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Some characteristics of microalgae isolated in Taiwan for biofixation of carbon dioxide

Ed-Haun Chang[1] and Shang-Shyng Yang*

Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan 10617

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Abstract. To contribute the biological mean of CO_2 fixation, more than 200 microalgal isolates were screened from lakes, ponds, sediments, hog wastewater, paddy fields, hot springs, and seawater in Taiwan. Two unicellular microalgae, *Chlorella* sp. NTU-H15 and *Chlorella* sp. NTU-H25, were isolated from hog wastewater. In the laboratory, they were able to grow up even in aeration containing CO_2 up to 40% and have growth rates of 0.21 to 0.22 g dry wt l^{-1} d^{-1} at 20% CO_2. Both algae had the same growth rate in the range from 5 to 40% CO_2 and had a similar light response between 190 and 589 μmol m^{-2} s^{-1}. *Chlorella* sp. NTU-H15 had a higher growth rate than *Chlorella* sp. NTU-H25 at pH 4.0 and ≥35°C. *Chlorella* sp. NTU-H15 was able to tolerate high concentrations of CO_2, high cell density, and a broad-range of temperature and pH. Each liter of *Chlorella* sp. NTU-H15 produced 1.8 g of dry cell. The maximum growth rate was 0.28 g dry wt l^{-1} d^{-1}, and the specific growth rate was 0.27 d^{-1} at 15% CO_2. Each mg of chlorophyll produced 1.6 mM O_2 min^{-1} at 700 μmol m^{-2} s^{-1} at 30°C and 10 mM $NaHCO_3$. While each liter of *Chlorella* sp. NTU-H25 produced 1.7 g of dry cell, the maximum growth rate was 0.27 g dry wt l^{-1} d^{-1}, and the specific growth rate was 0.27 d^{-1}. Both isolates are suitable for dense cultivation to fix CO_2 directly and to produce cell biomass.

Keywords: Biofixation; Carbon dioxide; *Chlorella*; Growth rate and microalga.

Introduction

Global warming induced by increasing concentrations of greenhouse gases in the atmosphere is a matter of great environmental concern. Carbon dioxide is the principal greenhouse gas. Atmospheric CO_2 has increased from 280 to 368 ppmv in the last 200 years and is responsible for about 50% enhancement in the greenhouse effect (Karube et al., 1992). Annual anthrophogenic emissions of CO_2 are estimated to be 2×10^{10} tons, primarily from combustion of fossil fuels in association with an increasing population and industrialization. Recently, many attempts have been made to reduce atmospheric CO_2. Physical and chemical treatments have been used to separate and recover CO_2. Microalgal photosynthesis has increasingly received attention as a means of reducing the emission of CO_2 into the atmosphere and producing industrially valuable compounds (Kodama et al., 1993; Kurano et al., 1995a; Lee et al., 1998; Yang et al., 2000).

Biofixation and utilization of CO_2 by microalgae are among the most productive biological methods of treating industrial waste emissions, and the yield of biomass per acre is three to fivefold greater than from typical crops (Law and Berning, 1991; Akimoto et al., 1994). Direct use of flue gas reduces the cost of pretreatment, but the high concentration of CO_2 and the presence of SOx and NOx inhibit the growth of cyanobacteria and microalgae (Kurano et al., 1995b). A few works have recently reported the isolation of highly CO_2-tolerant microalgae and cyanobacteria for biological fixation of CO_2, such as *Anacystis, Botryococcus, Chlamydomonas, Chlorella, Emiliania, Monoraphidium, Rhodobacter, Scenedesmus, Spirulina* and *Synechococcus* (Hanagata et al., 1992; Takeuchi et al., 1992; Kodama et al., 1993; Sawayama et al., 1995; Takano and Matsunaga, 1995; Watanabe and Hall, 1995; Yamada et al., 1995; Zeiler et al., 1995; Ike et al., 1996; Yun and Park, 1997; Sung et al., 1999). The goal of this study is to isolate microalgae in Taiwan which can tolerate high CO_2 concentrations and high temperatures in order to biofix carbon dioxide and discover the optimal conditions for biomass production.

Materials and Methods

Sources of Isolates

Microalgae were isolated from several samples taken from rivers, lakes, ponds, paddy fields, sediments, hog wastewater, ocean and hot springs in Taiwan (Table 1).

Culture Media

Medium BG-11 contained (g l^{-1}): $NaNO_3$, 1.5; $K_2HPO_4 \cdot 3H_2O$, 0.04; $MgSO_4 \cdot 7H_2O$, 0.075; $CaCl_2 \cdot 2H_2O$, 0.036; citric acid, 0.006, ferric ammonium citrate, 0.006; Na_2EDTA, 0.001; Na_2CO_3, 0.02 and trace metal solution 1 ml (including H_3BO_3 2.86 g, $MnCl_2 \cdot 4H_2O$ 1.81 g, $ZnSO_4 \cdot 7H_2O$ 0.222 g, $Na_2MoO_4 \cdot 2H_2O$ 0.390 g, $CuSO_4 \cdot 5H_2O$ 79 mg and $Co(NO_3)_2 \cdot 6H_2O$ 49.4 mg per liter) at pH 7.4. Me-

[1]Present address: Mackay Junior College of Nursing.
*Corresponding author. Tel: 886-2-23621519; Fax: 886-2-23679827; E-mail: ssyang@ccms.ntu.edu.tw

dium M4N contained (g l^{-1}): KNO_3, 5.0; KH_2PO_4, 1.25; $CaCl_2$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.003; $MgSO_4 \cdot 7H_2O$, 2.5 and A5 solution, 1 ml (containing H_3BO_3 2.86 g, $MnCl_2 \cdot 4H_2O$ 1.81 g, $CuSO_4 \cdot 5H_2O$ 80 mg, $ZnSO_4 \cdot 7H_2O$ 220 mg, Na_2MoO_4 210 mg and 1 drop of conc. H_2SO_4 per liter). Both media were used for the isolation of cyanobacteria and microalgae from fresh water (Rippka et al., 1979).

