Engineering in Life Sciences

www.els-journal.com

Athens Silaban[1]
Rong Bai[2]
M. Teresa Gutierrez-Wing[1]
Ioan I. Negulescu[3]
Kelly A. Rusch[1]

[1] Department of Civil and Environmental Engineering, Louisiana State University, Baton Rouge, LA, USA

[2] Cain Department of Chemical Engineering, Louisiana State University, Baton Rouge, LA, USA

[3] School of Human Ecology, Louisiana State University and Ag Center, Baton Rouge, LA, USA

Research Article

Effect of organic carbon, C:N ratio and light on the growth and lipid productivity of microalgae/cyanobacteria coculture

Current culture methods based on monocultures under phototrophic regimes are prone to contamination, predation, and collapse. Native cultures of multiple species are adapted to the local conditions and are more robust against contamination and predation. Growth, lipid and biomass productivity of a Louisiana native coculture of microalgae (*Chlorella vulgaris)* and cyanobacteria (*Leptolyngbya* sp.) in heterotrophic and mixotrophic regimes were investigated. Dextrose and sodium acetate at C:N ratios of 15:1 and 30:1 under heterotrophic (dark) and mixotrophic (400 μmol m^{-2} s^{-1}) regimes were compared with autotrophic controls. The carbon source and C:N ratio impacted growth and biomass productivity. Mixotrophic cultures with sodium acetate (C:N 15:1) resulted in the highest mean biomass productivity (156 g m^{-3} d^{-1}) and neutral lipid productivity (24.07 g m^{-3} d^{-1}). The maximum net specific growth rate (U) was higher (0.97 d^{-1}) in mixotrophic cultures with dextrose (C:N 15:1) but could not be sustained resulting in lower total biomass than in mixotrophic cultures with acetate (C:N 15:1), with a U of 0.67 d^{-1}. The ability of the Louisiana coculture to use organic carbon for biomass and lipid production makes it a viable feedstock for biofuels and bioproducts.

Keywords: Coculture / Lipid productivity / Microalgal productivity / Mixotrophic growth

Received: December 21, 2012; *revised:* March 12, 2013; *accepted:* May 5, 2013

DOI: 10.1002/elsc.201200219

1 Introduction

In the era of the uncertainty of fossil fuel sustainability and price instability, evidenced by the price fluctuation in the last 5 years from \$34 to \$145 bbl^{-1}, the interest in biofuels has increased. Cellulosic biofuels are projected to provide 61 billion liters of the 136 billion liters proposed by the U.S. EPA 2012 Renewable Fuel Standards 2. The remaining would come from other renewable sources. Microalgae (including cyanobacteria) are a promising feedstock for biofuels [1, 2]. Microalgae has several advantages, including their high productivity, capability of growing in non-productive land with various water sources, and the possibility of coproduction of biofuels and valuable coproducts without the use of food crops [2–6].

Although promising, the microalgae/cyanobacteria-based biofuel production cost is high. Biodiesel based on microalgal neutral lipids [3, 7, 8] has an estimated cost of \$3.21 L^{-1} with the current technologies according to an analysis by the U.S. Department of Energy (Document numbers ANL/ESD/12-4; NREL/TP-5100-55431; and PNNL-21437). The culture process is one of the main technical/economic obstacles in production of microalgal biofuels [6, 9]. One of the difficulties in large microalgal/cyanobacterial cultivation system is maintaining monocultures of the desired species in organisms other than extremophiles [10].

Several researchers have explored symbiotic relationships particularly between microalgae and bacteria [11–15] and microalgae with cyanobacteria [11, 14, 16]. Higher growth rates and nutrient uptake in mixed cultures has been reported [17, 18]. The exact mechanism of the microalgae and cyanobacteria interaction is currently unknown, but the benefits of this growth strategy could potentially be exploited for a sustained biomass production for biofuel.

In dense microalgal/cyanobacterial cultures, light may not be able to penetrate inside the cultures due to mutual shading by the cells [10, 19], impairing the growth of these photoautotrophic organisms. Some species overcome this limitation by utilizing organic carbon for heterotrophic growth in complete darkness or in mixotrophic regimes, using both CO_2 and organic carbon [19–23], a strategy that increases the growth rate and lipid productivity of the organisms [21, 24].

Correspondence: Dr. M.T. Gutierrez-Wing (mgutie5@lsu.edu), Department of Civil and Environmental Engineering Louisiana State University, Baton Rouge, LA 70803, USA.

Abbreviations: ANOVA, analysis of variance; **C:N ratio**, carbon to nitrogen ratio; **COD**, chemical oxygen demand; $\mathbf{P_v}$, Volumetric productivity; **U**, net specific growth rate

Significant increase in microalgal/cyanobacterial growth rate, biomass and lipid productivity with organic carbon addition, including glucose, dextrose, glycerol, and acetate has been observed and reported in literature [10, 20, 22, 24–29]. In the heterotrophic metabolism, the assimilation of organic carbon can be via multistep pathways such as aerobic glycolysis through the Embden–Meyerhof Pathway and Pentose Phosphate Pathway, and tricarboxylic acid cycle [10]. Glycerol is utilized in the Embden–Meyerhof Pathway pathway followed by the tricarboxylic acid cycle [30]. Meanwhile acetate is assimilated in a single-step catalyzed reaction to form acetyl-CoA [10, 30].

In this study, a Louisiana native microalgal/cyanobacterial coculture was tested for its ability to utilize organic carbon. This coculture was isolated from a local lake and proven to be resistant under extreme change of conditions such as pH, temperature (unpublished data). Although the reasons are still unclear, the growth of the coculture was higher than the same microalgae or cyanobacteria in monoculture. The objectives of this study were to: (i) Determine the ability of the Louisiana native microalgal/cyanobacterial coculture to be grown heterotrophically and mixotrophically, and to (ii) determine the effect of organic carbon addition on the growth, biomass productivity, lipid content and lipid productivity of the Louisiana native microalgal/cyanobacterial coculture.

2 Materials and methods

The effect of organic carbon addition (dextrose and sodium acetate) at two C:N ratios on the dry biomass concentration ($g\,m^{-3}$), biomass productivity (P_v), specific growth rate ($U; d^{-1}$), chlorophyll-a content in the biomass (mg [g biomass]$^{-1}$) and concentration in the culture ($mg\,m^{-3}$), lipid content (% of dry biomass) and lipid productivity ($g\,m^{-3}\,d^{-1}$) were investigated under dark (heterotrophic) and light (mixotrophic) growth conditions. The results were compared with those obtained in phototrophic cultures. This experiment provides baseline data for the Louisiana native microalgae/cyanobacteria investigated as a determinant of biotechnology application potential.

2.1 Microalgal species

The coculture examined was a mixture of a microalgal species and a cyanobacterial species referred to as the "Louisiana coculture" in this paper. The coculture was isolated from College Lake, Baton Rouge, Louisiana. Identification of the microalgae and the cyanobacteria by molecular methods was provided the UTEX Culture Collection of Algae. The microalgae were identified as *Chlorella vulgaris* and the cyanobacteria as *Leptolyngbya* sp. The composition of the coculture is approximately 97% *C. vulgaris* and 3% *Leptolyngbya* sp. by cell count based on flow cytometry.

