Biodegradation (2011) 22:805–814
DOI 10.1007/s10532-010-9419-z

ORIGINAL PAPER

Thermophilic, anaerobic co-digestion of microalgal biomass and cellulose for H_2 production

Sarah M. Carver · Chris J. Hulatt · David N. Thomas · Olli H. Tuovinen

Received: 19 April 2010 / Accepted: 14 September 2010 / Published online: 28 September 2010

Abstract Microalgal biomass has been a focus in the sustainable energy field, especially biodiesel production. The purpose of this study was to assess the feasibility of treating microalgal biomass and cellulose by anaerobic digestion for H_2 production. A microbial consortium, TC60, known to degrade cellulose and other plant polymers, was enriched on a mixture of cellulose and green microalgal biomass of *Dunaliella tertiolecta*, a marine species, or *Chlorella vulgaris*, a freshwater species. After five enrichment steps at 60°C, hydrogen yields increased at least 10% under all conditions. Anaerobic digestion of *D. tertiolecta* and cellulose by TC60 produced 7.7 mmol H_2/g volatile solids (VS) which were higher than the levels (2.9–4.2 mmol/g VS) obtained with cellulose and *C. vulgaris* biomass. Both microalgal slurries contained satellite prokaryotes. The *C. vulgaris* slurry, without TC60 inoculation, generated H_2 levels on par with that of TC60 on cellulose alone. The biomass-fed anaerobic digestion resulted in large shifts in short chain fatty acid concentrations and increased ammonium levels. Growth and H_2 production increased when TC60 was grown on a combination of *D. tertiolecta* and cellulose due to nutrients released from algal cells via lysis. The results indicated that satellite heterotrophs from *C. vulgaris* produced H_2 but the *Chlorella* biomass was not substantially degraded by TC60. To date, this is the first study to examine H_2 production by anaerobic digestion of microalgal biomass. The results indicate that H_2 production is feasible but higher yields could be achieved by optimization of the bioprocess conditions including biomass pretreatment.

Keywords *Dunaliella tertiolecta* · *Chlorella vulgaris* · Cellulose degradation · Anaerobic digestion · Hydrogen production

S. M. Carver (✉) · O. H. Tuovinen
Department of Microbiology, Ohio State University,
484 W. 12th Ave., Columbus, OH 43210, USA
e-mail: carver.84@gmail.com

S. M. Carver · O. H. Tuovinen
Department of Chemistry and Bioengineering,
Tampere University of Technology,
P.O. Box 541, 33101 Tampere, Finland

C. J. Hulatt · D. N. Thomas
School of Ocean Sciences, College of Natural Sciences,
Bangor University, Menai Bridge,
Anglesey LL59 5AB, UK

D. N. Thomas
Marine Research Centre, Finnish Environment Institute,
P.O. Box 140, 00251 Helsinki, Finland

Introduction

Microalgal biomass ties into multiple areas of bioenergy production, such as photosynthetic H_2 and anaerobic biogas production. Microalgae can produce H_2 by coupling photosynthesis with hydrogenases or

nitrogenases present in intracellular membranes (Benemann 2000; Melis and Happe 2001; Ghirardi et al. 2009). Similar biophotolysis can also occur in cyanobacteria. The efficiency of converting light energy into chemical energy in H_2 ranges between 3 and 15%, but under ambient environmental conditions conversions are less than 3% (Benemann 2000; Ghirardi et al. 2009). Direct H_2 production from microalgae as a source of sustainable energy is unlikely due to low efficiencies.

Multiple approaches have been tested for microalgae-coupled H_2 production. Some microalgae, such as the often studied green alga *Chlamydomonas reinhardtii*, can be grown under conditions known to encourage intracellular accumulation of starch (Ike et al. 1996, 1997; Kawaguchi et al. 2001). The biomass is then digested through acid or heat hydrolysis and fed to starch hydrolyzing heterotrophs, such as *Rhodobacter sphaeroides,* which can produce H_2 fermentatively (Ike et al. 1996). Kawaguchi et al. (2001) tested a three-phase approach whereby *C. reinhardtii* biomass was first grown to accumulate starch, followed by conversion of the starch to lactic acid by bacteria, and lastly, the fermentative production of H_2 from lactic acid by undefined bacteria. These approaches have produced H_2 yields too low to be feasible for industrial applications (Levin et al. 2004).