Medium ASN III contained (g l^{-1}): $NaNO_3$, 0.75; $K_2HPO_4 \cdot 3H_2O$, 0.002; $MgSO_4 \cdot 7H_2O$, 0.038; $CaCl_2 \cdot 2H_2O$, 0.018; citric acid, 0.003, ferric ammonium citrate, 0.003; Na_2EDTA, 0.0005; Na_2CO_3, 0.02, seawater, 750 ml and trace metal solution 1 ml at pH 8.3, and was used for the isolation of cyanobacteria and microalgae from sea water (Rippka et al., 1979). MN medium contained (g l^{-1}): KNO_3, 1; $MgSO_4 \cdot 7H_2O$, 0.25; NaCl, 0.1; Na_2EDTA, 0.016; $FeSO_4 \cdot 7H_2O$, 0.002 and trace element solution 1 ml (containing H_3BO_3, 2.86 g; $MnSO_4 \cdot 4H_2O$, 1.30 g; $CuSO_4 \cdot 5H_2O$, 1.8 g, $ZnSO_4 \cdot 7H_2O$, 0.22 g; Na_2MoO_4, 0.021 g per 1iter) and was used for the isolation of microalgae from lakes and ponds (Mayo, 1997). Modified Fitzgerald medium contained (g l^{-1}): $NaNO_3$, 0.496; KH_2PO_4, 0.039; $CaCl_2$, 0.036; Na_2EDTA, 0.001; $MgSO_4 \cdot 7H_2O$, 0.075, Na_2CO_3, 0.020; $Na_2SiO_3 \cdot 9H_2O$, 0.058; ferric citrate, 0.006; citric acid, 0.006 and Caffron solution 1 ml (containing H_3BO_3 3.1 g, $MnSO_4 \cdot 4H_2O$, 2.23 g; $ZnSO_4 \cdot 7H_2O$, 287 mg; $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$, 88 mg; $Co(NO_3)_2 \cdot 4H_2O$, 146 mg; $Na_2WO_4 \cdot 2H_2O$, 33 mg; KBr, 119 mg; KI, 83 mg; $Cd(NO_3)_2 \cdot 4H_2O$, 154 mg; $NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 198 mg; $VOSO_4 \cdot 2H_2O$, 20 mg; $Al_2(SO_4)_3 \cdot K_2SO_4 \cdot 24H_2O$, 474 mg and 0.05 M H_2SO_4 1 drop per liter), and was used for the isolation of microalgae from hot spring and fresh water (Takeuchi et al., 1992).

Table 1. Isolation sources of microalgae.

Area	Sources	No. of samples	No. of isolates	Code assigned
Taipei (121°27′-122°6′N 24°41′-25°38′E)	Hog wastewater	15	42	NTU-H1-H3, H5, H9, H15, H23, H25, H28, H36, H38-H41, H46, H48, H50
	Paddy field	4	2	NTU-M30, M31
	Pond	9	10	NTU-H4, H6, H8, M1, M5, M7, M12, M16, M17, M21
	Landfill leachate	6	10	NTU-F1, F6, F7, M24, M26, M27, M36-M39
	Seawater	4	-	
	Hot spring	2	2	NTU-F9, F10
Keelung (121°40′-122°47′N 24°50′-25°11′E)	Seawater	6	-	
	Pond	2	3	NTU-F26-F29
Taoyuan (121°08′-121°22′N 24°51′-25°12′E)	Hog wastewater	6	23	NTU-H10, H11, H14, H20, H24, H33, H37, H47, F11-F25
	Paddy field	1	2	NTU-H45, F8
	Pond	1	2	NTU-M45, M46
Hsinchu (121°12′-121°23′N 24°31′-24°52′E)	Wetland	3	4	NTU-AS1-AS4
	Pond	1	2	NTU-H16, H17
	Hog wastewater	5	22	NTU-H21, H31, H32, M51-M69
Maoli (120°11′-121°10′N 24°18′-24°57′E)	Pond	2	2	NTU-M25, M34
	Hog wastewater	10	32	NTU-H71-H102
Nantou (120°08′-121°10′N 23°51′-24°00′E)	Pond	8	11	NTU-H19, H22, H34, H42, H62, M3, M4, M6, M20, M23, M35
Tainan (120°00′-120°31′N 23°102′-23°28′E)	Pond	4	4	NTU-F22-F24, F30
Kaohsiung (120°12′-120°30′N 21°42′-23°28′E)	Fish pond	3	3	NTU-F3, F4, F5
Pingtung (120°21′-120°57′N 22°00′-22°50′E)	Pond	2	5	NTU-F2, M14, M28, M32, M33
	Seawater	5	6	NTU-AS6-AS12
Kingmen (118°10′-118°40′N 24°24′-24°33′E)	Pond	8	24	NTU-H26, H27, H44, H51-H60, M2, M9-M11, M13, M15, M18, M19, M29, M47, M48
	Seawater	1	1	NTU-AS14
	Lake	2	5	NTU-M22, M40-M44

H code was isolated with BG11 medium, M code with MN medium, F code with modified Fitzgerald, and AS code with ASN III medium.

Isolation of Carbon Dioxide-Fixing Microalgae

Samples were precultivated in an appropriate broth at 30°C and 392 μmol m^{-2} s^{-1} for 1 week and subcultivated for another week. Then the culture broth was smeared on different solid media and cultivated at 30°C and 392 μmol m^{-2} s^{-1} for 1 week. Colonies were picked and transferred to the same media for purification. To isolate the high CO_2-tolerant strains, the culture broth was aerated with 10% and 20% CO_2 at 30°C and 589 μmol m^{-2} s^{-1} for 1 week. Microbial growth was detected by the optical density at 680 nm, and isolates with optical densities higher than 2.0 after 4 days incubation were selected for further study.

