Characterization of a Native Algae Species *Chlamydomonas debaryana*: Strain Selection, Bioremediation Ability, and Lipid Characterization

Bo Zhang,[a] Lijun Wang,[a,*] Rifat Hasan,[b] and Abolghasem Shahbazi [a]

Native microalgal species may offer a favorable combination of both wastewater treatment and biofuel production. In this research, a green microalgae, *Chlamydomonas debaryana,* was isolated from a local lagoon, screened for its lipid content using flow cytometry, and further identified with microscopic observations and DNA sequence analysis. When using swine wastewater as a medium, the biomass yields were between 0.6 and 1.62 g/L, giving a median value of 1.11 g/L. By increasing mass transfer rates and providing sufficient light intensity, the microalgal growth was intrinsically enhanced. The growth of *C. debaryana* reduced most nutritional contents of the wastewater except iron. When combining the microalgal growth and nutrient removal, *C. debaryana* was able to utilize 1.3 to 1.6×10^3 mg COD (chemical oxygen demand)/g biomass, 55 to 90 ppm ammonia/g biomass, and 48 to 89 ppm phosphorous/g biomass, The lipid content of *C. debaryana* was 19.9 ± 4.3% of cell dry weight. The transesterified microalgal oil mostly consisted of 14 kinds of fatty acids, ranging from C5 to C22, which can be refined into renewable jet fuel or used as sources of omega-3 and omega-6 fatty acids.

Keywords: Microalgae; Strain selection; Chlamydomonas debaryana; Bioremediation; Swine wastewater; Lipid

Contact information: a: Department of Natural Resources and Environmental Design, North Carolina A&T State University, Greensboro, North Carolina, USA; b: Chemical, Biological and Bioengineering Department, North Carolina A&T State University, Greensboro, North Carolina, USA;
** Corresponding authors:lwang@ncat.edu*

INTRODUCTION

Microalgae are a diverse group of prokaryotic and eukaryotic photosynthetic microorganisms, typically found in freshwater and marine systems (Li *et al.* 2008). Algae are essential to global carbon, nitrogen, and sulfur cycling, with approximately 45% of the photosynthetic carbon assimilation being achieved by algae. Bioremediation of wastewater using algal strains such as *Chlorella* and *Dunaliella*, spans over 75 years (Abdel-Raouf *et al.* 2012). Algae bioremediation has been applied for a range of purposes, some of which are the removal of coliform bacteria, reduction of chemical and biochemical oxygen demand, the removal of N and/or P, and the removal of heavy metals. The growth of microalgae for wastewater treatment can further be used to supply feedstock for biofuel production. Microalgae are considered a promising feedstock for biofuel production. The oil yield of some algal stains is 1,000 to 4,000 gallons of oil/acre/yr whereas soybeans and other oil crops can generate 48 gallons of oil/acre/yr approximately (Zhang 2013). As an energy crop, microalgae do not compete with grain crops for limited arable lands because they may grow in water.

A number of researchers have reviewed algal strains and growth in municipal wastewater treatment effluent, livestock wastewater, agro-industrial wastewater, and industrial wastewater (Chevalier *et al.* 2000; García *et al.* 2006; Woertz *et al.* 2009). Algae cultivation requires three primary nutrients: carbon, nitrogen, and phosphorus. Micronutrients required in traceable amounts include silica, calcium, magnesium, potassium, iron, manganese, sulfur, zinc, copper, and cobalt, although the supply of these essential micronutrients rarely limits algal growth when wastewater is used (Hasan *et al.* 2014).

The growth characteristics and composition of microalgae are known to significantly depend on the environmental conditions, such as light intensity, photoperiod, temperature, CO_2 flow rate, nutrient composition, *etc.* (Becker 1988). However, there is no perfect algal strain for all climates and water types. Native microalgal species may have a competitive advantage under local geographical, climatic, and ecological conditions (Duong *et al.* 2012). Application of native microalgal species also reduces the risk of invasive algae species (Jarvis 2008).

Therefore our project first focused on the selection of suitable local microalgal strains for swine wastewater treatment that also had high lipid accumulation. The selection was done using a high throughput screening method based on flow cytometry, which has proven to be an invaluable tool in clinical, industrial, and research settings. The effect of cultivation conditions on biomass production of the selected strain was observed. To date, this is the first study to evaluate the wild type *Chlamydomonas debaryana* species for its bioremediation ability and the potential as a biofuel source.