The stock cultures of Louisiana coculture were maintained in Bold's Basal medium [31]. The stock cultures were maintained in 2 L Erlenmeyer flasks with 1 L volume of culture, under irradiance of 310 $\mu mol\,m^{-2}\,s^{-1}$ provided by 400 watt high-pressure sodium lamps. Temperature of 25 ± 2°C and continuous aeration at 0.47 L min^{-1} (1 SCFH) was maintained. Carbon dioxide was supplied daily (0.47 L min^{-1} for 1 min) to maintain the culture pH between 7 and 8. Approximately one-third of the stock culture was replenished weekly with Bold's Basal medium to maintain growth.

2.2 Experimental design

The batch experiment followed a randomized block three-factorial design (two light conditions × two organic carbon sources × two organic C:N ratios). The experiment was sequentially replicated three times. Two light conditions were tested: with continuous irradiance (light) and without irradiance (dark). The light conditions were used to test the possibility of mixotrophic (light) and heterotrophic (dark) growth of the Louisiana coculture. For the light conditions, continuous incident irradiance of 310 $\mu mol\,m^{-2}\,s^{-1}$ equivalent to the scalar irradiance of 400 $\mu mol\,m^{-2}\,s^{-1}$ was provided by 400 watt high-pressure sodium lamps. The selected irradiance was based on results of previous studies (unpublished data). Incident irradiance and scalar irradiance were measured using a Li-Cor LI-190 flat quantum sensor and a Li-Cor LI-193 spherical quantum sensor, respectively. The irradiance measurements were made in a specially prepared Erlenmeyer flask that allowed the positioning of the sensors at the surface of the culture for incident irradiance measurement, and completely submerged, just below the surface for the scalar irradiance measurement. The scalar irradiance was measured in the media without microalgae/cyanobacteria.

The two organic carbon sources tested were analytical grade dextrose (D-glucose) and sodium acetate. Dextrose and acetate were selected, as some authors [21, 24, 26, 32] report microalgal growth of several species including some from the genus *Chlorella* with these carbon sources. The organic C:N mass ratios tested were 15:1 and 30:1. These ratios are in the range reported in literature for mixotrophic microalgal growth [33, 34]. The nitrogen concentration was 41.16 $g\,m^{-3}$ as per the Bold's Basal medium. Dextrose concentrations of 1520 $g\,m^{-3}$ and 3040 $g\,m^{-3}$ and sodium acetate concentrations of 2120 $g\,m^{-3}$ and 4240 $g\,m^{-3}$ were added to obtain organic C:N mass ratios of 15:1 and 30:1 for each carbon source. The temperature was maintained at 25 ± 1°C by placing all of the treatment flasks in a circulating water bath. Previous to the start of the experiments, the inoculum was acclimated for 5 days to the initial experimental conditions for each treatment.

One-liter glass Erlenmeyer flasks were used for the cultures, capped with breathable stoppers (foam). The initial concentration of the Louisiana coculture was 100 $g\,m^{-3}$ with a culture volume of 600 mL. Aeration was maintained at 0.24 L min^{-1} (0.5 SCFH) for all treatments. No additional CO_2 (besides the atmospheric concentration) was added. Water loss by evaporation due to aeration was compensated by adding sterilized deionized water daily to maintain the original volumes. Cultures in Bold's Basal medium without organic carbon addition were maintained in light and dark conditions as controls.

2.3 Biomass productivity, net specific growth rate, and chlorophyll-a concentration

The growth rate of the Louisiana coculture was calculated from the dry biomass concentrations from each treatment flask measured every other day. Dry mass was measured from 5 mL

samples for each treatment and replicate. The measurements were done according to LSU Civil and Environmental Engineering Water Quality Lab SOP PA 200, adapted from Standard Method 2540D (APHA Standard Methods for the examination of Water and Wastewater, 22nd ed) with modification of the drying temperature (65°C) filter paper (Whatman GF/C, 1.2 μm pore size) and drying time (3 h) to avoid loss of volatile compounds. In a preliminary test, samples of the filtrate obtained following this method were analyzed by flow cytometry (Accuri™ C6) and showed no cell loss through the GF/C filter. The experiment ended when the culture reached stationary phase, determined by a null increase in biomass concentration in two consecutive measurements. The dry biomass concentrations were used to calculate daily volumetric productivity (P_v, g m^{-3} d^{-1}) and the net specific growth rate (U, d^{-1}). The decay rate was assumed negligible in this experiment as the cultivation time was relatively short. The daily volumetric productivity and specific growth rate presented is the mean value of the replicates.

Duplicate measurements of chlorophyll-a concentration (mg m^{-3}) for 10 mL of the initial and 5 mL of the final culture were made according to Standard Method 10200H (APHA Standard Methods for the Examination of Water and Wastewater, 22nd Ed.) using a Turner TD4000 fluorometer. The chlorophyll-a concentration provide information on the impact of organic carbon addition on the coculture's pigment production under different light conditions.

2.4 Chemical oxygen demand (COD)

Chemical oxygen demand (COD, g m^{-3}) was measured as a surrogate to the organic carbon in the sample. Measurements of COD were performed instead of total organic carbon since the process is relatively quicker than total organic carbon measurements. The COD can be calculated from the following Eq. [35]:

$$C_nH_aO_bN_c + \left(n + \frac{a}{4} - \frac{b}{2} - \frac{3c}{4}\right) O_2 \longrightarrow nCO_2 + \left(\frac{a}{2} - \frac{3c}{2}\right) H_2O + cNH_3 \quad (1)$$

For dextrose ($C_6H_{12}O_6$), Eq. (1) becomes:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O \quad (2)$$

And for sodium acetate ($NaCH_3COO$):

$$CH_3COO^- + 1.75O_2 \longrightarrow 2CO_2 + 1.5H_2O \quad (3)$$

Eqs. (2) and (3) can be used to estimate the initial COD of the culture media expected from the dextrose and sodium acetate concentrations added. The COD was measured every other day according to Standard Method 5220 (APHA Standard Methods for the Examination of Water and Wastewater, 22nd ed). Two mL samples from each experimental flask filtered through a 0.45-μm pore size nylon membrane filter were added to the HACH™ COD digestion vials and digested (HACH DR3800™) for 2 h at 150°C. The COD (g m^{-3}) concentration was determined by spectrophotometry (HACH DR/4000 UV/Vis) after cooling the vials to room temperature. The initial measured COD measurements were compared with the calculated values.