Measurement of Growth Rate

Microalgae was cultivated in 1 l flat bottles and at 30°C and 392 (or 589) μmol m^{-2} s^{-1} under aeration with air or air containing different concentrations of CO_2 at 0.15 vvm for 1 week. The growth rate of microalgae was measured by both the optical density at 680 nm and by the cell dry weight. Cells were harvested by Millipore (0.45 μm) filtration, washed twice with distilled water, and dried at 105°C overnight. Linear growth rate was calculated from the logarithmic growth over 2 to 4 days cultivation. Specific growth rate was calculated from the slope of growth rate and biomass yield at each cultivation condition.

Morphological Observation and Biochemical Test

Morphological properties of isolates were observed under a light microscope (Olympus BH-2, Japan). Biochemical and physiological characters used for the identification of isolates included nitrate reduction, mannitol utilization, vitamin requirement, acidity, salinity and temperature tolerance, gelatin liquefaction, and starch hydrolysis.

Carbon Dioxide Determination

Carbon dioxide concentration was analyzed by gas chromatography using a thermal conductivity detector as follows: Gas sample (1 ml) was injected into a Shimadzu 14A gas chromatograph (Shimadzu Co., Japan) with a glass column (0.26 mm × 2.0 m) packed with Porapack Q (80/100 mesh). The column temperature was set at 150°C, and the injection and the detector temperatures were set at 200°C. Carbon dioxide concentration was calculated with a standard curve from 0.1 to 1000 mg kg^{-1} (v/v) (Yang and Chang, 1997; Chang et al., 2001).

Effect of Carbon Dioxide on Cell Growth

Isolates were precultivated at 30°C and 589 μmol m^{-2} s^{-1} in a 500 ml conical flask with 300 ml BG-11 medium and bubbled with air or air containing 5 to 40% CO_2 at 0.15 vvm for 6 days. Then, the cultures were transferred to 1 l flat bottles with 800 ml medium at an initial algal concentration of 0.02 g l^{-1} (wet weight) and cultivated under the same conditions for 8 to 10 days. Microalgae growth was determined by both the optical density at 680 nm and by the cell dry weight.

Light Response

To measure the photosynthetic capacity of isolates, cells were harvested by centrifugation at 3,000 *g* for 10 min and then resuspended in 50 mM pH 7.0 Hepes buffer to give a density of 20 to 24 μg chl ml^{-1} in the presence of 10 mM $NaHCO_3$. Light was supplied by a quartz-halogen bulb in the range from 0 to 2,300 μmol m^{-2} s^{-1}, and the intensity was controlled with neutral filter. Light response curve was determined using a Clark-type electrode (Yellow Springs, USA) at 30°C.

Chemical and Physical Analyses

pH. Sample pH was determined directly with a pH meter (Mode Sentron 2001).

Nitrate content. Nitrate concentration was assayed according to Cawse (1967). A sample of 1 ml was mixed with alumina cream (4 ml) and then centrifuged at 3,000 *g* for 5 min. The supernatant (1 ml) was reacted with sulfuric acid solution (1 ml) for 2 min, then diluted with 5% perchloric acid to 10 ml, and the absorbance measured at 210 nm. Authentic potassium nitrate was used as a standard in the concentration range from 0 to 10,000 μM.

Phosphate content. Phosphate was assayed as described by Strickland and Parsons (1968). Phosphomolybdate complex in acid molybdate solution was reduced by ascorbic acid to a blue color, and the absorbance was measured at 885 nm. Authentic dibasic potassium phosphate was used as a standard in the range from 0 to 30 μM.

Light intensity. Light intensity was determined with Toshiba API-5 photometer.

Chlorophyll content. Chlorophyll was extracted by 95% ethanol, and the absorbance was measured at 649, 655 and 665 nm. The chlorophyll a and b content was calculated by the following equations:

C_a (μg ml^{-1}) = 13.7 A_{665nm} - 5.76 A_{649nm},

C_b (μg ml^{-1}) = 25.8 A_{649nm} - 7.60 A_{665nm}, and

C_{a+b} (μg ml^{-1}) = 1000/39.8 ×A_{655nm}) (Wintermans and De Mots, 1965; Liu et al., 1981).

Results and Discussion

Isolation of CO_2 Tolerant Microalgae

217 colonies of cyanobacteria and microalgae were isolated from 110 water samples taken from lakes, ponds, rivers, landfill leachates, hog wastewater, paddy fields, wetlands, hot springs, sediments, and seawater in Taiwan with the appropriate media (Yang et al., 2000). After a series of transfers, 98 isolates survived. Hog wastewater was the major source for the isolation of cyanobacteria and microalgae (119 isolates), followed by ponds (63 isolates), landfill leachates (10 isolates), and seawater (7 isolates). These isolates were primarily cultivated at 30°C, 392 μmol m^{-2} s^{-1}, and aerated with air at 0.15 vvm for 10 days. The culture broth had absorption peaks between 440 and 480 nm, between 680 and 690 nm, and a shoulder at 660 nm

Table 2. Optical density (OD) and cell dry weight of isolates cultivated at 30°C, 392 μmol m^{-2} s^{-1} under aeration with air at 0.15 vvm for 10 days or under aeration with 10% and 20% CO_2 at 0.15 vvm for 5 days.