EXPERIMENTAL

Microalgae Selection, Screening, and Identification

Every three months, water samples were collected from the lagoons at the North Carolina A&T State University (NC A&T) farm. The water samples were first diluted 10 times, and then enriched with different nutrients, such as proteose peptone. Microorganism isolation was carried out using a selective agar medium containing swine wastewater as the sole nutrient source, and 1.5% agar. In order to obtain unialgal strain cultures, ampicillin (100 mg/L) and the fungicide, carbendazim (50 mg/L), were also added into the selective medium. The cultures were incubated at 20 °C and 400 μmol $m^{-2}s^{-1}$ continuous cool-white fluorescent light illumination. For strain selection, the following parameters were considered: microalgal colonies that appeared within a 48- to 72-h period and large colonies at the end of incubation period (14 days) were selected.

High-throughput screening of algal strains with high lipid content was performed using flow cytometry. The BODIPY 505/515 (Invitrogen, USA) is a dye commonly used to detect lipids in bacteria. It stains neutral lipids and is membrane-soluble. Algae cell samples were collected by centrifuging, and the supernatant was discarded. Cells were diluted in 99 μL ddH_2O, and 1 μL of a BODIPY stock solution (100 μM in dimethyl sulfoxide) was added to attain a final concentration of 1 μM. Upon addition of the fluorochrome, the samples were vortexed and incubated at room temperature in darkness for 5 min. A Guava easyCyte 5HT flow cytometer (EMD Millipore Corporation, Billerica, MA) with a blue laser providing excitation source of 488 nm wavelength was utilized to determine the fluorescence intensity of the cells. The BODIPY and chlorophyll A fluorescence was determined using 525-nm and 690-nm band pass filters. The data of

the fluorescence intensity were compared to that of a commercial algal strain of *Chlorella vulgaris*.

Identification of the isolated strain was performed using morphological properties and sequence analysis of the 5.8s rDNA region and both internal transcribed spacer regions (ITS1 and ITS2). For morphological observation, algal cells were observed using a Zeiss Axio Scope A1 microscope (Carl Zeiss, Germany). The sequence analysis was performed by the Culture Collection of Algae at the University of Texas at Austin (UTEX). The DNA was extracted using a standard protocol (Blanca *et al.* 2010), and then PCR was performed. The primers used were 5'- GGGATCCGTTTCCGTAG-GTGAACCTGC -3' and 5'- GGGATCCATATGCTTAAGTTCAGCGGGT -3' (Goff and Moon 1993). The resulting gene sequences were aligned and compared to the nucleotide sequences of some known microorganisms in the GenBank database of the National Center for Biotechnology Information (NCBI) by using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov).

Microalgal Strains and Pre-cultured Conditions

Chlamydomonas debaryana AT24 was isolated from the lagoon at the farm of NC A&T. A microalgal strain of *Chlorella vulgaris* (UTEX 2714) was obtained from the Culture Collection of Algae at the University of Texas at Austin. *C. debaryana* was cultured in autoclaved wastewater, proteose medium, or soil extract medium. *C. vulgaris* was preserved in the proteose medium. When scaling-up the culture to a larger volume, a volume of 10% of algal seed was used.

Swine wastewater was collected from a swine growing facility at the NC A&T farm, and used as the substrate to cultivate microalgae. Pretreatment of swine wastewater was carried out by sedimentation and filtration with a Whatman quantitative filter paper with 8 μm pore size to remove large, non-soluble particulate solids. After filtration the wastewater was autoclaved for 15 min at 121 °C. For all 1.5% agar media, 15 g of agar was added into 1 L of medium.

Proteose medium consists of the following ingredients: $NaNO_3$ (2.94 mM), $CaCl_2.2H_2O$ (0.17 mM), $MgSO_4.7H_2O$ (0.3 mM), K_2HPO_4 (0.43 mM), KH_2PO_4 (1.29 mM), NaCl (0.43 mM), and proteose peptone (1g L^{-1}). Soil extract medium consists of the following ingredients: $NaNO_3$ (2.94 mM), $CaCl_2.2H_2O$ (0.17 mM), $MgSO_4.7H_2O$ (0.3 mM), K_2HPO_4 (0.43 mM), KH_2PO_4 (1.29 mM), NaCl (0.43 mM), and soil water (40 mL L^{-1}).

Culture of Microalgae with Swine Wastewater

The autoclaved wastewater was used accordingly as a medium for algal cultivation, and three kinds of instruments were used to grow microalgae in this study. In the first case, the microalgal culture of 200 mL was stored in 250 mL bottles and kept at room temperature under daily natural light.