2.5 Lipid content and productivity

The lipids of the coculture were extracted using the Folch's extraction method [36] modified by treatment of polar solvent before the addition of the nonpolar solvent instead of a simultaneous addition of both solvents. This method was used as it lower energy cost and higher efficiency than the Bligh and Dyer [37] method in samples with lipid content >2% [38]. Samples with a volume of 100 mL of final culture were collected and centrifuged at a relative centrifugal force (rcf) of 1103 × g for 10 min. The biomass pellet was collected and methanol (polar solvent) was added at a ratio 6:1 v/v solvent:biomass. The mixture was agitated for 20 min at room temperature and chloroform (nonpolar solvent) was added at a ratio of 12:1 v/v solvent:biomass, for a chloroform:methanol ratio of 2:1 v/v. The solvent and biomass mixture was shaken for an additional 20 min. After agitation, one part per water per five parts of mixture was added. The resulting mixture was centrifuged at an rcf of 1103 × g for 5 min. The mixture separated into two phases. The top phase was discarded, and the interphase was rinsed with methanol:water 1:1 v/v. The bottom layer that contained the lipid of interest with a small amount loss due to rinsing [36] was collected and the solvent was evaporated with assistance of rotary evaporator and final dry nitrogen flow. The weight of the lipids extracted was used to determine lipid content and productivity.

2.6 Statistical analyses

Three-way ANOVA (analysis of variance) of the dry biomass and lipid concentration and productivity, specific growth rate and chlorophyll-a content was performed using SAS (version 9.3) to detect any significant difference due to light conditions, organic carbon sources, and organic C:N ratios. Post-ANOVA comparisons were done by least significant difference. The treatments were compared with the controls through t-tests. All statistics were based on a 95% confidence level and $p<0.05$ was considered statistically significant.

3 Results and discussion

3.1 Biomass productivity, net specific growth rate, and chlorophyll-a concentration

The Louisiana coculture demonstrated the capability to growth under dark (heterotrophic) and light (mixotrophic) regimes. Representative examples of growth curves for acetate, dextrose, and control are presented in Fig. 1. The control without organic carbon did not grow under dark conditions. Sodium acetate addition, promote heterotrophic growth at both organic C:N ratios tested (15:1 and 30:1). For the cultures with dextrose growth were observed at organic C:N ratio of 15:1 but not at organic C:N ratio of 30:1.

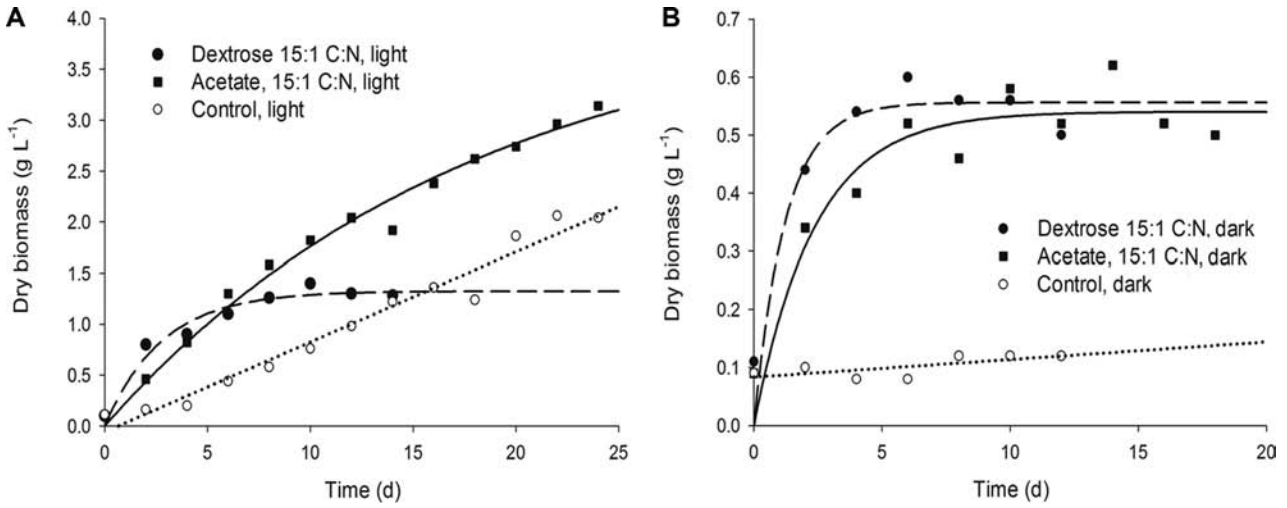

Figure 1. Representative growth curves of cultures. The examples represent treatments with dextrose and acetate at a 15:1 ratio of organic carbon: nitrate nitrogen and the controls with no organic carbon: (A) treatments under 400 μmol m^{-2} s^{-1}; (B) treatments in dark condition. The experiments were performed in triplicate following randomized three-factor design.

ANOVA analyses showed that light condition, organic carbon source, and organic C:N ratio had statistically significant effect on the final biomass concentration ($p = 0.002$, 0.0470, and 0.0018, respectively; Table 1). Cultures exposed to light/irradiance had higher final biomass concentrations compared to cultures in the dark. The organic carbon type and the organic C:N ratio also affected the final biomass concentration. Statistically significant lower growth and biomass concentration was observed for the cultures with dextrose at organic C:N 30:1. The highest average final biomass concentration of 2113 g m^{-3} was obtained with sodium acetate at organic C:N 15:1 under light (mixotrophic growth regime), but was not significantly higher than sodium acetate at organic C:N 30:1 under light or the control with no organic carbon due to the high variance of the replicate results (Table 1).

The cultures in dark conditions with organic carbon showed a higher final biomass concentration compared to the control, except the dextrose in the 30:1 organic C:N ratio (Table 1), demonstrating the capability of the Louisiana coculture for heterotrophic growth.

ANOVA results for the specific growth rate (Table 2) showed that the organic C:N ratio has a significant effect on the net specific growth rate ($p<0.0001$), but not the light condition and organic carbon source ($p = 0.0610$ and 0.2246, respectively). For the biomass productivity, both the organic carbon source and organic C:N ratio effects were significant ($p = 0.0109$ and 0.0090, respectively) but not the light condition ($p = 0.0894$). The net specific growth rate and biomass productivity of the coculture are of interest for the design of large scale continuous biomass production [39]. These two variables will impact the design, particularly flow rate, and harvest of the continuous flow systems.

The higher biomass productivity observed in mixotrophic (light) compared with heterotrophic (dark) cultures is consistent with the results reported by other authors in single species cultures [19, 21, 27]. The highest biomass productivity was obtained in mixotrophic culture with acetate 15:1 and 30:1. No net specific growth rate and biomass productivity increase was observed when the acetate organic C:N ratio was increased from 15:1 to 30:1. With dextrose, growth rate, and biomass productivity were inhibited with an increase of organic C:N ratio from 15:1 to 30:1. The inhibition of microalgal growth has been reported by several authors when organic carbon was supplied in high concentration [19, 32, 34, 40]. With higher concentrations of organic carbon, the ATP demand for organic carbon assimilation may inadvertently limit the photosynthetic carbon reduction resulting in growth inhibition [40]. Chen [32] suggested that the high sodium concentration may be a factor in the inhibition when using sodium acetate.