Code of isolates	OD under aeration with air	OD under aeration with 10% CO_2	Cell dry weight (g l^{-1}) under aeration with 10% CO_2	OD under aeration with 20% CO_2*
NTU-H1	0.65±0.22	1.41±0.46	0.28±0.10	
NTU-H2	1.21±0.12	3.06±0.05	0.42±0.07	1.62±0.02
NTU-H3	0.81±0.22	2.97±0.18	0.52±0.01	2.23±0.01
NTU-H4	2.00±0.33	1.77±0.21	0.44±0.06	
NTU-H5	1.25±0.21	1.87±0.90	0.48±0.28	
NTU-H6	0.63±0.12	2.62±0.06	0.63±0.08	1.60±0.02
NTU-H8	1.26±0.34	2.29±0.10	0.64±0.07	1.25±0.04
NTU-H9	0.61±0.12	0.44±0.16	0.30±0.10	
NTU-H10	0.75±0.33	1.75±0.05	0.53±0.04	1.15±0.10
NTU-H11	1.73±0.01	1.63±0.69	0.49±0.22	1.52±0.10
NTU-H12	1.50±0.11	2.02±0.04	0.65±0.01	1.50±0.03
NTU-H14	0.45±0.10	1.78±0.08	0.57±0.01	0.75±0.02
NTU-H15	1.40±0.22	2.31±0.25	0.46±0.02	2.12±0.09
NTU-H16	1.00±0.30	1.94±0.10	0.54±0.06	1.50±0.12
NTU-H19	1.53±0.21	2.32±0.02	0.55±0.04	1.25±0.00
NTU-H20	0.50±0.16	2.21±0.16	0.58±0.03	1.11±0.05
NTU-H21	1.23±0.01	2.67±0.20	0.23±0.09	
NTU-H22	1.30±0.01	1.82±0.42	0.37±0.49	
NTU-H23		2.79±0.08	0.12±0.12	1.85±0.04
NTU-H24	0.60±0.00	1.44±0.15	0.32±0.31	1.22±0.08
NTU-H25	0.50±0.01	2.90±0.30	0.61±0.03	2.00±0.01
NTU-H27	0.31±0.01	1.69±0.11	0.53±0.23	
NTU-H29	1.05±0.25	2.32±0.06	0.47±0.32	
NTU-H30	0.51±0.98	1.92±0.16	0.48±0.05	
NTU-H31	1.25±0.16	2.52±0.23	0.79±0.17	1.58±0.07
NTU-H32	1.21±0.01	1.62±0.09	0.53±0.00	1.31±0.10
NTU-H33	1.11±0.35	2.47±0.57	0.44±0.33	2.81±0.07
NTU-H34	0.52±0.11	0.06±0.26	0.28±0.29	
NTU-H51		1.00±0.72	0.30±0.11	
NTU-H52		2.13±0.19	0.55±0.21	1.00±0.00
NTU-H53		0.73±0.06	0.29±0.02	
NTU-H54		0.02±0.01	0.13±0.08	
NTU-H55		0.09±0.02	0.21±0.01	
NTU-H56		0.21±0.02	0.30±0.03	
NTU-H57		2.67±0.05	0.59±0.00	1.41±0.01
NTU-H58		2.11±0.02	0.45±0.01	1.32±0.15
NTU-H59		1.55±0.57	0.45±0.12	
NTU-H60		1.59±0.17	0.41±0.04	
NTU-M1		2.68±0.10	0.60±0.01	2.30±0.07
NTU-M2		1.71±0.39	0.54±0.16	0.48±0.02
NTU-M3		1.39±0.12	0.44±0.06	
NTU-M4		0.67±0.33	0.29±0.07	
NTU-M5		2.75±0.17	0.56±0.05	2.25±0.08
NTU-M6		1.14±0.22	0.41±0.17	
NTU-M8		2.14±0.05	0.44±0.01	0.90±0.00
NTU-M9		1.75±0.22	0.47±0.01	
NTU-M10		0.57±0.04	0.30±0.04	
NTU-M11		1.58±0.34	0.30±0.13	0.25±0.08
NTU-M12		0.63±0.11	0.21±0.01	
NTU-M13		0.68±0.06	0.45±0.04	
NTU-M14		2.98±0.05	0.69±0.03	1.51±0.07
NTU-M15		2.12±0.34	0.39±0.10	1.24±0.07
NTU-M16		0.93±0.07	0.22±0.01	
NTU-M17		1.01±0.02	0.43±0.05	
NTU-M18		2.60±0.48	0.51±0.15	0.08±0.11
NTU-M19		1.60±0.70	0.22±0.03	
NTU-M20		0.04±0.00	0.06±0.03	
NTU-M21		2.56±0.06	0.60±0.07	2.00±0.10
NTU-M23		2.02±0.19	0.55±0.06	1.21±0.01

*NTU-H2~NTU-H32 only incubated for 4 days.
Means±S.D. (n=3)

(data not shown). There was a linear correlation between the optical density at 680 nm and the cell dry weight. The log of optical density and the dilution factors were Y= 9.748X - 0.069 (R^2=0.999), Y=9.339X - 0.008 (R^2=0.999), Y= 8.138X - 0.053 (R^2=0.999) and Y= 9.146X - 0.052 (R^2=0.997) for isolates NTU-H15, NTU-H25, NTU-H47 and NTU-M1, respectively, where Y is the log of optical density and X is the dilution factor. Therefore, the optical density at 680 nm was used as the growth parameter of the isolated cyanobacteria and microalgae.

