RESEARCH ARTICLE

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Isolation and Heterotrophic Growth Characterization of Freshwater Microalgae

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Heterotrophic growth of microalgae could be an option to shorten their growing time and allow faster harvesting of their biomass. Suitable strains of freshwater microalgae have potential for offshore cultivation. Algae strains were isolated from rice paddies in Taiwan for heterotrophic culture. In this study, ten single strains were separated for phylogenetic and molecular evolutionary analyses. The 18S *r*RNA and RubisCO gene sequences were aligned and compared with the GenBank database of the National Center for Biotechnology Information (NCBI). Ten strains in five genera were identified as *Leptolyngbya*, *Scenedesmus*, *Dictyosphaerium*, *Chlorella*, and *Micractinium*. Under heterotrophic growth conditions, an isolated strain of *Scenedesmus deserticola* ICL grew faster in a high glucose concentration. The maximum concentration of *S. deserticola* ICLwas 3,030 mg/L and the crude lipid content was 20.5% (dry base) when the additional glucose was 20 g/L in a basic BG-11 medium. In a dark environment, the microalgae were grown using organic carbon with respiration. Organic carbon increased the specific growth rate of the microalgae efficiently.

Keywords: Blast, *Scenedesmus Deserticola*, Lipid Content, Specific Growth Rate

1. INTRODUCTION

Renewable materials from environment[1] are good natural supplies for the development of sustainable strategy. Algal biomass is a good renewable bioresource. Microalgae have several useful characteristics such as a simple structure, high photosynthesis efficiency, and a short growth period.[2] Microalgae are good raw biomaterial for food supplements, fermentation and bioenergy, and could be cultivated artificially. In the last decade, bio-oil production from algal lipids[3] and the bio-alcohol from algal cellulose[4] have been evaluated as biofuel.

For stable harvesting of high-quality algae biomass in large quantities, several conditions must be considered, including algae strains, illumination, nutrients, water quality, and the temperature of the cultivated environment. High photon flux density could increase the uptake rate of phosphate and algal biomass.[5] In autotrophic growth, microalgae can use an inorganic carbon source; in the contrast, an organic carbon source can be used for heterotrophic growth with respiration.[6] The characteristics of algal biomass resulting from heterotrophic growth is different from that resulting from autotrophic growth. Quality of phytogenic lipid is important.[7] Under heterotrophic conditions, the composition of the polyunsaturated fatty acids of *Chlorella sacharophila* are high.[8] The yield of bio-oil from heterotrophic green algae is 3–4 times higher than from autotrophic cells.[3] An algal-biomass product resulting from heterotrophic growth also has the potential to produce docosahexaenoic acid as a daily nutrient.[9] In wastewater treatment, microalgae could use soluble organics as a carbon source to decrease the pollution in wastewater.[10] The nitrogen and phosphates in wastewater can be assimilated by the microalgae to polish the water.[11,12] Suitable microalgal strains can produce biodiesel in photobioreactor.[13]

However, most heterotrophic and autotrophic cultivation was focused on marine microalgae, where growth sites are limited to the near-shore field.[14,15] Marine microalgae cannot be grown well in fresh water. Hence, the isolation and identification of heterotrophic microalgae strains in freshwater could enhance cultivation in different sites. This study presents a discussion of additional organic and inorganic carbon for microalgae. The effect of lighting periods was also controlled to estimate the growth of each species.

2. MATERIALS AND METHODS

2.1. Sampling, Isolation and Identification of Algal Strains

The samples were collected from ten rice paddies with an approximate area of 10 km^2 centered on E120°27′22.1″ and N23°33′8.48″ in Chiayi County, Taiwan. Sterile bottles with samples were stored at 4 °C, and the samples were isolated using the spread-plate technique. To obtain autotrophic colonies from

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the mixed cultures in a Petri dish, a basic BG-11 medium[16] with 15 g/L agar was adopted for isolation of pure cultures. Every plate was inoculated and then cultivated with light at 28 °C for 5 to 7 days. Each single colony was picked up and transferred to a BG-11 agar slant that represented a single algal species, which was stored at 28 °C with light. Stored strains were transferred every 30 days into new BG-11 agar slants.