Mixotrophic cultures with sodium acetate at organic C:N 15:1 and 30:1 and dextrose 15:1 showed a higher specific growth rate than the control under light, with a maximum value for dextrose 15:1 (Table 2). This demonstrates possibility of the Louisiana coculture to grow in mixotrophic conditions. Although the maximum specific growth rate was higher for the treatments with organic carbon, their biomass productivity was not statistically different with the light control. This may be due to a fast consumption of the carbon that did not sustain the high growth rates for long periods of time. For heterotrophic cultures, significantly higher maximum net specific growth rate and biomass productivity compared to dark control with sodium acetate and dextrose 15:1 but not for the 30:1 treatments.

The comparison of total dry biomass and net specific growth rate from this work with values reported in literature are represented in Table 3. Literature values vary widely, but all report significant increase of total dry biomass and growth rate with the addition of organic carbon [19–23, 27], with glucose as the most common source. The result from this experiment shows a higher total dry biomass with acetate as the organic carbon compared to dextrose (D-glucose). Some authors report higher total dry biomass than the results obtained in this work with higher glucose concentrations, for example, Xie et al. [23] (3680 g m^{-3} with 24 g L^{-1} glucose) and Heredia-Arroyo et al.

Table 1. Final biomass concentration* (g m^{-3}) of the Louisiana coculture at different light condition, organic carbon source, and organic C:N ratios and controls at each light condition

Light condition	Organic carbon	Organic C:N ratio	*p*-Value for final biomass	Final biomass concentration* (g m^{-3})	Comparison with control***
**Final biomass concentration for control, light = 1580 ± 530 g m^{-3}*					
Light	Acetate	15:1	0.4397	2113 ± 938[a]	*NS*
Light	Acetate	30:1	0.8897	1153 ± 92[ab]	*NS*
Light	Dextrose	15:1	0.4034	1293 ± 42[b]	*NS*
Light	Dextrose	30:1	0.0096	153 ± 31[d]	*Lower, S*
**Final biomass concentration for control, dark = 140 ± 20 g m^{-3}*					
Dark	Acetate	15:1	0.0011	433 ± 58[c]	*Higher, S*
Dark	Acetate	30:1	0.0003	440 ± 40[c]	*Higher, S*
Dark	Dextrose	15:1	0.0014	433 ± 61[c]	*Higher, S*
Dark	Dextrose	30:1	0.7247	133 ± 23[c]	*NS*

*Significant difference (S) is detected when *P*-value is <0.05.
**Mean ± standard error. Different superscript letters represent significant difference ($p<0.05$) among treatments.
***NS = Not significant, S = significant.

Table 2. Net specific growth rate* (U, d^{-1}) and biomass productivity* (P_v, g m^{-3} d^{-1}) of the Louisiana coculture at different light condition, organic carbon, and organic C:N ratio levels. The light and dark controls were maintained with no organic carbon

Light condition	Organic carbon	Organic C:N ratio	Maximum net specific growth rate (U, d^{-1})	Volumetric biomass productivity (g m^{-3} d^{-1})
Light	*Control*	—	*0.23 ± 0.02*	*91 ± 30*
Light	Acetate	15:1	0.67 ± 0.10[b]$_1$	134 ± 19[a]$_2$
Light	Acetate	30:1	0.56 ± 0.08[b]$_1$	136 ± 11[a]$_2$
Light	Dextrose	15:1	0.97 ± 0.06[a]$_1$	113 ± 15[ab]$_2$
Light	Dextrose	30:1	0.03 ± 0.01[d]$_1$	4 ± 2[d]$_1$
Dark	*Control*	—	*0.14 ± 0.07*	*19 ± 6*
Dark	Acetate	15:1	0.54 ± 0.11[b]$_1$	66 ± 1[c]$_1$
Dark	Acetate	30:1	0.34 ± 0.15[c]$_2$	66 ± 51[c]$_2$
Dark	Dextrose	15:1	0.66 ± 0.05[b]$_1$	93 ± 15[bc]$_1$
Dark	Dextrose	30:1	0.02 ± 0.01[d]$_2$	3 ± 2[d]$_1$

*Mean ± SD. Different superscript letters represent significant difference ($p<0.05$) among treatments. Subscript numbers represent the results of the *t*-tests between the control and the treatments: 1 = statistically significant difference; 2 = no statistically significant difference. The lines in italics represent the control for each light condition.

[22] (4000 g m^{-3} with 40 g L^{-1} glucose), compared with 1.5 g L^{-1} of dextrose (1293 g m^{-3}). The Louisiana coculture had higher total dry biomass (2110 g m^{-3} with 2.1 g L^{-1} of sodium acetate) compared to the result by Liang et al. [19] with *C. vulgaris* (987 g m^{-3} with 10 g L^{-1} acetate) and higher biomass productivity (134 g m^{-3} d^{-1} compared to 87 g m^{-3} d^{-1}). The net specific growth rates from this experiment are comparable to those obtained by Garcia et al. [21] and Heredia-Arroyo et al. [22] (Table 3). The growth rate and productivity results show that the Louisiana coculture can use dextrose and acetate for mixotrophic growth.

The chlorophyll-a concentration (Table 4) was measured as indicator of the physiological state of the coculture. No analyses were done for the control under dark and with dextrose 30:1 as little to no growth were observed in this treatment. There chlorophyll-a concentration and content of mixotrophic cultures with acetate 30:1 and dextrose 15:1 had lower values compared to the control under light. The treatment of acetate 15:1 had no significant difference with the light control. Under heterotrophic conditions, a significant increase in chlorophyll-a content with sodium acetate 15:1, 30:1 and dextrose 15:1 addition compared to control under light (phototrophic) was observed. Although the chlorophyll-a pigments are produced to capture light energy during autotrophic growth, the production increases in low light [41]. Contrary to the results of this work, some authors have found a decrease of the chlorophyll content in the dark [42] after 1 or 2 days for several species. Grant and Hommersand [43] found that *Chlorella protothecoides* cells were "bleached" yellow after transferring from autotrophic to heterotrophic conditions, and proposed that chlorophylls are catabolically degraded and result in excretion of phycoerythrin. The differences observed in the coculture may result from the capability of the cyanobacteria to can survive and grow in lower light conditions [44]. Some microalgal and cyanobacterial species can produce large quantities of other photosynthetic pigments such as lutein [45], astaxanthin [46], and phycocyanin [33, 47] under low light conditions.