When comparing growth rates of *C. debaryana* in different media, microalgae were cultivated in 250-mL shake flasks containing 100 mL of medium and incubated in an AlgaeTron AG 130-ECO chamber with an orbital shaker (Photon Systems Instruments, Czech Republic) at 20 °C, 400 μmol $m^{-2}s^{-1}$, and 150 rpm.

To investigate the effects of environmental factors on microalgae growth, an AlgaeTron Multi-Cultivator MC 1000 photobioreactor (Photon Systems Instruments, Czech Republic) was used. It consists of eight 100-mL tubular reactors. The temperature of the reactors is controlled centrally, and each reactor is independently illuminated by an

array of white LEDs. Air is bubbled through each tube at the flow rate of 100 mL/min to provide CO_2 and increase the mass transfer rate. Typically, 72 mL of swine wastewater and 8 mL of seed microalgae suspension were introduced into each photobioreactor. In all cases, microalgae were cultured in the swine wastewater for 30 days with varying light intensities (300, 600, or 900 μmol $m^{-2}s^{-1}$). All experiments and analyses were performed in triplicate or duplicate. The statistical significance of the light intensity on the algal cell density was assessed using ANOVA F-test in Microsoft® Excel 2007 (Redmond, WA). The statistical significance level cut-off was chosen as $P = 0.05$.

Determination of Microalgae Growth and Nutrient Analysis

For each sampling, 5 mL of microalgal broth was collected from the photobioreactor or the shake flask, and centrifuged at 2600 × G and 20 °C for 15 min. To determine microalgae growth, the collected microalgal cells were dried at 50 °C until the sample reached equilibrium moisture content. Supernatants were separated to determine the nutrient removal from wastewater. Supernatants were filtered using a 0.45-mm nylon membrane filter. Then, the filtrates were appropriately diluted and analyzed for the chemical oxygen demand (COD), ammonia, and total phosphorus according to the Lamotte Smart 3 colorimeter manual (LaMotte 2011).

Lipid Extraction and Fatty Acid Methyl Ester (FAME) Content Analysis

Lipid extraction was performed at the end of each culture period. A modified Christie method (Christie 1993) was applied to quantify the amount of total lipid content. Approximately 0.1 to 0.2 g dried microalgae were weighed into clean screw-top glass tubes and 5 mL of a 2:1 chloroform-methanol (v/v) mixture was added. The tubes were incubated at 65 °C for 1 h. Afterwards, the mixture was centrifuged at 4000 × G for 10 min, the supernatant was collected, and the pellet was re-extracted following the same procedures until the supernatant became colorless. All supernatants were pooled and then evaporated by blowing nitrogen gas through the tube. The lipid was gravimetrically quantified.

Extracted algal lipids (~20 mg) were weighed into clean, 20-mL screw-top glass tubes, to which 4 mL of fresh solution of a mixture of methanol, concentrated sulfuric acid, and chloroform (1.7:0.3:2.0 v/v/v) was added. The bottles were closed tightly with Teflon tape to avoid leakage and then weighed. For transesterification, tubes were placed inside a heating block at temperatures of 90 °C and heated for 60 min. On completion of the reaction, the tubes were cooled to room temperature and weighed again to identify any leaking samples. Then, 1 mL of distilled water was added to the mixture, which was thoroughly vortexed for 1 min. After the formation of two phases, the lower phase containing FAME was transferred to a 1.5-mL gas chromatography vial (Indarti *et al.* 2005). Samples were stored in a freezer (-20 °C) until gas chromatography-mass spectrometry (GC-MS) analysis.

The chemical compositions of the liquid products were identified using an Agilent 7890A gas chromatograph/5975c mass spectrometer with a HP-5MS capillary column (Santa Clara, CA). The GC was programmed at 60 °C for 4 min and then increased at 10 °C/min to 280 °C, and finally held for 5 min. The injector temperature was 250 °C, and the injection size was 1 μL. The flow rate of the carrier gas (helium) was 1 mL/min. The ion source temperature was 230 °C for the mass selective detector. The compounds were identified by comparison with the NIST Mass Spectral Database (Zhang *et al.* 2006).

Elemental and Compositional Analysis of Microalgae

A Perkin-Elmer CHN/S analyzer (Waltham, MA) was used to analyze the elemental compositions of microalgal strains. The protein content of microalgae was determined by the Dumas method (Jung *et al.* 2003). The moisture content of the biomass was determined by the method of LAP #001 (Sluiter *et al.* 2008). The ash content of the biomass was determined using the method of LAP #005 (Zhang *et al.* 2012).