To obtain sufficient algal biomass for DNA extraction, each strain was cultivated in a sterilized bottle with a BG-11 medium and collected by centrifugation. The genomic DNA of each algal strain was extracted using the TANBead Plant DNA Auto Kit (Taiwan Advanced Nanotech Inc. Taiwan). Two primers, NS1 (5′-GTAGTCATATGCTTGTCT-3′) and NS2 (5′-GGCTGCTGGCACCAGACTTGC-3′), were adopted amplify the 18S *r*RNA of eukaryotic microalgae.[17] For the prokaryotic algae, two primers, cbbL-F (5′-GACTTCACCAAAGACGACGA-3′) and cbbL-R (5′-TCGAACTTGATTTCTTTCCA-3′), were used to amplify the ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO).[18, 19] For the eukaryotic microalgae, the genomic DNA template was mixed with a polymerase chain-reaction (PCR) mixture containing 10× PCR buffer, 2.5 mM dNTP, 10 μM of NS1, 10 μM of NS2 primers and 0.2 U Taq polymerase, then denatured at 96 °C for 5 min. The PCR program was run for 30 s at 95 °C, and 30 s at 50 °C, 30 s at 72 °C for 35 cycles with a 5-min final extension at 72 °C. For the prokaryotic algae, the template was mixed with a PCR mixture containing 10× PCR buffer, 2.5 mM dNTP, 10 μM of cbbL-F, 10 μM of cbbL-R primers and 0.2 U Taq polymerase, then denatured at 96 °C for 5 min. The PCR program was run for 30 s at 95 °C, 30 s at 50 °C and 60 s at 72 °C for 35 cycles with a 5-min final extension at 72 °C.

The 18S *r*RNA and RubisCO gene sequences were aligned and their sequences compared using the Basic Local Alignment Search Tool (BLAST) in the GenBank database of the National Center for Biotechnology Information (NCBI). Phylogenetic and molecular-evolutionary analyses and a neighbor-jointing tree were conducted using MEGA version 5.[20]

2.2. Cultivation and Production of Algae

For the growth of microalgal strains when testing the effects of carbon types, sodium bicarbonate and glucose were used for autotrophic and heterotrophic growth, respectively. A low-concentration (2 g/L) or high-concentration (20 g/L) carbon source was added to a basic BG-11 medium. Several strains that grew well in the pretest were picked for the growth experiment; furthermore, a strain of *Nannochloropsis oculata* obtained from Tungkang Biotechnology Research Center, Fisheries Research Institute, Taiwan, was also adopted. The blank was filled with a basic BG-11 medium to contrast the effect of different carbon sources. In a serum bottle sealed with elasticity silica gel, the BG-11 medium was filled to approximately 600 mL and inoculated with a microalgal strain with an initial concentration of 10 mg/L in a laminar flow cabinet. The vessel was then placed on a shaker at 120 rpm in a cultivated chamber at 28 °C for 14 days. In addition to the carbon-source effects, lighting effects were estimated with a wholly dark and a lighted period. In the wholly dark situation, the lighting sources were closed. In the lighting-period situation, the light strength was 3,565 lux and the dark/light period was 12 h:12 h in the cultivated chamber. The algal concentration was obtained using the dry-weight method. The mixture was filtered through a glass-fiber membrane, dried in an oven at 105 °C, and weighted. The specific growth rate (μ_{net}, day^{-1}) could be calculated as:

$$\mu_{net} = \frac{(\ln X_1 - \ln X_0)}{\Delta t} \quad (1)$$

where Δt is days between the starting and designating day; X_0 and X_1 are the biomass concentration of the starting and designating day, respectively.