Effect of CO_2 Concentration on Cell Growth

To investigate the effect of CO_2 concentration on the growth of the isolated cyanobacteria and microalgae, the isolates were incubated at 30°C and 589 μmol m^{-2} s^{-1} under aeration with different concentrations of CO_2 at 0.15 vvm for 6 to 10 days. It was shown that most microalgae grew slowly in air aeration conditions; only isolate NTU-H4 had an optical density at 680 nm higher than 2.0 (Table 2). When these isolates were incubated under aeration with 10% CO_2, 25 isolates had an optical density at 680 nm higher than 2.0, and 22 isolates had cell dry weight higher than 0.5 g l^{-1} among 59 tested isolates over 5 days cultivation. The maximal optical density was 3.06 while the minimal value was only 0.02. The maximal cell dry weight was 0.79 g l^{-1}, and the minimal one was 0.06 g l^{-1} (Table 2). When incubated under aeration with 20% CO_2 for 5 days, 13 of the 47 tested isolates had an optical density at 680 nm that was higher than 2.0. The maximal optical density was 2.81, and the minimal value was 0.08. From the cell growth under aeration with 10% and 20% CO_2, isolates NTU-H3, NTU-H15, NTU-H25, NTU-H33, NTU-H36, NTU-H39, NTU-H40, NTU-H47, NTU-M1, NTU-M5 and NTU-M21 were selected for further studies. Flue gas contains 13 to 15% CO_2, meaning these isolated microalgae were incubated at 30°C and 589 μmol m^{-2} s^{-1} under aeration with 15% CO_2. Cell dry weight increased on incubation. Five isolates had a cell dry weight higher than 1.6 g l^{-1}, and the maximal growth rate was more than 0.18 g l^{-1} d^{-1} (Table 3). The specific growth rates of these five isolates were between 0.177 and 0.271 d^{-1} during 2 to 8 days incubation. Isolates NTU-H15 and NTU-25 had high cell biomass and appropriate specific growth rate during 2 to 6 days cultivation (0.268 to 0.271 d^{-1}). Cell biomass of *Chlorella* sp. K35 and *Oocystis* sp. was only 1.0 g l^{-1} when they were cultivated in the broth under aeration with 10 to 20% CO_2 for 10 days (Hanagata et al., 1992; Takeuchi et al., 1992). Isolates NTU-H15 and NTU-H25 were screened from the hog wastewater. They could tolerate high concentration of salts and had high growth rates at 15% CO_2. Therefore, these two algae were used for this CO_2 fixation study.

The effect of CO_2 concentration on the cell growth of *Chlorella* sp. NTU-H15 and NTU-H25 is shown in Figure 1. Aeration with addition of CO_2 stimulated cell growth, and both strains had a maximal growth at 5% CO_2, which decreased gradually with increasing CO_2 concentration. A long lag period was observed under aeration with 40% CO_2, and the cell growth decreased significantly. Both strains had a maximal linear growth rate under aeration with 5% CO_2 (between 0.28 and 0.31 g l^{-1} d^{-1}). This rate decreased slightly under aeration with 15 to 20% CO_2 (from 0.21 to 0.27 g l^{-1} d^{-1}), fell moderately under aeration with 40% CO_2 (between 0.15 and 0.18 g l^{-1} d^{-1}), and plunged under aeration with 60% CO_2 (between 0.06 and 0.07 g l^{-1} d^{-1}) (Table 4). Kodama et al. (1993) reported that *Chlorococcum littorale*, a highly CO_2-tolerant microalgal strain, had growth rates of 0.4, 0.3, 0.2 and 0 g l^{-1} d^{-1} at 5, 20, 40 and 70% CO_2, respectively. Hanagata et al. (1992) indicated that two CO_2-tolerant microalgae, *Scenedesmus* sp. and *Chlorella* sp., had growth rates between 0.15 and 0.18 g l^{-1} d^{-1} at 10 to 40% CO_2, and they could not grow at 60% CO_2. Sung et al. (1999) showed that *Chlorella* KR-1 had growth rates of 1.1, 0.8, 0.6 and 0.1 g l^{-1} d^{-1} under aeration with 10, 30, 50, and 70% CO_2, respectively. Sivla and Pirt (1984) reported that CO_2 at $P_{CO2} \geq 0.6$ atm had a significantly inhibitory effect on the growth of *Chlorella*. The same phenomenon was also found in this study. The growth rates of our isolates were lower than those of *Chlorococcum littorale* and *Chlorella* KR-1, but they had higher growth rates than those of *Scenedesmus* sp. and

Table 3. Biomass, maximum growth rate, specific growth rate and sources of microalgal isolates incubated at 30°C, 589 μmol m^{-2} s^{-1} under aeration with 15% CO_2 for 10 days.

Code of isolates	Source	Cell dry weight (g l^{-1})	Maximum growth rate (g l^{-1} d^{-1})	Specific growth rate (d^{-1})
NTU-H3	Hog wastewater	1.65±0.34	0.20±0.01	0.197
NTU-H15	Hog wastewater	1.80±0.05	0.28±0.02	0.271
NTU-H25	Hog wastewater	1.71±0.25	0.27±0.03	0.268
NTU-H33	Hog wastewater	0.75±0.24	0.08±0.01	0.072
NTU-H36	Hog wastewater	1.34±0.12	0.18±0.02	0.179
NTU-H39	Hog wastewater	1.41±0.14	0.18±0.08	0.152
NTU-H40	Hog wastewater	0.48±0.08	0.06±0.00	0.057
NTU-H47	Hog wastewater	1.65±0.05	0.22±0.02	0.205
NTU-M1	Pond	1.62±0.12	0.20±0.01	0.177
NTU-M5	Pond	1.02±0.21	0.26±0.03	0.239
NTU-M21	Pond	0.47±0.25	0.15±0.01	0.131

Means±S.D. (n=3)

Chlorella sp. This might be due to the CO_2-tolerance and different culture conditions of *Chlorococcum* and *Chlorella* KR-1. The growth rate of isolates of *Chlorella* sp. NTU-H15 and NTU-H25 might be improved by the adaptation of carbon dioxide enrichment and by the adjustment of culture conditions.