Microalgae have been used in wastewater treatment, specifically during secondary treatment processes to assimilate nutrients into biomass (Kojima and Lee 2001). Several decades ago, bulk microalgal biomass from wastewater treatment was recognized as a readily accessible feedstock for anaerobic digestion (Golueke et al. 1957; Oswald and Golueke 1960). Hernández and Córdoba (1993) demonstrated that biogas could be produced from *Chlorella vulgaris* biomass upon anaerobic digestion. Of the total biogas, 68–76% was CH_4 and total gas yields ranged between 0.40 and 0.45 l/g COD removed. Yen and Brune (2007) showed the feasibility of anaerobic co-digestion of waste paper and microalgal sludge, yielding up to 1.6 l (ca. 70 mmol) CH_4/l·d. Effluents from anaerobic digesters, especially those in the olive oil industry, have also been used as a medium for microalgal production (Hodaifa et al. 2008; Cordóba et al. 2008). Currently, the economic sustainability of biodiesel production from microalgal lipids is believed to be dependent on anaerobic digestion of residual biomass for additional biofuels (Sialve et al. 2009).

Biodiesel production from microalgae has four major phases: mass growth of microalgal biomass in photobioreactors, dewatering of biomass, lipid extraction, and processing of the lipid fraction for biodiesel production (Lardon et al. 2009; Mata et al. 2010). Residual biomass following lipid extraction has no further use for biodiesel production but could provide a feedstock for additional, sustainable energy production via anaerobic digestion processes. The ratio of carbon to nitrogen in the microalgal biomass is relatively low (<10) and could prove to be an issue for anaerobic digestion (Parkin and Owen 1986). Therefore, additional biodegradable C-rich compounds may be beneficial as a co-substrate with spent algal biomass (Hernández and Córdoba 1993; Yen and Brune 2007). Plant biomass, especially cellulose, provides an abundant feedstock that has been extensively studied and promoted in the sustainable energy field (Perlack et al. 2005; Department of Energy 2007). As cellulose feedstock is C-rich, it provides an ideal co-substrate for anaerobic digestion of microalgal biomass. Anaerobic digestion can be directed toward CH_4 or H_2 production but only H_2 provides a potential energy source that is sustainable and carbonless (Ren et al. 2009).

The purpose of this study was to examine the feasibility of H_2 production via anaerobic digestion of microalgal biomass. *Dunaliella tertiolecta* and *Chlorella vulgaris*, both green algae, were grown for mass harvest in this study. A thermophilic, cellulolytic microbial consortium was initially enriched with mixtures of the feedstocks, cellulose and microalgal biomass, before final testing for biogas and metabolite production.

Materials and methods

Cultivation and harvesting of microalgae

The freshwater microalga *Chlorella vulgaris* (strain 211/11B, Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, Oban, Argyll, UK) and marine *Dunaliella tertiolecta* (strain 13.86, Sammlung von Algenkulturen des Instituts für Pflanzenwissenschaften der Universität Göttingen, Germany) were selected for this study. Both green algae have been the subject of recent research endeavors and discussed as potential sources of biofuel (Hulatt and Thomas 2010).

C. vulgaris has a typical eukaryotic algal cell wall, whereas, *D. tertiolecta* is devoid of a rigid cell wall (Sialve et al. 2009; Ben-Amotz et al. 2010).

The algae were cultured autotrophically in 20 l photobioreactors. Photobioreactors were cylindrical (∅ 0.16 m), constructed of polyethylene, and sparged with 0.3 μm filtered air at 0.5 l/l·min (Whatman Hepa-Vent). Light was provided by cool white fluorescent tubes and the incident photosynthetic photon flux density averaged 225 μmol photons of photosynthetically active radiation m^2/s. *C. vulgaris* was grown in Jaworski's medium (http://www.ccap.ac.uk/media/documents/JM.pdf) prepared with Milli-Q water. *D. tertiolecta* was cultured in Walne's medium (http://www.ccap.ac.uk/media/documents/Walnes.pdf) made of sterilized seawater (~3.5% salinity).

Microalgal biomass was harvested via flocculation and centrifugation. For *D. tertiolecta*, the pH was adjusted with NaOH to approximately pH 9.5 for flocculation (Horiuchi et al. 2003). *C. vulgaris* was flocculated by adding 0.08 g chitosan/l and adjusting the pH to 7.0. Biomass was concentrated by centrifugation at 1,000×*g* for 10 min and removing the supernatant. The thick slurry was stored at −20°C. The pH of each slurry was adjusted to pH 7.0. Slurry volatile solid (VS) concentrations were 0.094 and 0.14 g/l for *D. tertiolecta* and *C. vulgaris*, respectively.