The crude lipid contents of the microalgae were also measured. Microalgae were collected by centrifugation and raised by deionized water three times, then lyophilized to create dried-algae powder. For getting crude lipids, a solvent with methanol and chloroform (1:2, v/v) was added to extract the weighted dried powder in the tube.[21] The top mixed liquors were removed, and chloroform was added to re-extract the powder three times. The residue in the tube was dried with nitrogen stream and weighted to obtain the crude-lipid contents.

3. RESULTS AND DISCUSSION

3.1. Identification of Algal Strains

After several reiterations of the separation technique, ten single strains were isolated from the samples. The BLAST program showed the alignment and comparison results of the gene sequences, 18S *r*RNA and RubisCO, to indicate the phylogenic relationship. Five genera were identified and are listed in Table I. Figure 1 shows six groups of genera found using analysis with the Neighbor-Jointing tree. Three strains of 1-3(2), RA, and 1-1(2) are *Chlorella* spp. that were collected from different sites. One prokaryotic blue-green alga, *Leptolyngbya* sp. GP, was identified in a site that was fertilizing on the sampling day.

3.2. Cultivation of Algae

After identification of the algal species of the isolated microalgae, three separated strains, *Leptolyngbya* sp. GP, *Chlorella* sp. RA, and *Scenedesmus deserticola* ICL, were used to growth-test with the purchased *N. oculata*. The experiment was processed with different lighting periods. For the lighting period of 12 L:12 D, the control test of basic BG-11 medium was cultivated. Additionally, extra glucose and $NaHCO_3$ with different concentrations were added to the basic BG-11 medium. The additional glucose was also added to the medium for the 0 L:24 D lighting period.

The variation of biomass concentrations with days is shown in Figures 2 to 4. In the basic BG-11 medium, the maximum microalgae concentration of every tested strain is approximately 95 to 105 mg/L over the full 14 days. In Figure 2, the biomasses are raised rapidly with a higher organic carbon concentration and lighting period of 12 L:12 D for the strains of *N. oculata* and *S.*

Table I. Molecular phylogenetic of separated strains in this study.

Taxon	Strain	Taxon	Strain
Genus *Leptolyngbya*		Genus *Chlorella*	
Leptolyngbya sp.	GP	*Chlorella* sp.	RA
Genus *Scenedesmus*		*Chlorella* sp.	1-3(2)
Scenedesmus deserticola	ICL	*Chlorella sorokiniana*	1-1(2)
Genus *Dictyosphaerium*		Genus *Micractinium*	
Dictyosphaerium sp.	2-2(3)	*Micractinium* sp.	5-3(2)
Dictyosphaerium sp.	3-3(3)	*Micractinium* sp.	1-1(1)
Dictyosphaerium sp.	3-5(3)		

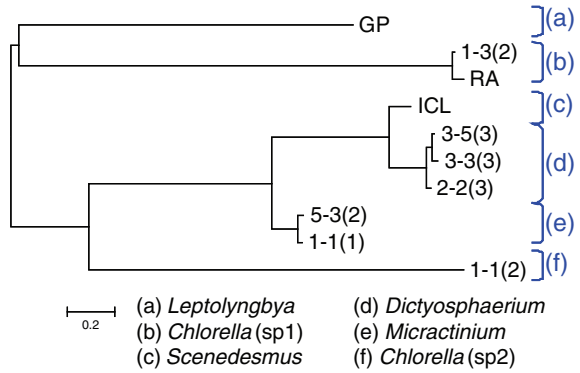

Fig. 1. The phylogenetic tree of isolated algal strains using 18S *r*RNA and RubisCO.

deserticola ICL. In Figure 2(b), the maximum concentration of microalgae is 1,225 and 3,030 mg/L for the *N. oculata* and *S. deserticola* ICL, respectively. These two strains could use organic carbon to increase biomass. For the additional inorganic carbon source, the higher $NaHCO_3$ concentration led to higher biomass of the *S. deserticola* ICL, as shown in Figure 3. Compared to the inorganic carbon source, additional glucose could lead to a higher biomass concentration for the strain of *S. deserticola* ICL. In the dark environment, some strains were grown by respiration as shown in Figure 4. The *N. oculata* grew well in a lower-glucose concentration. By the contrast, the *L.* sp. GP and *S. deserticola* ICL grew well in a higher organic carbon–carbon condition.