Table 3. Comparison of other microalgae growth experiments with organic carbon addition

Species	Condition	Organic carbon	Cultivation days	Net Specific growth rate (U, d^{-1})	Reference
Platymonas subcordiformis	Mixotrophic, 95 μmol m^{-2} s^{-1} in 5-L bioreactor	Glucose, 24 g L^{-1}	14	0.55	[23]
Nannochlorop-sis sp.	Mixotrophic, 73 μmol m^{-2} s^{-1} in 250-mL Erlenmeyer flask	Glucose, 5.4 g L^{-1}	8	0.30	[27]
Phaeodactylum tricornutum	Mixotrophic, 165 μmol m^{-2} s^{-1} in 1-L Erlenmeyer flask	Glucose, 5 g L^{-1}	21	0.96	[21]
Spirulina platensis	Mixotrophic, 45.5 μmol m^{-2} s^{-1} in 2-L Erlenmeyer flask	Molasses, 0.75 g L^{-1}	25	0.064	[20]
Chlorella vulgaris	Mixotrophic, fluorescent light in 1-L bottle	Acetate, 1% w/v	12	0.37	[19]
		Glucose, 1% w/v	12	0.25	
Chlorella protothecoides	Mixotrophic, fluorescent light in 250-mL flask	Glucose, 15 g L^{-1}	6	0.96	[22]
Louisiana Coculture	Mixotrophic, 400 μmol m^{-2} s^{-1}	Acetate, 2.1 g L^{-1}	20	0.67	This work
		Dextrose, 1.5 g L^{-1}	12	0.97	

Table 4. Chlorophyll-a concentration* (mg m^{-3}) and content* (mg (g biomass)$^{-1}$) of the Louisiana coculture for all experimental treatments. The control was maintained in light and with no organic carbon added

Light condition	Organic carbon	Organic C:N ratio	Chlorophyll-a concentration (mg m^{-3})	Chlorophyll-a content (mg [g biomass^{-1}])
Light	Acetate	15:1	$2179 \pm 1724^{c,d}{}_2$	$1.0 \pm 0.9^{d}{}_2$
Light	Acetate	30:1	$854 \pm 854^{de}{}_1$	$0.5 \pm 0.5^{d}{}_1$
Light	Dextrose	15:1	$186 \pm 30^{e}{}_1$	$0.1 \pm 0.0^{d}{}_1$
Dark	Acetate	15:1	$4593 \pm 2{,}163^{b}{}_2$	$11.8 \pm 2.2^{b}{}_1$
Dark	Acetate	30:1	$3533 \pm 498^{bc}{}_2$	$8.1 \pm 1.6^{c}{}_1$
Dark	Dextrose	15:1	$7035 \pm 970^{a}{}_1$	$16.3 \pm 2.2^{a}{}_1$

*Mean $\pm$ standard error. Different superscript letters represent significant difference ($p<0.05$) among treatments. Subscript numbers represent the results of the *t*-tests between the control and the treatments: 1 = statistically significant difference; 2 = no statistically significant difference.

These pigments can represent a source of valuable compounds that could potentially reduce the economic pressure in the production of microalgal biofuels.

3.2 COD

The measured initial COD (Table 5) had no significant difference compared to the initial COD calculated from the amount of organic carbon added ($p = 0.170$), showing that COD is a good estimate of the organic carbon in the microalgal culture.

For the culture with dextrose at a ratio C:N of 15:1, the COD was exhausted early in the culture (day 2–4) for both heterotrophic and mixotrophic culture. With dextrose addition, the COD consumption rate for mixotrophic was 697 g m^{-3} d^{-1} (the first 2 days) and for heterotrophic was 311 g m^{-3} d^{-1} (the first 4 days). The higher COD consumption rate in the early days might coincide with a high specific growth rate that was achieved by mixotrophic culture with dextrose addition. This result are opposite of reported in literature, where dextrose metabolic flux was higher in dark compared to light conditions [10]. These apparent contradictions may be because the irradiance level effect is lower for cyanobacteria [48]. Cyanobacteria are part of the Louisiana coculture.

The COD of heterotrophic culture with sodium acetate (for both C:N 15:1 and 30:1) was also exhausted in the early stage of the culture. The COD consumption rate for heterotroph culture with sodium acetate C:N 15:1 was 323 g m^{-3} d^{-1} (the first 4 days) and for C:N 30:1 COD consumption rate was 438 g m^{-3} d^{-1} (the first 4 days). Meanwhile, for the mixotrophic culture with sodium acetate addition, both C:N 15:1 and 30:1 had lower but steadier COD consumption throughout the cultivation duration. Mixotrophic culture with sodium acetate C:N 15:1 had a COD consumption rate of 67 g m^{-3} d^{-1} (for 14 days), and cultures with sodium acetate C:N 30:1 had a COD consumption rate of 108 g m^{-3} d^{-1} (for 20 days). These results indicate that although the Louisiana coculture can use sodium acetate for growth, the

Table 5. Comparison between the initial COD values calculated from Eqs. (2) and (3) and from the actual measurement by the digestion method

Organic carbon source added	Organic carbon (g m^{-3})	Initial COD (g m^{-3})	
		Calculated	Actual*
1520 g m^{-3} Dextrose (C:N 15:1)	617	1646	1458 ± 25
3040 g m^{-3} Dextrose (C:N 30:1)	1235	3292	2971 ± 55
2120 g m^{-3} Sodium acetate (C:N 15:1)	617	1441	1380 ± 96
4240 g m^{-3} Sodium acetate (C:N 30:1)	1235	2882	2793 ± 166

*Mean ± SD.

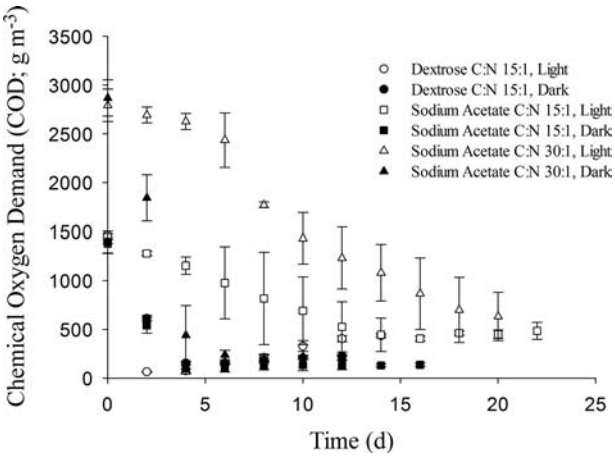

Figure 2. Chemical oxygen demand (COD) versus time for dextrose and acetate as organic carbon sources at C:N ratios of 15:1 and 30:1. The experiments were performed in triplicate following randomized three-factor design. Results for light (400 μmol m^{-2} s^{-1}) and dark conditions in triplicate are presented. COD values decrease with time indicating that the organic carbon is being consumed by the microalgae/cyanobacteria coculture.

metabolic cost may be higher than with dextrose, shifting the preferred carbon source to CO_2.