RESULTS AND DISCUSSION

Screening and Identification of the Algal Strains

Over two hundred strains (named S1, S2, *etc.*) were isolated from the lagoons at the NC A&T farm between October 2011 and November 2012. Among all isolated strains, eight green algal strains were found to accumulate higher amounts of neutral lipid and chlorophyll A than *C. vulgaris*, which is reported to have a lipid content of 15 to 17% w/w in dry biomass weight. One algal strain, S24 was chosen to be further characterized with light microscope observations and sequence analysis of ITS1, ITS2, and 5.8s rDNA regions. Microscopic examination demonstrated green spherical to oval cells 5 to 10 µm in diameter. Most cells showed morphology consistent with *Chlamydomonas* spp. in a palmaloid stage. Motile cells with two flagella were also observed. The DNA was successfully extracted, and PCR was performed using primers designed to amplify the 5.8s rDNA region and both internal transcribed spacer regions. Analysis was performed using the consensus sequence of a bidirectional sequence pair. The BLAST results comparing the sequenced region with sequences in the NCBI database showed the identified strain of S24 as most similar to *Chlamydomonas debaryana*. The sequence analysis results were consistent with the microscopic observations. The identified strained was renamed as *Chlamydomonas debaryana* AT24.

Growth in Wastewater and Synthetic Media

The swine wastewater collected in December 2012 was employed as a medium for algal cultivation. Swine wastewater contains high levels of nitrogen and phosphates, which may limit the growth of microalgal species. To study the effect of nutrient levels on the growth, the wastewater was diluted to four different concentrations (*i.e.*, 20%, 50%, 75%, and 100%). *C. debaryana* AT24 was inoculated into 250-mL bottles containing 200 mL wastewater. The cultivations were conducted at room temperature under daily natural light for 60 days, and the bottles were manually agitated at least once a day. The growth curves of *C. debaryana* are shown in Fig. 1. Under these culture conditions, microalgae grown in 25% wastewater showed the shortest lag phase, and entered into the stationary phase after 21 days. For the remaining three concentrations, microalgae started to grow exponentially after 7 days, and entered into the stationary phase after 50 days. The microalgae biomass yields were 0.26 ± 0.03 g/L, 0.48 ± 0.04 g/L, 0.58 ± 0.05 g/L, and 0.61 ± 0.07 g/L for 25%, 50%, 75%, and 100% swine wastewater, respectively.

Further, the growth rate of *C. debaryana* in the wastewater was compared with those in the proteose medium and the soil extract medium. For this purpose, microalgae were cultivated in shake flasks containing 100 mL of medium for 15 days and incubated in an AlgaeTron Chamber with an orbital shaker at 20 °C, 400 µmol $m^{-2}s^{-1}$, and 150 rpm.

The microalgal biomass yields were 1.62 ± 0.08 g/L, 1.44 ± 0.05 g/L, and 1.25 ± 0.03 g/L for the wastewater, proteose, and soil extract media, respectively.

The results of microalgal growth showed that diluting the wastewater may relieve the algal growth inhibition caused by high nutrient content and decrease the lag phase. However, adding more water into a wastewater treatment process is not practical, and lowering the content of nutrients will result in a lower biomass yield. The results from the incubator cultivation suggested that the microalgae growth rate is governed mainly by environmental factors. High biomass yield could be achieved by optimizing the operation parameters, and the swine wastewater could serve as an alternative medium for microalgae.

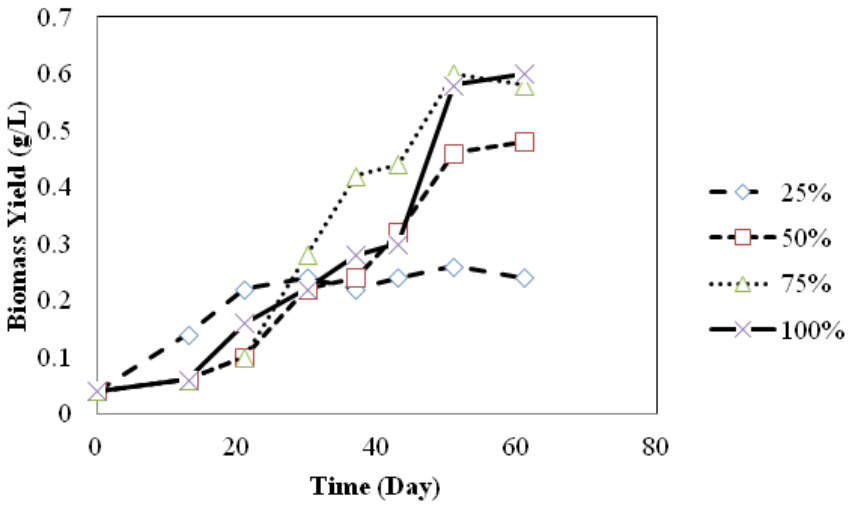

Fig. 1. Growth curves of *C. debaryana* AT24 grown at 20 °C under daily natural light in 250-mL bottles containing 200 mL of wastewater as medium