Light/dark cycle, nutrient types and species of microalgae affect the concentration of biomass in literatures. *Chlamydomonas globosa*, *Chlorella minutissima* and *Scenedesmus bijuga* could grow well under BG-11 with glucose in dark conditions which were isolated from wastewater.[22] Moreover, the chlorophyll contents/biomass in dark condition decreases 3 to 20 times than the light environment. The nutrients, such as glycerol, acetate, glucose,[23] poultry litter extract and wastewater[22] affect the growth of microalgae. In this study, the algal color of 0 h:24 h was whiter than the 12 h:12 h which could be affected by light. Furthermore, microalgae cultivate in organic carbon with light will obtain higher biomass concentration.

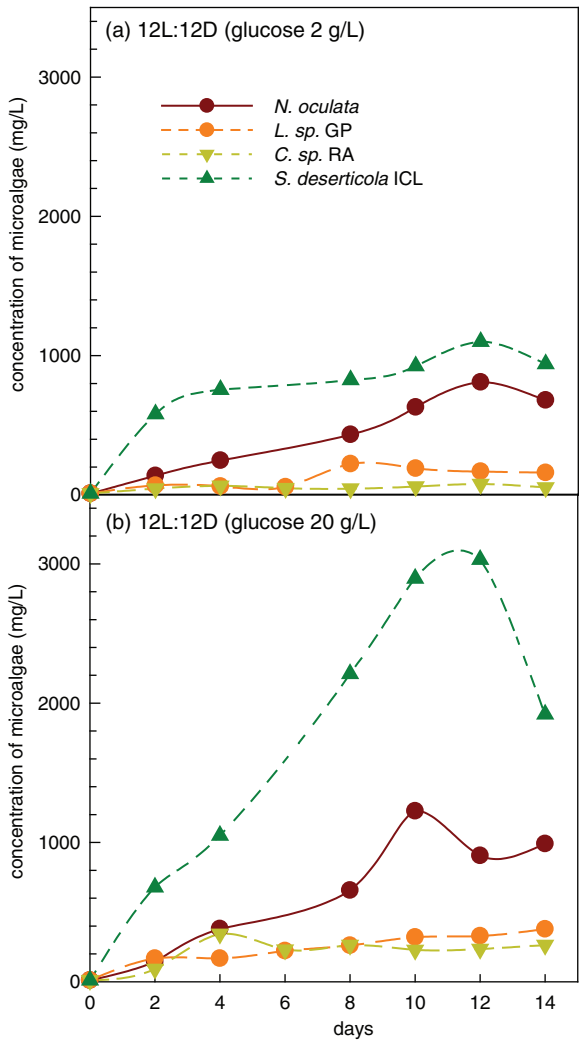

Fig. 2. The biomass concentration variation of four strains with 12L:12D lighting period and glucose: (a) 2 g/L; (b) 20 g/L.