In this experiment, organic carbon source was added only at the start of the experiment and was exhausted early (Fig. 2), especially with the addition of dextrose. The high growth rates observed in the initial stages of the culture suggest that a steady supply of organic carbon, instead of only an initial dose could maintain the culture growth rate for longer periods. Continuous organic carbon dosing has been used for the heterotrophic growth of microalgae [47, 49, 50] showing improved total dry biomass production and growth rate. The objective of fed-batch culture systems, where the substrate (in this case organic carbon) is dosed after the initial substrate is consumed to maximize the growth and biomass of microalgae while avoiding the inhibition that can occur when organic carbon is present in high concentrations at the start of the culture. In this experiment, addition of sodium acetate at ratio C:N 30:1 resulted in lower total dry biomass and growth rate compared to the 15:1 ratio, indicating that some inhibition occurred when sodium acetate was added at higher amounts. With fed-batch culture, sodium acetate can be added at lower amounts during a period of time, thus preventing inhibition that could occur. Adding organic carbon at lower amounts could also prevent bacterial contamination due to the availability of organic matter for heterotrophs. In some microalgal cultures grown with a light/dark cycle, organic carbon are added only during daytime hours, as bacteria could grow faster compared to microalgae under dark heterotrophic conditions [51, 52].

Finding organic carbon sources that are available for little or no cost can reduce the overall cost of mixotrophic/heterotrophic cultivation. Municipal wastewater, which contains organic carbonaceous matter, can be utilized for the growth of mixotrophic microalgae and has been reported in various research articles [53–56]. The COD amount of wastewater varies between sources. For comparison, the synthetic wastewater regulated by ASTM has COD value of 3500 g m^{-3}, the primary effluent collected from Baton Rouge south wastewater treatment plant in November 2010 has a COD value of 1865 g m^{-3}, and the primary effluent collected from Saint Paul (Minnesota) metropolitan wastewater treatment plant has COD value of 224 g m^{-3} [55]. Further investigation for the growth of Louisiana coculture in wastewater effluent containing organic carbon should be done. If the Louisiana coculture is able to utilize organic carbon in the wastewater effluent it will be more interesting as it combines two processes together, the coculture cultivation concurrently with wastewater treatment. A preliminary experiment was performed to investigate the ability of the Louisiana coculture to grow in the primary and final effluent collected from Baton Rouge South wastewater treatment plant. The preliminary results showed that the Louisiana coculture cultivated in the primary and final effluent was able to achieve growth and final biomass concentration similar to culture in Bold's Basal medium.

3.3 Lipid content and productivity

Based only on the biomass productivity, addition of organic carbon to the Louisiana coculture is not justified as mixotrophic growth did not yield significantly higher biomass productivity compared to phototrophic growth. To determine if the extra cost is justified, the lipid content and productivity were calculated and compared with those obtained in phototrophic growth (Table 6). No analysis was done for the control in the dark and dextrose 30:1 as no sufficient biomass was available to perform the extraction in biomass for this treatment, as the culture did not grow. The lipid content for culture with dextrose C:N 15:1 was not significantly different than the control under light for

Table 6. Lipid content* and productivity* of the Louisiana coculture at different light condition, organic carbon, and organic C:N ratio levels. The control was maintained in light and with no organic carbon added

Light condition	Organic carbon	Organic C:N ratio	Lipid content (% of dry mass)	Lipid productivity ($g\ m^{-3}\ d^{-1}$)
Light	*Control*	—	*8.67 ± 0.93*	*7.7 ± 2.2*
Light	Acetate	15:1	$18.20 \pm 3.42^{a}{}_{1}$	$24.1 \pm 2.3^{a}{}_{1}$
Light	Acetate	30:1	$13.13 \pm 3.21^{b}{}_{2}$	$18.0 \pm 5.1^{b}{}_{1}$
Light	Dextrose	15:1	$8.67 \pm 0.21^{c}{}_{2}$	$9.8 \pm 1.1^{c}{}_{2}$
Dark	Acetate	15:1	$19.60 \pm 2.29^{a}{}_{1}$	$12.9 \pm 1.6^{bc}{}_{1}$
Dark	Acetate	30:1	$13.59 \pm 2.70^{b}{}_{1}$	$9.2 \pm 7.4^{c}{}_{2}$
Dark	Dextrose	15:1	$8.77 \pm 0.12^{c}{}_{2}$	$8.2 \pm 1.3^{c}{}_{2}$

*Mean ± standard error. Different superscript letters represent significant difference ($p<0.05$) among treatments. Subscript numbers represent the results of the *t*-tests between the control and the treatments: 1 = statistically significant difference; 2 = no statistically significant difference.

both mixotrophic and heterotrophic growth regime. Meanwhile with sodium acetate, the lipid content had significantly higher values for both mixotrophic and heterotrophic growth regime and C:N 15:1 and 30:1 (13.13 to 19.60% of dry biomass) compared to the control under light (8.67%). No significant difference was detected between the C:N ratios for acetate, with the lipid content at C:N 15:1 (18.90% average) higher than at C:N 30:1 (13.36% average). The lipid productivity was significantly increased with sodium acetate 15:1 under mixotrophic condition to 24.1 g m^{-3} d^{-1}, which was 3.1 times higher than the control under light. However, no difference was detected for mixotrophic cultures with dextrose 15:1 and control under light.

Increase of lipid content has been reported for several microalgae species with addition of organic carbon including such as *Nannochloropsis* sp. with glucose and ethanol [27], *C. vulgaris* with glycerol [19], or *Chlorella protothecoides* with acetate [22]. One of the theories for mixotrophic growth is that the energy requirements for cell growth are mostly met by the photosynthetic process by fixing inorganic carbon, leaving the excess organic carbon in the medium to be directed more toward intracellular lipid storage [57]. Based on this experiment, addition of sodium acetate resulted in higher lipid content compared to the addition of dextrose. The metabolism of acetyl-CoA might be the key of the increase of fatty acid content that is the basic building block of fatty acid synthesis. Acetate can be directly activated to acetyl-CoA by a one-step reaction catalyzed by acetyl-CoA synthase, while glucose/dextrose needs several steps to reach the same point [10, 40]. The dextrose consumed by the coculture may not be used for fatty acid synthesis, as Tanner [58] reports that more than 85% of glucose assimilated by microalgae is converted to sucrose and polysaccharides (mainly starch).

As the mixotrophic culture with sodium acetate addition enhanced the lipid content and productivity, it is the choice of organic carbon for the production of the Louisiana coculture as a biodiesel feedstock. Although the cost of microalgae-based biodiesel production depends on various factors such as infrastructure, nutrients, harvesting, oil extraction, etc., the cultivation component of the cost could be reduced by utilizing relatively inexpensive and available agro-industrial coproducts containing organic carbon (including acetate) that contributes in enhancing the lipid production of the Louisiana native coculture. Increased lipid content can also reduce the extraction cost as more lipids will be obtained in the same process.

4 Concluding remarks

This study demonstrated the capability of the Louisiana native coculture to grow in either heterotrophic or mixotrophic regime with the addition of dextrose or sodium acetate. However, only mixotrophic growth with sodium acetate addition at C:N 15:1 had higher biomass productivity (134 g m^{-3} d^{-1}) and lipid productivity (24.07 g m^{-3} d^{-1}) compared to autotrophic growth of the control (1.47 and 3.10 times, respectively). The Louisiana coculture lipid content was also significantly higher for mixotrophic growth with sodium acetate addition (18.20%) compared to autotrophic growth (8.67%). Thus, based on this experiment, mixotrophic growth with sodium acetate (C:N 15:1) is the preferred cultivation conditions for the Louisiana coculture for lipid production.