Microbial consortium

The microbial consortium, designated TC60, originated from the interior of a compost pile and subcultures had been maintained with cellulose. The culture can also grow on hemicellulose, pectin and starch. The identification of the dominant species based on 16S rRNA gene sequences is presently in progress. Based on qualitative PCR-DGGE analysis, the dominant species vary with substrate and other incubation conditions.

The TC60 culture was maintained anaerobically (N_2 headspace) in medium that contained (per liter): 2 g trypticase, 1 g yeast extract, 4 g Na_2CO_3, 0.23 g K_2HPO_4, 0.18 g KH_2PO_4, 0.36 g NH_4Cl, 0.04 g NaCl, 0.09 g $MgSO_4{\cdot}7H_2O$, 0.06 g $CaCl_2{\cdot}2H_2O$, 0.25 g cysteine-HCl, 0.25 g $Na_2S{\cdot}9H_2O$, 2 mg $CoCl_2{\cdot}6H_2O$, 0.16 mg Na_2SeO_4, and 0.09 mg $NiCl_2{\cdot}6H_2O$. Cellulose (Sigmacell, Type 20) was purchased from Sigma-Aldrich. Microalgal biomass and cellulose were added to a combined concentration of 4 g volatile solids (VS)/l. Cultures (50 ml) in 125 ml serum bottles were inoculated (10% v/v) and degassed prior to incubation at 60°C with 180 rev/min. Samples were withdrawn anaerobically, centrifuged at 16,000×*g* for 10 min, and the fractions were stored at −20°C until further analysis.

Four consecutive enrichment passages were completed before the fifth enrichment was analyzed in detail. The initial four enrichment stages were incubated in duplicate as follow: *D. tertiolecta* to cellulose (1:1 VS/VS), *D. tertiolecta* only, and the same two conditions with *C. vulgaris*. Appropriate controls included *D. tertiolecta* without TC60, *C. vulgaris* without TC60, TC60 with cellulose, and TC60 with medium only. The fourth enrichment of 1:1 microalgal biomass to cellulose was used to inoculate 1:2, 2:1, and 1:0 (VS/VS) ratios of substrate in the fifth enrichment. Each condition was tested in duplicate. At the end of the incubation, total and volatile solids (TS and VS, respectively) were measured according to the Standard Methods (Eaton et al. 2005). For the fifth enrichment, TS and VS were measured prior to incubation and after 10 days.

Analytical methods

Supernatants for HPLC analysis of short chain fatty acids (SCFAs) were cleaned via solid phase extraction (C18-T), diluted with Milli-Q water, and filtered through a 0.2 μm PTFE filter (Pall). A guard column, 5 cm × 4.6 mm ID (Supelguard H) and a cation-exchange column, 30 cm × 7.8 mm ID (Supelcogel C-610H) were used with an autosampler (Spectra-Physica AS 3000) and a UV detector set to 210 nm (Spectra-Physics SP100). A Beckman 114 M HPLC pump maintained a flow rate of 0.5 ml/min of the mobile phase, 0.1% *o*-phosphoric acid (Peu et al. 2004). Run times were 65 min per sample with 100 μl injections at 70 min intervals. Chromatographs were analyzed through a computer interface equipped with the Clarity Chromatography Station (DataApex).

Overpressures were measured with a sterile syringe immediately after removal from the incubator and headspace samples were manually injected into the GC. Gases were analyzed with a Shimadzu GC-2014 equipped with a thermal conductivity detector and a Porapak N (2 m length × 2 mm ID) column (Sigma-Aldrich). The carrier gas, nitrogen, was maintained at