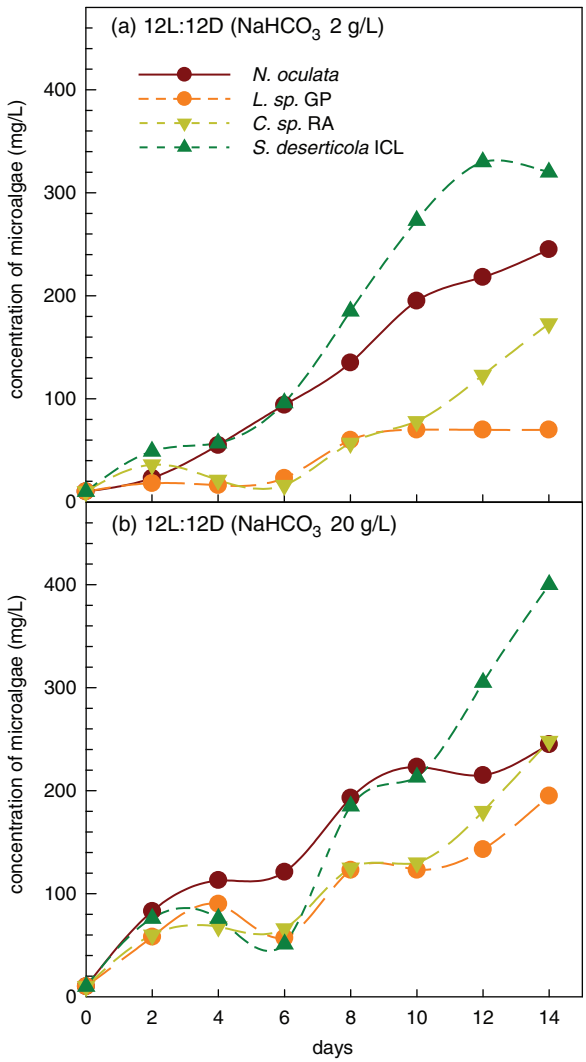

Fig. 3. The biomass concentration variation of four strains with 12L:12D lighting period and sodium bicarbonate: (a) 2 g/L; (b) 20 g/L.

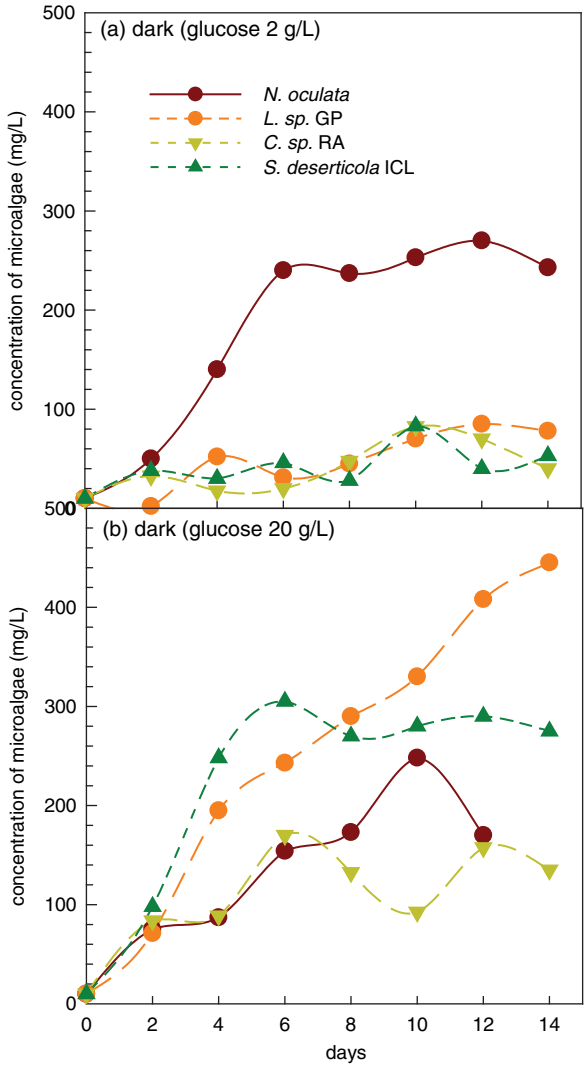

Fig. 4. The biomass concentration variation of four strains in dark environment with organic carbon: (a) 2 g/L; (b) 20 g/L.