Culture pH decreased dramatically at the initial growth stage, increased gradually with cultivation, and reached a plateau of pH 6.9 after more than 8 days incubation. Cell mass increased with cultivation for 8 days (Figure 2). Phosphate is one of the major nutrients for the normal growth of algae and plays an important role in most cellular processes (Vonshak, 1986). Phosphate and nitrate concentrations plummeted at the early growth stage. After 5 days of cultivation, phosphate concentration was below detection, and the cell concentration increased slowly. Average phosphate and nitrate uptake rate was about 60 μM d^{-1} and 2 mM d^{-1}, respectively. Kurano et al. (1995a) reported that 17 mM nitrate was consumed completely and phosphate uptake ceased after 50 h cultivation of *Chlorococcum littorale*. The average phosphate uptake of *Chlorococcum littorale* was about 300 μM d^{-1}. When *Oocystis* sp. was cultivated under aeration with 10 and 20% CO_2, it exhausted 6 mM nitrate at Day 6 and Day 13 (Takeuchi et al., 1992). Healey (1982) reported that phosphate and nitrogen metabolism were closely related and changed with the N/P ratio of lakes and the phytoplanktonic population. Phosphate might be a limiting factor for *Chlorella* sp. NTU-H15 growth in BG11 medium. Cell dry weight increased from 1.14 to 1.52 g l^{-1} after 7 days incubation when the phosphate concentration was doubled (400 μM) (data not shown).

Table 4. Effect of CO_2 concentration and light intensity on specific growth rate of *Chlorella* sp. NTU-15 and NTU-H25 cultivated for 3-5 days.

Culture conditions		NTU-H15	NTU-H25
30°C, and 589 μmol m^{-2} s^{-1} under aeration with	0.04% CO_2	0.044	0.033
	5% CO_2	0.311	0.284
	15% CO_2	0.271	0.268
	20% CO_2	0.225	0.211
	40% CO_2	0.180	0.152
	60% CO_2	0.072	0.056
30°C, and under aeration with 15% CO_2 at	190 μmol m^{-2} s^{-1}	0.174	0.185
	293 μmol m^{-2} s^{-1}	0.248	0.234
	589 μmol m^{-2} s^{-1}	0.271	0.268
	912 μmol m^{-2} s^{-1}	0.260	0.274

Table 5. Morphological and biochemical properties of isolated microalgae and *Chlorella* strains.

Item	*Chlorella vulgaris* 211-11p	*Chlorella sorokiniana* 211-8k	Isolates				
			NTU-H15	NTU-H25	NTU-H47	NTU-M1	NTU-M5
Cell form	Unicellular & spherical	Unicellular & spherical	Unicellular & spherical	Unicellular & spherical	Unicellular & spherical	Unicellular & spherical	Unicellular & spherical
Cell size (μm)	3-6	3-6	3-6	3-6	3-6	3-6	3-6
Acid tolerant (pH limit)	4.0	4.0	4.0	4.0	4.0		
Salt tolerant (% NaCl)	2.5	2.0	2.5	2.0	2.0		
Pyrenoid	+	+	+	+	+	+	+
Chloroplast	Cup-shape	Cup-shape	Cup-shape	Cup-shape	Cup-shape	Cup-shape	Cup-shape
Motility	-	-	-	-	-	-	-
Flagella	-	-	-	-	-	-	-
Chl a & chl b	+	+	+	+	+	+	+
Gelatin liquefication	-	-	-	-	-		
Starch hydrolysis	-	-	-	-	-		
Nitrate reduction	+	+	+	+	+		
Thiame requirement	-	-	-	-	-		
Growth Temp.							
30°C	+	+	+	+	+		
38°C	-	+	+	+	+		
40°C	-	±	+	+	+		
42°C	-	-	-	-	-		
Temp. limit	< 38°C	≒ 38°C	≒ 40°C	≒ 40°C	≒ 40°C		
Thermophily	-	+	+	+	+		

Identification of Isolated Microalgae

Some morphological and chemical characteristics of these five isolates and authentic strains are summarized in Table 5. All of them were unicellular spheroids with a diameter of 3-6 μm. Each cell had one cup-shaped chloroplast with a distinctive pyrenoid. Chlorophylls a and b were presented. No sexual reproduction was observed. They formed autospores inside the cell and multiplied to two, four, or more individuals. In addition, all isolates had a cell growth limit at pH 4.0, 2.0% NaCl and 40°C. Nitrate reduction activity was observed, but starch hydrolysis and gelatin liquefaction were not detected. On the basis of these characters and thermo-tolerance, these five isolates were fundamentally identical and belonged to the genus *Chlorella* (Harold and Michael, 1978; Kessler, 1985). *Chlorella sorokiniana* was able to grow at temperatures up to 38-42ºC (Kessler, 1985). The enzyme activity and molecular phylogeny of these isolates were analyzed for species identification as Kessler (1985) and Huss et al. (1999) described. The results of this species identification and molecular phylogeny will be published and discussed hereafter (Chang and Yang, unpublished data).

