.

2.6. Growth, biomass and total lipid measurements

Every 24 h, the optical density (OD) of each culture was determined at 750 nm [1] using a 722 s-type spectrophotometer, and the growth curves were drawn based on the OD_{750} measurements. The specific growth rate was calculated according to the daily changes of absorbance during the logarithmic phase (Fig. S1). For biomass determination, 200 ml of each culture was collected at stationary phase (Fig. S1), and then centrifuged at 4000 ×*g* for 10 min to harvest the cells. The algal pellets were rinsed with 40 ml deionized water, centrifuged again, freeze-dried at −50 °C, and then the dry weight was determined gravimetrically. The algal powders were stored at −20 °C for total lipid analysis.

Total lipid was extracted according to the protocol of Bligh and Dye [41] with some modification. For each sample, about 25 mg algal powder (W) was mixed with 2 ml chloroform and 1 ml methanol by vortex for 2 min, and then kept at room temperature for 24 h. Then the mixture was centrifuged at 4000 ×*g* for 10 min, and the supernatant was transferred into a pre-weighed vial (W_1). The algal residue was mixed with another 1 ml of chloroform/methanol (2:1, *v*/*v*) by vortex, and then centrifuged as described above. After that, the supernatants were combined and dried in an oven at 70 °C to a constant weight (W_2). The lipid content of each sample was measured gravimetrically and calculated as follows: total lipid (% dry weight) = $(W_2 - W_1) / W \times 100$.

2.7. Statistical analysis

Statistical analyses were performed using the SPSS 16.0 software. The growth rate, biomass and lipid content data for freshwater/marine microalgal strains belonging to different genera were analyzed using a one-way analysis of variance (ANOVA). Correlation of growth rate and biomass with lipid content was analyzed using bivariate correlations, and the Pearson's correlation coefficients are given with their significance levels. Differences are considered significant at $P < 0.05$. All data throughout the paper are given as mean ± SE.

3. Results and discussion

3.1. Isolation and identification of microalgal strains

To date, over 1200 single-cell clones of unicellular microalgae have been isolated in our laboratory. Among them, five freshwater strains and 32 marine strains were identified and used for further analysis. By alignment and comparison of the ITS1-5.8S-ITS2 sequences with other

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