Removal of Nutrients from Swine Wastewater

Swine wastewater contains a considerable amount of nutrients, to include COD, nitrogen, phosphorus, copper, iron, chlorine, chromium, and sulfate. Microalgal growth reduced most nutritional contents of the wastewater except iron (Table 1). Figure 2 shows the concentration profiles of COD, ammonia, and phosphorous, and the pH profile during a typical 60-day culture in which *C. debaryana* AT24 was grown at 20 °C under daily natural light in 250-mL bottles containing 200 mL of wastewater as medium. The initial pH, concentrations of COD, ammonia, and phosphorous were 9.3, 2300 mg/L, 50.2 ppm, and 130 ppm, respectively.

The values of all four parameters decreased at a higher rate until the 20th day. After that, the COD, ammonia, and phosphorous contents decreased very slowly, while pH fluctuated. The final concentration of COD, ammonia, and phosphorous were 520 mg/L, 4.9 ppm, and 89.2 ppm, respectively. When compared with the microalgal growth curve in Fig 1, the initial nutrient consumption may mainly contribute to cell maintenance and facilitate microalgae adaptation to the new environment. The pH fluctuation between days 20 and 60 may indicate newly produced chemicals by algae, and/or chemical removal.

Table 1. Properties of Swine Wastewater Before and After a Typical 60-day Culture

Nutrients	Autoclaved swine wastewater	After cultivation
COD (mg/L)	2300	590
Ammonia (ppm)[a]	50.2	6.9
Total Phosphorous (ppm)	130	105
pH	9.3	8.3 to 8.5
Iron (ppm)	2.4	2.4
Copper (ppm)	0.4	0.1
Free chlorine (ppm)	0.2	0.05
Total chlorine (ppm)	1.1	0.1
Chromium (ppm)	4.1	2.1
Sulfate (ppm)	240	130

[a] The total nitrate content in the swine wastewater is negligible.

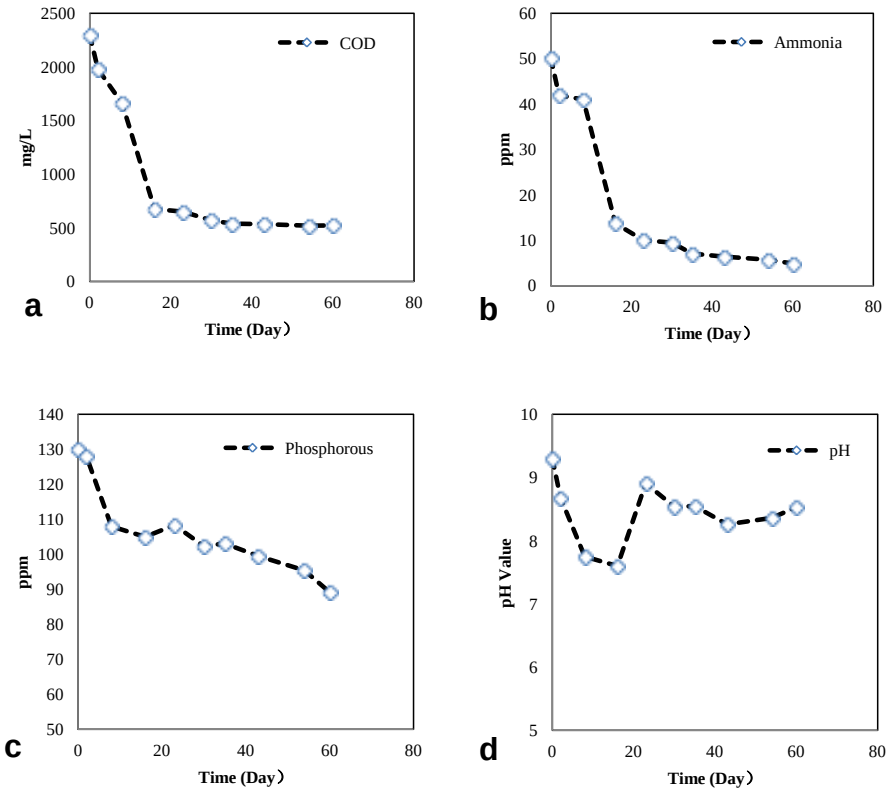

Fig. 2. The concentration profiles of (a) COD, (b) ammonia, (c) phosphorous, and (d) pH profile during a 60-day culture. *C. debaryana* AT24 was grown at 20 °C under daily natural light in 250-mL bottles containing 200 mL of wastewater as medium