The COD available is consumed faster in heterotrophic cultures than in mixotrophic cultures. In mixotrophic cultures, the COD in the microalgal cultures with sodium acetate was consumed steadier throughout the cultivation duration compared to the microalgal cultures with dextrose that are exhausted at the earlier culture phase. A daily dosing of organic carbon (batch-fed) may be better than adding a single dose of organic carbon at the beginning of the cultivation to minimize contamination and exhaustion of the organic carbon source. It could also prevent inhibition of culture growth as experienced when sodium acetate was increased from C:N 15:1 to C:N 30:1.

Further analysis on the ability of the Louisiana coculture growth in the presence of inexpensive and readily available agro-industrial coproducts as organic carbon source in larger scale and continuous production should be executed as an opportunity to reduce the cost of the Louisiana native coculture cultivation for biodiesel production.

This work was funded through the National Science Foundation Energy for Sustainability Program, award number 0853483 and a Sun Grant DOT-RITA Seed grant. The authors will like to thank Marjan Mohtashamian and Todd Crawford for their help in the laboratory.

The authors have declared no conflict of interest.

Practical application

The use of native cocultures in mixotrophic regimes for the production of biofuels and bioproducts can overcome some of the problems observed in traditional monocultures. Mixed species cultures (cocultures) can have higher resistance to contamination and can use a wider range of resources compared with monocultures. The use of mixed species cocultures native to the production area reduces the risks associated with nonindigenous species. The coculture studied is capable of photoautotrophic and mixotrophic growth, thereby, opening the door for the use of organic carbon waste streams as a supplement to CO_2 injection.

5 References

[1] Li, Y., Horsman, M., Wu, N., Lan, C. Q. et al., Biofuels from Microalgae. *Biotechnol. Prog.* 2008, *24*, 815–820.
[2] Parmar, A., Singh, N. K., Pandey, A., Gnansounou, E. et al., Cyanobacteria and microalgae: A positive prospect for biofuels. *Bioresour. Technol.* 2011, *102*, 10163–10172.
[3] Chisti, Y., Biodiesel from microalgae. *Biotechnol. Adv.* 2007, *25*, 294–306.
[4] Pienkos, P. T., Darzins, A., The promise and challenges of microalgal-derived biofuels. *Biofuels, Bioprod. Bior.* 2009, *3*, 431–440.
[5] Gouveia, L., Microalgae as a feedstock for biofuels. (Ed.), *Springer Briefs in Microbiology*, Springer, Heidelberg 2011, pp. 1–68.
[6] Brennan, L., Owende, P., Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. *Renew. Sust. Energy Rev.* 2010, *14*, 557–577.
[7] Fukuda, H., Kondo, A., Noda, H., Biodiesel fuel production by transesterification of oils. *Soc. Biotechnol. Jpn.* 2001, *92*, 405–416.
[8] Meher, L. C., Sagar, D. V., Naik, S. N., Technical aspects of biodiesel production by transesterification—a review. *Renew. Sustain. Energy Rev.* 2006, *10*, 248–268.
[9] Larkum, A. W. D., Ross, I. L., Kruse, O., Hankamer, B., Selection, breeding and engineering of microalgae for bioenergy and biofuel production. *Trends Biotechnol.* 2012, *30*, 198–205.
[10] Perez-Garcia, O., Escalante, F. M. E., de-Bashan, L. E., Bashan, Y., Heterotrophic cultures of microalgae: Metabolism and potential products. *Water Res.* 2011, *45*, 11–36.
[11] de-Bashan, L. E., Schmid, M., Rothballer, M., Hartmann, A. et al., Cell-cell interaction in the eukaryote-prokaryote model of the microalgae *Chlorella vulgaris* and the bacterium *Azospirillum brasilense* immobilized in polymer beads. *J. Phycol.* 2011, *47*, 1350–1359.
[12] Ueda, H., Otsuka, S., Senoo, K., Community composition of bacteria co-cultivated with microalgae in non-axenic algal cultures. *Microbiol. Cult. Coll.* 2009, *25*, 21–25.
[13] Park, Y., Je, K.-W., Lee, K., Jung, S.-E. et al., Growth promotion of *Chlorella ellipsoidea* by co-inoculation with *Brevundimonas* sp. isolated from the microalga. *Hydrobiologia* 2008, *598*, 219–228.
[14] Imase, M., Watanabe, K., Aoyagi, H., Tanaka, H., Construction of an artificial symbiotic community using a *Chlorella*-symbiont association as a model. *FEMS Microbiol. Ecol.* 2008, *62*, 273–282.
[15] Gonzales, L. E., Bashan, Y., Increased growth of the microalga *Chlorella vulgaris* when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium *Azospirillum brasilense*. *Appl. Environ. Microbiol.* 2000, *66*, 1527–1531.
[16] Graham, L. E., Wilcox, L. W. (Ed.), *Algae* Prentice Hall, Upper Saddle River 2000, pp. 60–139.
[17] Cheirsilp, B., Suwannarat, W., Mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* for lipid production from industrial wastes and its use as biodiesel feedstock. *New Biotechnol.* 2011, *110*, 510–516.
[18] Silva-Benavides, A. M., Torzillo, G., Nitrogen and phosphorus removal through laboratory batch cultures of microalga *Chlorella vulgaris* and cyanobacterium *Planktothrix isothirx* grown as monoalgal and as co-cultures. *Appl. Phycol.* 2012, *24*, 267–276.
[19] Liang, Y., Sarkany, N., Cui, Y., Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnol. Lett.* 2009, *31*, 1043–1049.
[20] Andrade, M. R., Costa, J. A. V., Mixotrophic cultivation of microalga *Spirulina platensis* using molasses as organic substrate. *Aquaculture* 2007, *264*, 130–134.
[21] Garcia, M. C. C., Miron, A. S., Sevilla, J. M. F., Grima, E. M. et al., Mixotrophic growth of the microalga *Phaedactylum tricornutum* influence of different nitrogen and organic carbon sources on productivity and biomass composition. *Process Biochem. (Amsterdam Neth.)* 2005, *40*, 297–305.
[22] Heredia-Arroyo, T., Wei, W., Hu, B., Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides*. *Appl. Biochem. Biotechnol.* 2010, *172*, 1978–1995.
[23] Xie, J., Zhang, Y., Li, Y., Wang, Y., Mixotrophic cultivation of *Platymonas subcordiformis*. *J. Appl. Phycol.* 2001, *13*, 343–347.
[24] Liu, X., Duan, S., Li, A., Xu, N. et al., Effects of organic carbon sources on growth, photosynthesis, and respiration of *Phaedactylum tricornutum*. *Appl. Phycol.* 2009, *21*, 239–246.
[25] Chen, F., Johns, M. R., Effect of C/N ratio and aeration on the fatty acid composition of heterotrophic *Chlorella sorokiniana*. *J. Appl. Phycol.* 1991, *3*, 203–209.
[26] Yang, X., Zeng, X., Ji, Y., Liu, Q., Effects of sodium nitrate and sodium acetate concentrations on the growth and fatty acid composition of *Brachiomonas submarina*. *Ocean Univ. Qingdo* 2003, *2*, 75–78.
[27] Fang, X., Wei, C., Zhao-Ling, C., Fan, O., Effects of organic carbon sources on cell growth and eicosapentaenoic acid content of *Nannochloropsis* sp. *Appl. Phycology* 2004, *16*, 499–503.
[28] Fujita, T., Aoyagi, H., Ogbonna, J. C., Tanaka, H., Effect of mixed organic substrate on α-tocopherol production by *Euglena gracilis* in photoheterotrophic culture. *Appl. Microbiol. Biotechnol.* 2008, *79*, 371–378.
[29] Xu, H., Miao, X., Wu, Q., High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. *Biotechnology* 2006, *126*, 499–507.