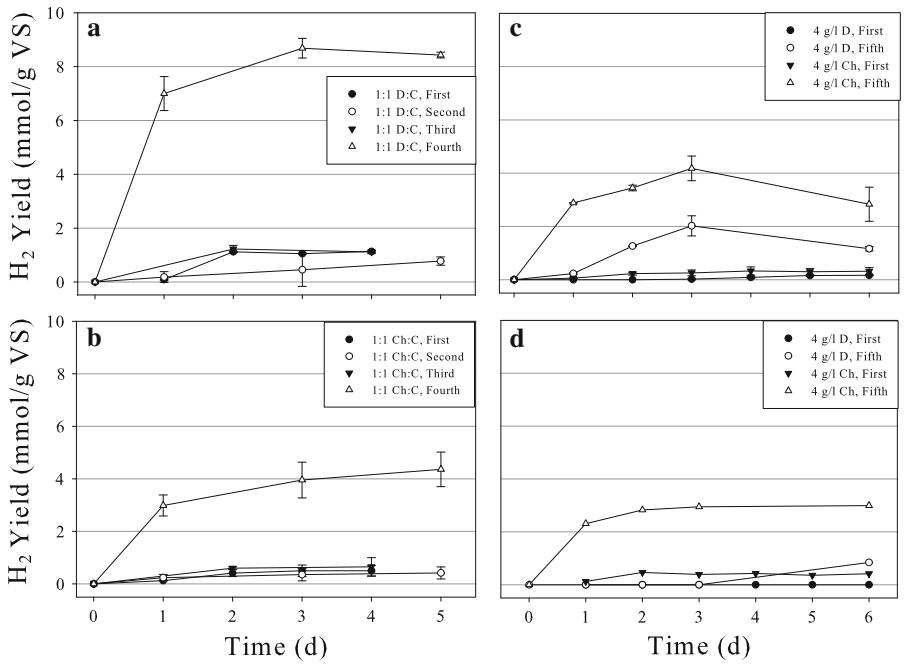

Fig. 1 Improvement of H_2 yields over sequential enrichment cultures. **a** 1:1 (VS/VS) *D. tertiolecta* to cellulose with TC60. **b** 1:1 *C. vulgaris* to cellulose with TC60; **c** First and fifth enrichments of TC60 with only microalgal biomass. **d** first and fifth enrichments of microalgal biomass without TC60. *Vertical bars* indicate the standard error. *C* cellulose, *Ch C. vulgaris*, *D D. tertiolecta*

20 ml/min. Temperatures were 110°C for the injector and detector while the column oven was kept at 80°C. The chromatographs were analyzed with GC Solution Analysis software (Shimadzu).

The ammonium concentration in supernatants was measured fluorimetrically according to Holmes et al. (1999). Samples were diluted with Milli-Q water and analysis was conducted using a Hitachi F2000 fluorometer. Excitation was measured at 360 nm and emission at 420 nm.

Solids for C and N analysis were dried at 80°C for 72 h followed by measurement with a Thermo Electron FlashEA 1112 analyzer (Thermo Scientific). The instrument was calibrated using sulfanilamide, 2,5-bis(5-*tert*-butyl-benzoxazol-2-yl)thiophene, L-cystine, and DL-methionine as standards.

Results and discussion

Enrichment improves H_2 production

By the fourth enrichment, headspace H_2 and CO_2 levels had increased (Fig. 1). CH_4 was not detected under any experimental conditions in this work. Slight changes in gas yields were seen between TC60 enrichments when grown on cellulose. The TS and VS levels were relatively constant from one enrichment to another (16.0–17.8 g TS/l and 8.2–9.3 g VS/l for *Dunaliella*; 9.2–11.2 g TS/l and 3.8–5.3 g VS/l for *Chlorella*). Following enrichment, H_2 production increased from 0.2 to 2.1 mmol H_2/g VS for *D. tertiolecta* and 0.3 to 4.2 mmol H_2/g VS for *C. vulgaris* when inoculated with TC60 (Fig. 1c). H_2 levels also increased when the microalgal biomass was not inoculated with TC60 (Fig. 1d). Autoclaved anaerobic controls showed no gas production, ruling out abiotic H_2 production.

Microscopic examination revealed a diverse microbial population associated with the microalgal biomass controls after enrichment. Both the *Dunaliella* and *Chlorella* slurries contained bacteria and protozoa, which included microorganisms capable of producing H_2 under the thermophilic conditions. Heterotrophic contamination may have been associated with the original stock cultures or introduced during the cultivation and handling of microalgal biomass.