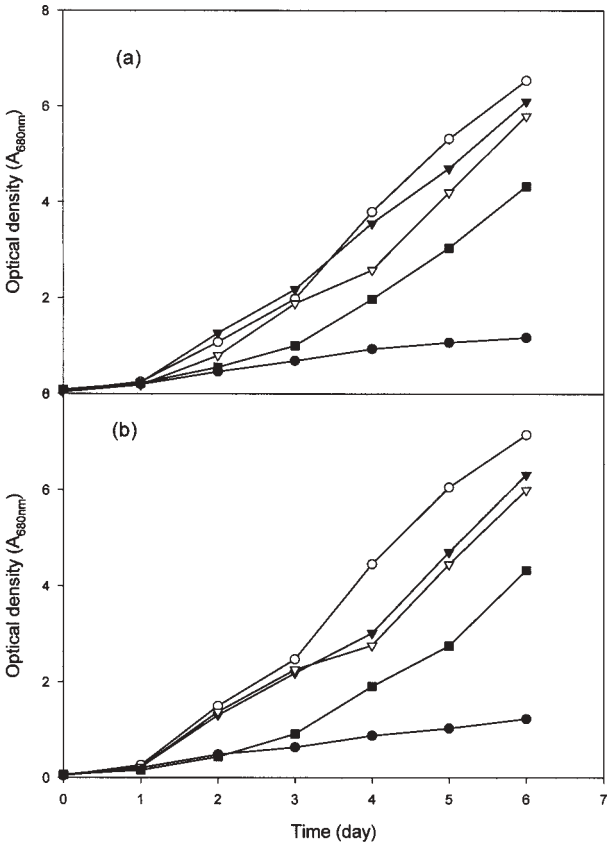

Figure 1. Effect of carbon dioxide concentration on the growth rate of *Chlorella* isolates at 30°C and 589 μmol m^{-2} s^{-1}. (a) *Chlorella* sp. NTU- H15; (b) *Chlorella* sp. NTU- H25. ●, 0.035% CO_2; ○, 5% CO_2; ▼, 15% CO_2; ▽, 20% CO_2; ■, 40% CO_2.

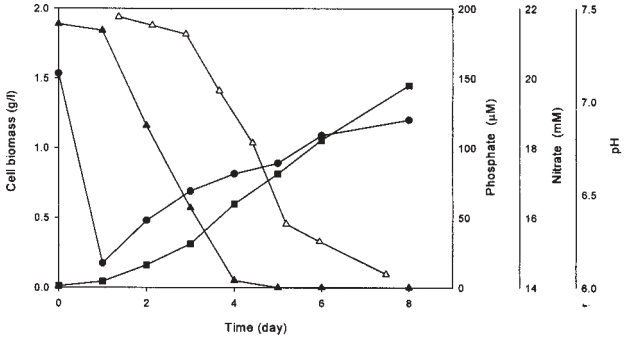

Figure 2. Cell growth and nutrient profile of *Chlorella* sp. NTU-H15 aerated with 15% CO_2. ■, cell mass; ●, pH; ▲, phosphate; △, nitrate.

Effect of Cultivation Temperature

The growth rates of the *Chlorella* isolates showed significant inhibition at incubation temperatures ≤ 28°C or ≥ 42°C. *Chlorella* sp. NTU-H15 had a high growth rate at 35 and 39°C ($A_{680\,nm}$ = 1.50) while *Chlorella* sp. NTU-H25 had a high value at 30°C ($A_{680\,nm}$ = 1.51). The optical density was only 0.05 to 0.20 when they were cultivated at 25, 28 or 42°C. Kessler (1985) reported that *C. sorokiniana* had an upper growth limit at 38 to 42°C, and temperature tolerance appeared as a species-specific character in the genus *Chlorella*. Hanagata et al. (1992) indicated that *Chlorella* sp. strain 35 had a five-day lag phase when it was cultivated at 40°C. Sung et al. (1999) showed that 30°C was the maximal growth temperature of *Chlorella* KR-1. The isolate *Chlorella* sp. NTU-H15 had high thermostability at 39°C and was able to tolerate a high CO_2 concentration (40%). Therefore, this isolate might have a high potential for biofixation of CO_2 emitted from coal-fired thermal power plants.

Effect of pH

The linear growth rate of *Chlorella* sp. NTU-H15 was 0.12 g l^{-1} d^{-1} at an initial pH of 3.5, increased gradually to 0.24 g l^{-1} d^{-1} at an initial pH of 6.0, and remained constant at an initial pH of 7.0. The cell growth of *Chlorella* sp. NTU-H15 was inhibited at an initial pH below 3.0. Kodama et al. (1993), Sung et al. (1999) and Morita et al. (2000) also reported that the growth of *Chlorococcum littorale*, *Chlorella* KR-1, and *Chlorella sorokiniana* was not affected by culture pH when the value was higher than pH 4.0, and the growth rate was inhibited drastically at pH 3.0. In this study, *Chlorella* sp. NTU-H15 was able to grow at pH 3.5 to 7.0. This characteristic is very important and suitable for stack gases using the cultivation of *Chlorella* for biomass production.

Effect of Light Intensity

Chlorella sp. NTU-H25 had a high growth rate at a light intensity of 589 to 912 μmol m^{-2} s^{-1}, and the growth rate decreased at 190 to 293 μmol m^{-2} s^{-1}. *Chlorella* sp. NTU-H15 had the highest growth rate at a light intensity of 589

μmol m^{-2} s^{-1}, a lower rate at 912 and 293 μmol m^{-2} s^{-1}, and the lowest rate at 190 μmol m^{-2} s^{-1} (Figure 3). *Chlorella* sp. NTU-H25 had a high growth rate at light intensity of 912 and 589 μmol m^{-2} s^{-1}, followed by 293 and 190 μmol m^{-2} s^{-1}. Saturating light intensity for the tested *Chlorella* strains was about 589 μmol m^{-2} s^{-1}. Low light intensity reduced the growth rate and biomass production.