When considering all cultivation conditions used in this study, if *C. debaryana* AT24 was grown to treat the swine wastewater, the removal efficiency of COD, ammonia, and phosphorous were 42 to 60%, 85 to 99%, and 32 to 50%, respectively. If combining the microalgal growth and nutrient removal, *C. debaryana* was able to utilize

1.3 to 1.6×10^3 mg COD/g biomass, 55 to 90 ppm ammonia/g biomass, and 48 to 89 ppm phosphorous/g biomass, respectively.

Effect of Light Intensity

To investigate the effect of light intensity on microalgae growth, *C. debaryana* AT24 was grown at 20 °C under varying light intensities in the AlgaeTron tubular photobioreactor for 30 days. The light intensity range of 300 to 900 μmol $m^{-2}s^{-1}$ was chosen because it is easily achieved in North Carolina. Moreover, light intensities of 600 μmol $m^{-2}s^{-1}$ and 900 μmol $m^{-2}s^{-1}$ were used to study the effect of photoinhibition on microalgae growth.

Figure 3 shows the growth curves of *C. debaryana* AT24 under different light intensities. Because the tubular photobioreactor could provide better mass transfer rates and high light intensity, the lag phase was shorter for all cultures. The final biomass yields after 30-day cultivation were 1.11 ± 0.11 g/L, 1.06 ± 0.33 g/L, and 1.15 ± 0.04 g/L for 300, 600, and 900 μmol $m^{-2}s^{-1}$, respectively. The P-values for the light intensity was 0.549. If a statistical significance level cut-off was chosen as P = 0.05, it is concluded that the light intensity was not a significant factor. The results also indicated that *C. debaryana* is not sensitive to photoinhibition. To further investigate the effect of light intensity on the saturation of photosynthesis in this species, a light intensity range of 50-1000 μmol $m^{-2}s^{-1}$ is chosen in our ongoing research.

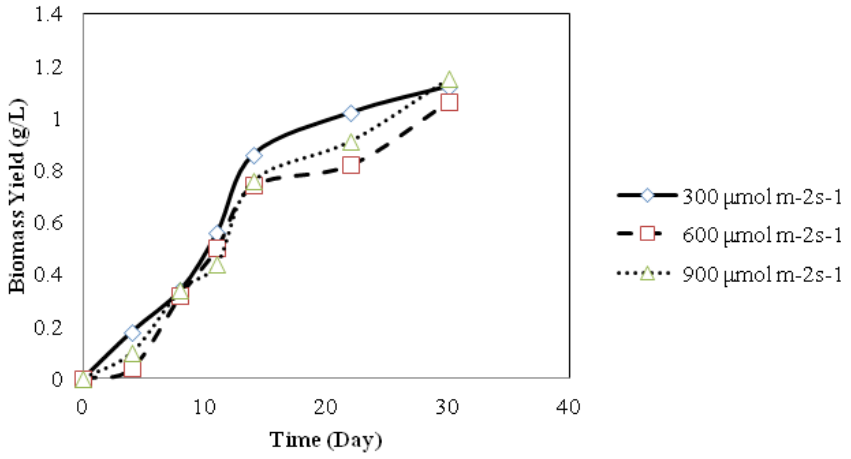

Fig. 3. Growth curves of *C. debaryana* AT24 under various light intensities

Lipid Extraction and Characterization

Microalgal lipids were extracted and characterized according to the methods described in the Experimental section, and the results of elemental and composition analysis of *C. debaryana* before/after extraction are listed in Table 2. The protein content and ash content of *C. debaryana* were approximately 59% and 7.9% of total dry weight, respectively. After the solvent extraction of lipids, the nitrogen content was reduced by over 50%, indicating that the protein degradation also happened during the extraction process.