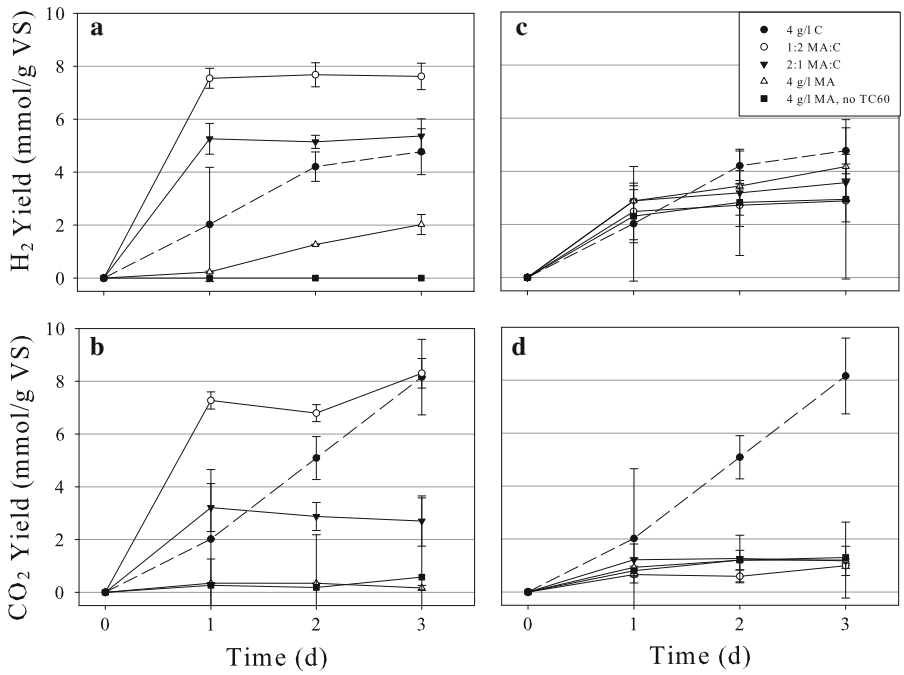

Fig. 2 Cumulative gas yields during the first three days of the fifth enrichment of TC60 with different substrate conditions. **a** H_2 and **b** CO_2 yields for samples with *D. tertiolecta;* **c** H_2 and **d** CO_2 yields for samples with *C. vulgaris.* As a reference, gas yields for cellulose-fed TC60 are included (*dashed line*). H_2 and CO_2 yields are included for uninoculated microalgal biomass. *C* cellulose; *MA* microalgal biomass

Analysis of the fifth enrichment

The fourth enrichment of the 1:1 (VS/VS) ratio of microalgal biomass to cellulose was used to inoculate the fifth enrichment, which received 1:2, 2:1, or 1:0 ratios of microalgal biomass to cellulose. Most gas production occurred within the first three days under all conditions. In addition to H_2, CO_2 was monitored in order to observe the growth of TC60. Direct measurement of microalgal or bacterial biomass was not possible due to unknown quantities of incomplete hydrolysis products and lysed cells.

As shown in Fig. 2, cellulose-fed TC60 yielded 5.2 mmol H_2 and 8.9 mmol CO_2/g VS by day 3. When grown on a ratio of 1:2 *D. tertiolecta* biomass and cellulose, the H_2 levels increased whereas the CO_2 levels were relatively constant and similar to those in cellulose-fed cultures (Fig. 2a, b). The H_2:CO_2 ratio increased from 0.8 with cellulose to 1.1 (Table 1). With a 2:1 ratio, the gas yields were comparable to those in cellulose-fed cultures (7.7 mmol H_2 and 8.6 mmol CO_2/g VS) and the H_2:CO_2 ratio increased to 1.8. TC60 fed only *D. tertiolecta* without cellulose yielded a very high H_2:CO_2 ratio but individual gas yields were relatively low (Table 1; Fig. 2). When *D. tertiolecta* biomass was not inoculated, both the H_2 and CO_2 yields remained low. These results indicated that *D. tertiolecta* biomass serves as an additional source of nutrients for TC60 and enhanced H_2

Table 1 H_2:CO_2 ratios (± standard error) for TC60 grown on various substrates

Condition	H_2:CO_2	
	Day 2	Day 6
No added substrate	1.98	1.16
4 g/l cellulose	0.83 ± 0.02	0.61 ± 0.01
1:2 *Dunaliella*:cellulose	1.13 ± 0.01	0.90 ± 0.02
2:1 *Dunaliella*:cellulose	1.81 ± 0.25	1.24 ± 0.20
4 g/l *Dunaliella*	3.69 ± 0.03	1.93 ± 0.10
4 g/l *Dunaliella* (no TC60)	0.00	0.82
1:2 *Chlorella*:cellulose	4.71 ± 0.59	1.67 ± 0.40
2:1 *Chlorella*:cellulose	3.03 ± 1.45	1.41 ± 0.07
4 g/l *Chlorella*	3.00 ± 0.84	1.60 ± 0.02
4 g/l *Chlorella* (no TC60)	2.35	2.32

production when cellulose was present. The heterotrophs associated with the *D. tertiolecta* biomass were relatively inactive under conditions in this study. Heterotrophs in the *D. tertiolecta* culture are most likely halophiles or at least highly salt-tolerant (~3.5% salinity) and their activity would be negligible in low salinity medium.