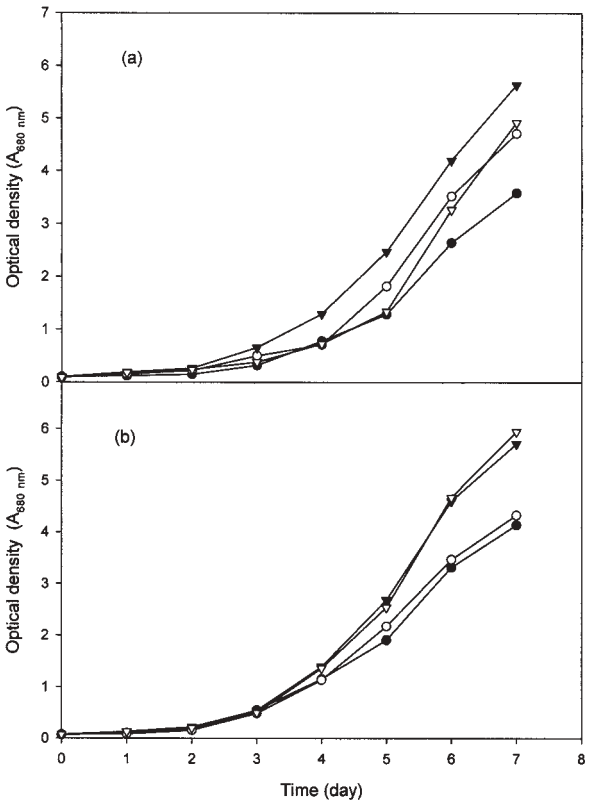

Figure 3. Effect of light intensity on algal growth rate of *Chlorella* aerated with 15% CO_2. (a) *Chlorella* sp. NTU-H15; (b) *Chlorella* sp. NTU-H25. ●, 190 μmol m^{-2} s^{-1}; ○, 293 μmol m^{-2} s^{-1}; ▼, 589 μmol m^{-2} s^{-1}; ▽, 912 μmol m^{-2} s^{-1}.

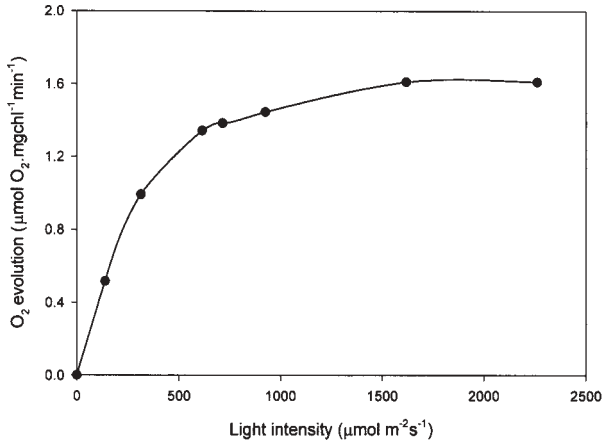

Figure 4. Effect of light intensity on photosynthetic O_2 evolution of *Chlorella* sp. NTU-H15 at 30°C and 10 mM $NaHCO_3$.

The effect of light intensity on the photosynthetic oxygen evolution of *Chlorella* sp. NTU-H15 is shown in Figure 4. Oxygen evolution increased with the increasing of light intensity, becoming saturated at around 700 μmol m^{-2} s^{-1} at 30ºC and 10 mM $NaHCO_3$. Each mg of chlorophyll produced 1.6 μM O_2 min^{-1}. The light intensity saturation of *Chlorella* sp. NTU-H15 was higher than that of *Oocystis* sp. (around 300 μmol m^{-2} s^{-1}) (Takeuchi et al., 1992), *Chlorella* sp. and *Scenedesmus* sp. (around 200 μmol m^{-2} s^{-1}) (Hanagata et al., 1992), and was equivalent to that of *Chlorella* sp. isolated from hot spring (about 800 μmol m^{-2} s^{-1}) (Murakami et al., 1998). Therefore, *Chlorella* sp. NTU-H15 and NTU-H25 had a higher growth rate than *Chlorella* sp. or *Scenedesmus* sp. due to the high light intensity saturation. But the oxygen evolution of chlorophyll in *Chlorella* sp. NTU-H15 was lower than that of *Oocystis* sp. (each mg produced 2.5 μM O_2 min^{-1}) (Takeuchi et al., 1992), perhaps due to the high light intensity saturation in *Chlorella* sp. NTU-H15. During incubation of *Chlorella* sp. NTU-H15, the light intensity was only around 600 μmol m^{-2} s^{-1}; therefore, the growth rate of *Chlorella* sp. NTU-H15 might be improved if the cultures were grown under high light intensity and a sufficient nutrient supply.

In conclusion, the local isolates *Chlorella* sp. NTU-H15 and NTU-H25 grew well at high temperature, high cell density, high CO_2 concentration, and over a broad-range of pH values. They are suitable strains for large-scale, dense cultivation with industrial discharge gases to fix CO_2 directly to reduce global warming and create a cell biomass for producing industrially valuable compounds.

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以台灣分離微藻行二氧化碳生物固定之一些特性

張義宏　楊盛行

國立台灣大學農業化學系

由台灣之湖泊、池塘、底泥、豬糞尿廢水、水田、溫泉和海水中分離出二百餘株微藻可以固定二氧化碳。其中由豬糞尿廢水中分離之單細胞微藻 *Chlorella* sp. NTU-H15 和 *Chlorella* sp. NTU-H25 可以在 40% CO_2 中生長。在 20% CO_2 下，生長速率介於 0.21 至 0.22 g dry wt $l^{-1}d^{-1}$。此二分離株在 5~40% CO_2 和 190-589 µmol $m^{-2}s^{-1}$ 下有相似之光反應。*Chlorella* sp. NTU-H15 生長速率高於 *Chlorella* sp. NTU-H25。並且 *Chlorella* sp. NTU-H15 可以耐受高濃度 CO_2，高細胞濃度、寬廣 pH 和培養溫度，每升可得 1.8 克乾重菌體，在 15% CO_2 下，最大生長速率 0.28 g dry wt $l^{-1}d^{-1}$ 和比生長速率 0.27 d^{-1}。每 mg 葉綠素在 30°C，700 µmol $m^{-2}s^{-1}$ 和 10 mM $NaHCO_3$ 可以產生 1.6 mM O_2。至於每升 *Chlorella* sp. NTU-H25 可以產生 1.7 g 乾重菌體，最大生長速率 0.25 g dry wt $l^{-1}d^{-1}$ 和比生長速率 0.27 d^{-1}。可見此兩株微藻適合於高菌體濃度下固定 CO_2 生產菌體。

關鍵詞：生物固定；生長速率；微藻；*Chlorella*。