[30] Neilson, A. H., Lewin, R. A., The uptake and utilization of organic carbon by algae: An essay in comparative biochemistry. *Phycologia* 1974, *13*, 227–264.
[31] Bold, H. C., The morphology of *Chlamydomonas chlamydogama*, sp. nov. *Bull. Torrey Bot. Club* 1949, *76*, 101–108.
[32] Chen, F., Johns, M. R., Heterotrophic growth of *Chlamydomonas reinhardtii* on acetate in chemostat culture. *Process Biochem. (Amsterdam, Neth.)* 1996, *31*, 601–604.
[33] Sloth, J. K., Wiebe, M. G., Eriksen, N. T., Accumulation of phycocyanin in heterotrophic and mixotrophic cultures of the acidophilic red alga *Galdieria sulphuraria*. *Enzyme Microb. Technol.* 2006, *38*, 168–175.
[34] Ogawa, T., Aiba, S., Bioenergetic analysis of mixotrophic growth in *Chlorella vulgaris* and *Scenedesmus acutus*. *Biotechnol. Bioeng.* 1981, *23*, 1121–1132.
[35] Tchobanoglous, G., Burton, F. L., Stensel, H. D., in: Metcalf & Eddy, (4th Ed.), *Wastewater Engineering, Treatment and Reuse*, McGraw-Hill, New York 2003, pp. 93–94.
[36] Folch, J., Lees, M., Stanley, G. H. S., A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 1957, *226*, 497–509.
[37] Bligh, E. G., Dyer, W. J., A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 1959, *37*, 911–917.
[38] Iverson, S. J., Lang, S. L. C., Cooper, M. H., Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. *Lipids* 2001, *36*, 1283–1287.
[39] Koller, M., Salerno, A., Tuffner, P., Koinigg, M. et al., Characteristics and potential of micro algal cultivation strategies: A review. *J. Clean. Prod.* 2012, *37*, 377–388.
[40] Heifetz, P. B., Forster, B., Osmond, C. B., Giles, L. J. et al., Effects of acetate on facultative autotrophy in *Chlamydomonas reinhardtii* assessed by photosynthetic measurements and stable isotope analyses. *Plant Physiol.* 2000, *122*, 1439–1445.
[41] Ellis, R., Spooner, T., Yakulis, R., Regulation of chlorophyll synthesis in the green alga Golenkinia. *Plant Physiol.* 1975, *55*, 791–795.
[42] Sánchez, S., Martínez, M., Espejo, M., Pacheco, R. et al., Mixotrophic culture of *Chlorella pyrenoidosa* with olive-mill wastewater as the nutrient medium. *J. Appl. Phycol.* 2001, *13*, 443–449.
[43] Grant, N. G., Hommersand, M. H., The respiratory chain of *Chlorella protothecoides*. *Plant Physiol.* 1974, *54*, 50–56.
[44] Mullineaux, C. W., Emyln-Jones, D., State transitions: An example of acclimation to low-light stress. *J. Exp. Bot.* 2004, *56*, 389–393.
[45] Shi, X.-M., Zhang, X.-W., Chen, F., Heterotrophic production of biomass and lutein by *Chlorella protothecoides* on various nitrogen sources. *Enzyme Microb. Technol.* 2000, *27*, 312–318.
[46] Ip, P.-F., Chen, F., Production of astaxanthin by the green microalga *Chlorella zofingiensis* in the dark. *Process Biochem. (Amsterdam, Neth.)* 2005, *40*, 733–738.
[47] Schmidt, R. A., Wiebe, M. G., Eriksen, N. T., Heterotrophic high cell-density fed-batch cultures of the phycocyanin-producing red alga *Galdieria sulphuraria*. *Biotechnol. Bioeng.* 2005, *90*, 77–84.
[48] Yang, C., Hua, Q., Shimizu, K., Integration of the information from gene expression and metabolic fluxes for the analysis of the regulatory mechanisms in *Synechocystis*. *Appl. Microbiol. Biotechnol.* 2002, *58*, 813–822.
[49] Li, Y., Zhao, Z., Bai, F., High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. *Enzyme Microb. Technol.* 2007, *41*, 312–317.
[50] Ganuza, E., Anderson, A., Ratledge, C., High-cell-density cultivation of *Schizochytrium* sp. in an ammonium/pH-auxostat fed-batch system. *Biotechnol. Lett.* 2008, *30*, 1559–1564.
[51] Abeliovich, A., Weisman, D., Role of heterotrophic nutrition in growth of the alga *Scenedesmus obliquus* in high-rate oxidation ponds. *Appl. Environ. Microbiol.* 1978, *35*, 32–37.
[52] Lee, K., Lee, C.-G., Effect of light/dark cycles on wastewater treatments by microalgae. *Biotechnol. Bioproc. Eng.* 2001, *6*, 194–199.
[53] Ogbonna, J. C., Yoshizawa, H., Tanaka, H., Treatment of high strength organic wastewater by a mixed culture of photosynthetic microorganisms. *Appl. Phycology* 2000, *12*, 277–284.
[54] Kong, Q.-x., Li, L., Martinez, B., Chen, P. et al., Culture of microalgae *Chlamydomonas reinhardtii* in wastewater for biomass feedstock production. *Appl. Biochem. Biotechnol.* 2010, *160*, 9–18.
[55] Wang, L., Min, M., Li, Y., Chen, P. et al., Cultivation of green algae *Chlorella* sp. in different wastewaters from municipal wastewater treatment plant. *Appl. Biochem. Biotechnol.* 2010, *162*, 1174–1186.
[56] Pittman, J. K., Dean, A. P., Osundeko, O., The potential of sustainable algal biofuel production using wastewater resources. *Bioresour. Technol.* 2011, *102*, 17–25.
[57] Mitra, D., van Leeuwen, J., Lamsal, B., Heterotrophic/mixotrophic cultivation of oleaginous *Chlorella vulgaris* on industrial co-products. *Algal Res.* 2012, *1*, 40–48.
[58] Widmar, T., The *Chlorella* hexose/H^+-symporters. *Int. Rev. Cytol.* 2000, *200*, 101–141.