In the *C. vulgaris*-fed TC60 culture, growth remained low according to the CO_2 levels, and the H_2 levels under all conditions were within standard error measurements, averaging approximately 3.0 mmol H_2 and 2.5 mmol CO_2/g VS (Fig. 2d). These H_2 levels were comparable to those observed when TC60 was fed cellulose. Microscopic examination showed a diverse prokaryotic population in the *C. vulgaris* slurry. H_2 was produced without inoculation of TC60, indicating anaerobic activity of satellite heterotrophs associated with *C. vulgaris*. Due to the comparable H_2 yields regardless of the substrate ratio, it is concluded that these heterotrophs used organic compounds in the medium, e.g., yeast extract, tryptic-case, and microalgal excreta.

The ammonium and SCFA concentrations were also monitored in the anaerobic digestion experiments. Formation of ammonium from protein degradation has been cited as a possible concern for microalgal digestion (Tam and Wong 1996; Yen and Brune 2007; Sialve et al. 2009). However, ammonium concentrations, up to 17.7 mM, did not have adverse effects on growth of TC60 and gas production (data not shown). When TC60 was fed cellulose, the concentrations of ammonium remained below 10 mM (Table 2). Without cellulose or microalgal biomass, the ammonium yields increased to 30 mM. Ammonium concentrations increased with the concentration of *D. tertiolecta* biomass, indicating enhanced ammonification of N-containing compounds in the medium. When the ratio of *D. tertiolecta* to cellulose was 1:2, the ammonium concentration was 16.3 mM and increased to 21.5 mM when TC60 was supplied with *D. tertiolecta* without cellulose. With *C. vulgaris* biomass, ammonium concentrations (24.3–27.4 mM) were relatively high under all experimental conditions. These results indicated ammonification by satellite heterotrophs regardless of TC60 inoculation.

The concentrations and trends in SCFA profiles varied with experimental conditions. When TC60 was grown only on cellulose, the dominant SCFAs were lactate (22.1 mM), butyrate (18.1 mM) and acetate (10.9 mM) (Table 2). In the presence of *D. tertiolecta* or *C. vulgaris* biomass, lactate concentration was <4 mM, whereas both acetate and butyrate levels increased (Table 2; Fig. 3). Lactic acid fermentation was suppressed by the presence of microalgal biomass, therefore allowing for increased H_2 yields. In the presence of *D. tertiolecta* and cellulose, the SCFA profiles shifted towards acetate and butyrate pathways which are coupled with hydrogenases.

Total SCFA concentrations increased with the amount of *C. vulgaris* biomass (Table 2). This association appeared to be the result of heterotrophic microorganisms present in the microalgal slurry rather than TC60. The controls without TC60 showed acid production at levels four times higher with *C. vulgaris* than with the *D. tertiolecta* control (Fig. 3). The

Table 2 Maximum lactate, acetate, butyrate, and ammonium concentrations (± standard error) during the 5th enrichment

Condition	Maximum metabolite concentration (mM)				
	Lactate	Acetate	Butyrate	Total	Ammonium
No added substrate	0.24	7.53	17.64	37.65	28.89
4 g/l cellulose	22.06 ± 2.86	10.93 ± 2.74	18.10 ± 0.69	67.33	9.84 ± 5.70
1:2 *Dunaliella*:cellulose	3.75 ± 1.55	16.34 ± 3.32	19.75 ± 1.97	66.33	16.31 ± 1.88
2:1 *Dunaliella*:cellulose	1.38 ± 0.89	15.48 ± 0.80	17.89 ± 0.74	50.71	18.28 ± 1.44
4 g/l *Dunaliella*	1.06	8.17 ± 0.84	16.37 ± 0.40	50.97	21.52 ± 0.59
4 g/l *Dunaliella* (no TC60)	1.13	5.28	3.51	23.90	14.86
1:2 *Chlorella*:cellulose	0.70	8.82 ± 1.12	22.61 ± 2.80	53.62	24.31 ± 2.82
2:1 *Chlorella*:cellulose	0.82 ± 0.02	10.43 ± 2.13	29.42 ± 2.11	63.30	26.84 ± 2.45
4 g/l *Chlorella*	1.88	11.32 ± 1.49	35.36 ± 0.85	72.80	27.45 ± 3.80
4 g/l *Chlorella* (no TC60)	0.97	12.68	34.09	80.19	25.76