The maximum biomass concentration appeared mostly within 12 days. The specific growth rate was calculated by Equation 1, and is shown in Table II. For higher additional carbon, μ_{net} are higher than the lower additional carbon. The strain of *S. deserticola* ICL obtained a the maximum μ_{net} of 0.476 which was 4 times greater than *Phaeodactylum tricornutum*.[23] Organic carbon could thus increase the biomass production of microalgae.

Table II. The specific growth rate with different additional carbon sources and lighting period.

Light:dark period (hours)	μ_{net} (day^{-1})						
	12 L:12 D					0 L:24 D	
Additional carbon sources (g/L)	—	$NaHCO_3$		Glucose		Glucose	
	0	2	20	2	20	2	20
Nannochloropsis oculata	0.194	0.257	0.256	0.366	0.375	0.275	0.236
Leptolyngbya sp. GP	0.053	0.162	0.222	0.235	0.291	0.178	0.309
Chlorella sp. RA	0.177	0.209	0.241	0.171	0.263	0.162	0.229
Scenedesmus deserticola ICL	0.120	0.291	0.285	0.391	0.476	0.116	0.280

Table III. The crude lipid contents in different additional carbon sources and lighting period.

Light:dark period (hours)	Crude lipid content (%, w/w dry weight)			
	12 L:12 D			0 L:24 D
Additional carbon sources (g/L)	—	$NaHCO_3$	Glucose	Glucose
	0	20	20	20
Nannochloropsis oculata	7.2	8.5	8.3	10.3
Leptolyngbya sp. GP	10.1	14.6	9.6	10.0
Chlorella sp. RA	9.6	12.9	10.1	9.9
Scenedesmus deserticola ICL	6.3	6.4	20.5	13.7
Chlorella saccharophila[10]	–	–	23.4	–

3.3. Crude Lipid Content of Algae

For the production of lipids, the lighting period and carbon type affect the results, which are shown in Table III. In the additional organic carbon environment, the strains of *N. oculata* and *L.* sp. GP accumulated larger lipids in a dark environment. For lighting of 12 L:12 D, the inorganic carbon could lead to a higher concentrations of crude lipids with *N. oculata, L.* sp. GP, and *Chlorella* sp. RA than the organic sources. In this study, the strain with the highest lipid content (20.5%, w/w dry base) was *S. deserticola* ICL with organic carbon and lighting environment. The maximum lipid production and lipid productivity is calculated as 0.621 g-lipid/L and 0.052 g-lipid/(L day) respectively for the *S. deserticola* ICL. The lipid productivity with organic carbon is similar to *Chlorella vulgaris*.[24]

When the additional glucose was 2.5 g/L, the crude lipid content was 46.7% for *Chlorella saccharophila*. However, when the additional glucose was 20 g/L, it decreased to 23.4%.[8] This indicates that varying conditions between strains leads to different results. For *Scenedesmus* sp. in basic BG-11 medium, higher temperature obtained higher biomass concentration and lower lipid content.[25] For *Scenedesmus obliquus*, highest biomass concentration (2.0 g/L) and lipid content (1.05 g-lipid/L) obtain in the poultry litter combined with municipal secondary settling tank discharges.[26] The cyanobacteria, such as *Leptolyngbya* and *Spirulina*, synthesize lipid in heterotrophic environment.[27, 28]

4. CONCLUSION

Heterotrophic cultures of microalgae have potential for producing of biofuel and polyunsaturated fatty acids.[29] We isolated and identified 10 microalgal strains from freshwater environments that have potential for heterotrophic growth. The isolated strain of *S. deserticola* ICL can produce more biomass and accumulate more lipids in a higher organic carbon and light environment. Higher additional organic carbon concentration raises the specific growth rate. In the dark environment, the microalgae grew by using organic carbon with respiration. The heterotrophic growth of microalgae is a possible method for obtaining higher biomass and lipids if suitable strains are chosen.

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