The lipid content of *C. debaryana* AT24 was 19.9 ± 4.3% of the cell dry weight, while the lipid content of *C. vulgaris* was determined as 15.2 ± 3.9% of the cell dry weight. The components of microalgal oils (lipids) were determined by GC-MS analysis. The transesterified microalgal oil consisted of over 60 fatty acids ranging from C4 to C24. Approximately 70% of the total peak area was composed of methyl esters of

following 14 fatty acids: levulinic acid (C5), nonanoic acid (C9), nonanedioic acid (C10), tetradecanoic acid (C14), hexadecanoic acid (C16), heptadecanoic acid (C17), octadecanoic acid (C18), 6,9-octadecadienoic acid (C18), 9,12-octadecadienoic acid (Z,Z)- (C18), 9-cis,11-trans- octadecanoic acid (C18), nonadecanoic acid (C19), 2-octyl-cyclopropaneoctanoic acid (C19), eicosanoic acid (C20), and docosanoic acid (C22). Fatty acids with a carbon number distribution of 8 to 16 can then be refined into renewable jet fuel using conventional hydro-processing technology applied in petroleum refineries. The microalgal oil also contained a large number of unsaturated fatty acids, which could be used as sources of omega-3 and omega-6 fatty acids.

Table 2. Elemental and Composition Analysis of *C. debaryana* AT24 (Moisture Free Basis, % by Weight)

Composition	*C. debaryana* AT24	After lipid extraction
C	51.2	36.5
H	7.2	6.21
N	9.5	4.1
S	1.1	0.8
ash	7.9	10.1
protein	59.4	N/A

In addition, the polysaccharide content of this species is ~10% of the dry weight. The algal polysaccharides mainly consist of cellulose, hemicelluloses, and neutral polysaccharides, and are usually underutilized. The utilization of algal biomass is largely limited by its high water content, which is often over 85% (w/w) after harvesting. We are currently developing a green biorefinery approach (Xiu *et al.* 2014) to convert the algal polysaccharides to bioethanol.

CONCLUSIONS

1. In this study, the green microalgae *Chlamydomonas debaryana* was isolated from a local lagoon, screened for its lipid content using flow cytometry, further identified with light microscope observations and sequence analysis of ITS1, ITS2, and 5.8s rDNA regions, and named *C. debaryana* AT24.
2. When using swine wastewater as a medium for cultivation of the microalgae, the biomass yields were between 0.6 and 1.62 g/L, giving a median value of 1.11 g/L. By increasing mass transfer rates and providing sufficient light intensity, the microalgae growth was enhanced. The results indicated that *C. debaryana* was not sensitive to photoinhibition.
3. The growth of *C. debaryana* reduced most nutritional contents of the wastewater, except iron. The removal efficiency of COD, ammonia, and phosphorous were 42 to 60%, 85 to 99%, and 32 to 50%, respectively. When combining the microalgal growth and nutrient removal, *C. debaryana* was able to utilize 1.3 to 1.6×10^3 mg

COD/g biomass, 55 to 90 ppm ammonia/g biomass, and 48 to 89 ppm phosphorous/g biomass.

4. The lipid content of *C. debaryana* AT24 was 19.9 ± 4.3% of the cell dry weight. The transesterified microalgal oil mostly consisted of 14 fatty acids, ranging from C5 to C22, which hold high potential as a source of biofuels and food supplements.

ACKNOWLEDGMENTS

This publication was made possible by Grant Number NC.X-269-5-12-130-1 and NC.X2013-38821-21141 from the U.S. Department of Agriculture (USDA-NIFA). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institute of Food and Agriculture. The authors would like to thank Dr. David R. Nobles, Jr. at the University of Texas at Austin for assistance with microalgal strain identification.

REFERENCES CITED

Abdel-Raouf, N., Al-Homaidan, A. A., and Ibraheem, I. B. M. (2012). "Microalgae and wastewater treatment," *Saudi Journal of Biological Sciences* 19(3), 257-275. DOI: 10.1016/j.sjbs.2012.04.005

Becker, E. W. (1988). "Micro-algae for human and animal consumption," in: *Micro-Algal Biotechnology*, M. M. Borowitzka and L. J. Borowitzka (eds.), Cambridge University Press, Cambridge, pp. 222-256.

Blanca, G., Duncan, G., Agarkova, I., Borodovsky, M., Gurnon, J., Kuo, A., Lindquist, E., Lucas, S., Pangilinan, J., Polle, J., *et al*. (2010). "The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex," *Plant Cell* 22(9), 2943-2955. DOI: 10.1105/tpc.110.076406

Chevalier, P., Proulx, D., Lessard, P., Vincent, W. F., and de la Noüe, J. (2000). "Nitrogen and phosphorus removal by high latitude mat-forming cyanobacteria for potential use in tertiary wastewater treatment," *Journal of Applied Phycology* 12(2), 105-112. DOI: 10.1023/A:1008168128654

Christie, W. W. (1993). "Preparation of lipid extracts from tissues," in: *Advances in Lipid Methodology - Two*, W. W. Christie (ed.), Oily Press, Dundee, pp. 195-213.