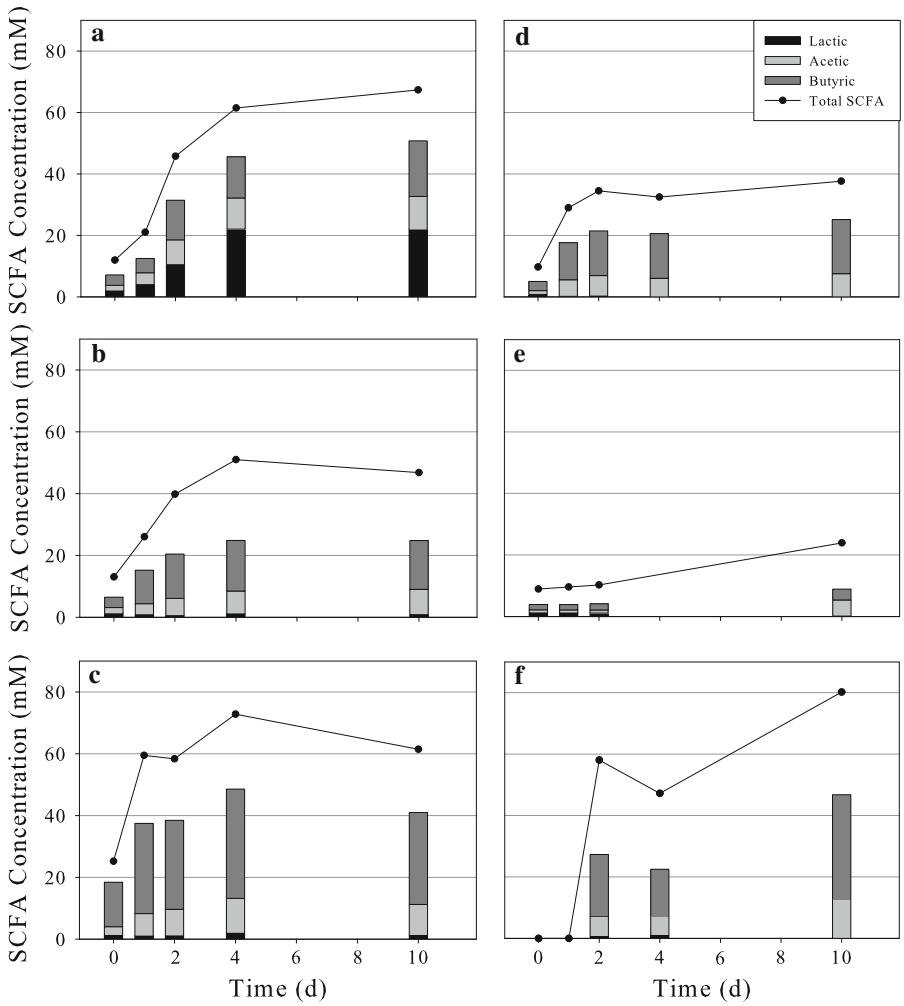

Fig. 3 Changes in lactate, acetate, butyrate and total SCFA concentrations over time for single substrate conditions. **a** 4 g/l cellulose with TC60. **b** 4 g/l *D. tertiolecta* with TC60. **c** 4 g/l *C. vulgaris* with TC60. **d** TC60 without added substrate. **e** 4 g/l *D. tertiolecta* without TC60. **f** 4 g/l *C. vulgaris* without TC60

relatively high levels of SCFAs in the presence of *C. vulgaris*, even without TC60 inoculation, suggest that the heterotrophs in the algal slurry were active in producing butyrate (50% of total SCFA). *D. tertiolecta* without TC60 had lower acid production than TC60 grown in medium only, indicating utilization of acetate and butyric acid by the satellite organisms.