Duong, V. T., Li, Y., Nowak, E., and Schenk, P. M. (2012). "Microalgae isolation and selection for prospective biodiesel production," *Energies* 5(6), 1835-1849. DOI: 10.3390/en5061835

García, J., Green, B. F., Lundquist, T., Mujeriego, R., Hernández-Mariné, M., and Oswald, W. J. (2006). "Long term diurnal variations in contaminant removal in high rate ponds treating urban wastewater," *Bioresource Technology* 97(14), 1709-1715. DOI: 10.1016/j.biortech.2005.07.019

Goff, L. J., and Moon, D. A. (1993). "PCR amplification of nuclear and plastid genes from algal herbarium specimens and algal spores," *Journal of Phycology* 29(3), 381-384. DOI: 10.1111/j.0022-3646.1993.00381.x

Hasan, R., Zhang, B., Wang, L., and Shahbazi, A. (2014). "Bioremediation of swine wastewater and biodiesel production by using *Chlorella vulgaris*, *Chlamydomonas*

reinhardtii, and *Chlamydomonas debaryana*," *Journal of Petroleum & Environmental Biotechnology* 5(3), 175. DOI: 10.4172/2157-7463.1000175

Indarti, E., Majid, M. I. A., Hashim, R., and Chong, A. (2005). "Direct FAME synthesis for rapid total lipid analysis from fish oil and cod liver oil," *Journal of Food Composition and Analysis* 18(2-3), 161-170. DOI: 10.1016/j.jfca.2003.12.007

Jarvis, E. E. (2008). "Aquatic species program (ASP): Lessons learned," *http://www.nrel.gov/biomass/pdfs/jarvis.pdf*. [Accessed on July 17, 2014]

Jung, S., Rickert, D. A., Deak, N. A., Aldin, E. D., Recknor, J., Johnson, L. A., and Murphy, P. A. (2003). "Comparison of kjeldahl and dumas methods for determining protein contents of soybean products," *Journal of the American Oil Chemists' Society* 80(12), 1169-1173. DOI: 10.1007/s11746-003-0837-3

LaMotte (2011) "Lamotte Smart 3 colorimeter manual," *http://www.geotechenv.com/Manuals/LaMotte_Manuals/smart3_colorimeter_operators_manual.pdf*. [Accessed on July 17, 2014]

Li, Y., Horsman, M., Wu, N., Lan, C. Q., and Dubois-Calero, N. (2008). "Biofuels from microalgae," *Biotechnology Progress* 24(4), 815-820. DOI: 10.1021/bp070371k

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Wolfe, J. (2008). "Determination of total solids in biomass and total dissolved solids in liquid process samples, laboratory analytical procedure," Technical Report NREL/TP-510-42621, *http://www.nrel.gov/docs/gen/fy08/42621.pdf*. [Accessed on July 17, 2014]

Woertz, I., Feffer, A., Lundquist, T., and Nelson, Y. (2009). "Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock," *Journal of Environmental Engineering* 135(11), 1115-1122. DOI: 10.1061/(ASCE)EE.1943-7870.0000129

Xiu, S., Shahbazi, A., and Boakye-Boaten, N. A. (2014). "Effects of fractionation methods on the isolation of fiber-rich cake from alfalfa and ethanol production from the cake," *BioResources* 9(2), 3407-3416. DOI: 10.15376/biores.9.2.3407-3416

Zhang, B. (2013). "New golden age of the algal technology," *Journal of Petroleum & Environmental Biotechnology* 4, e120. DOI: 10.4172/2157-7463.1000e120

Zhang, B., Carlson, R., and Srienc, F. (2006). "Engineering the monomer composition of polyhydroxyalkanoates synthesized in *Saccharomyces cerevisiae*," *Applied and Environmental Microbiology* 72(1), 536-543. DOI: 10.1128/AEM.72.1.536-543.2006

Zhang, B., Shahbazi, A., Wang, L., Whitmore, A., and Riddick, B. A. (2012) "Fermentation of glucose and xylose in cattail processed by different pretreatment technologies," *BioResources* 7(3), 2848-2859. DOI: 10.15376/biores.7.3.2848-2859

Article submitted: June 24, 2014; Peer review completed: August 9, 2014; Revised version received and accepted: August 12, 2014; Published: August 26, 2014.