Assessment of microalgal biomass digestion

To assess the biodegradability of microbial biomass, samples of solids were analyzed for C, N, TS, and VS concentrations. These results showed relatively little change in either the TS or VS over incubation. The initial C concentration in the solids was comparable across all substrate ratios in *C. vulgaris*-fed samples, suggesting that the biomass remained more or less intact under all conditions. In contrast, the initial C content of *D. tertiolecta*-fed samples varied (Fig. 4). *D. tertiolecta* cells were prone to lysis as seen microscopically, releasing soluble nutrients and lowering the C concentration in the solid fraction. Therefore, the *Dunaliella*-fed TC60 cultures contained cytosolic compounds which contributed to the increased gas production and reduced lag time. The lack of a rigid cell wall and tendency of lysis of *Dunaliella* biomass when removed from high salinity medium are useful attributes for anaerobic digestion.

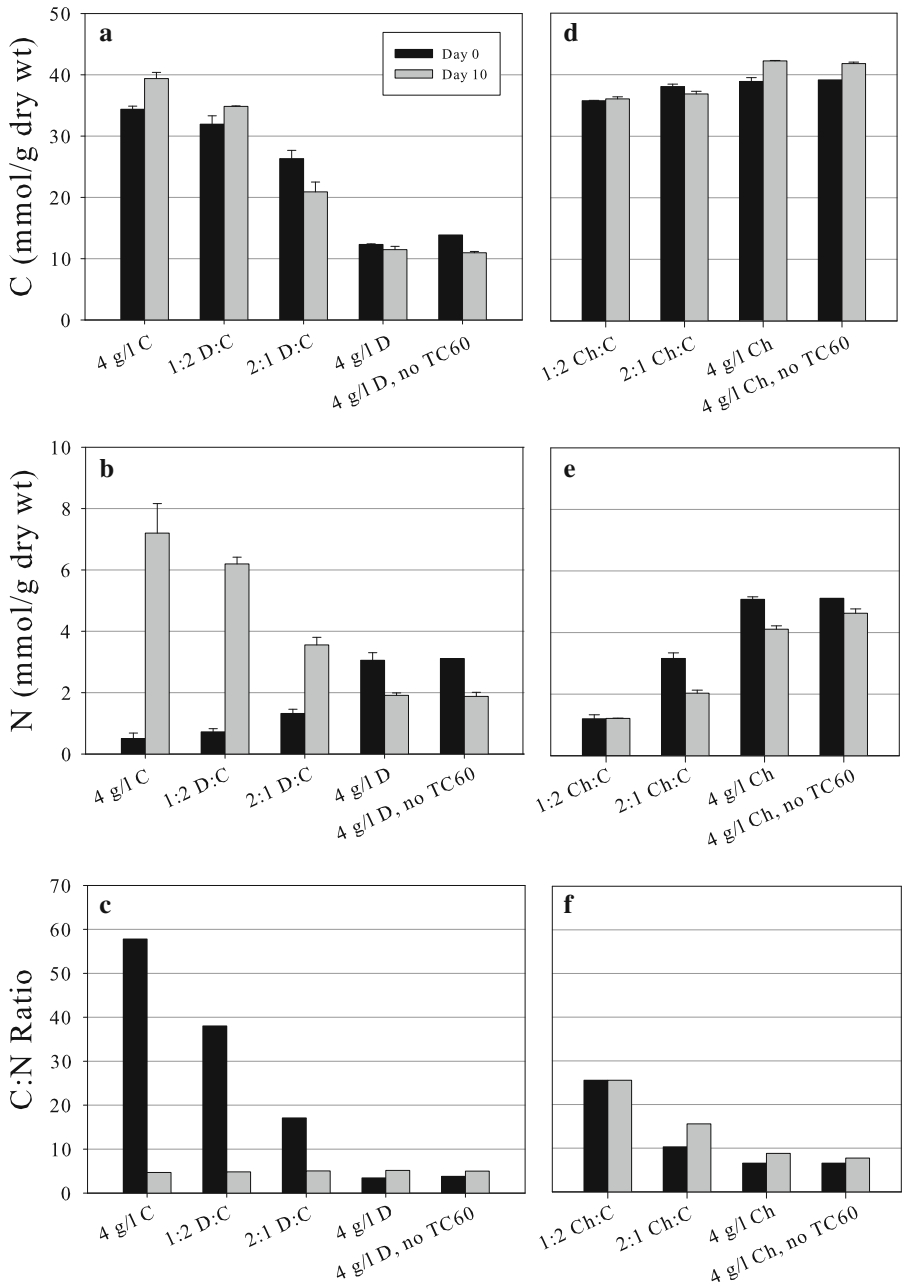

Fig. 4 C and N analysis of solid fractions and the corresponding C:N ratios for *D. tertiolecta* (**a–c**) and *C. vulgaris* (**d–f**). The cultures were inoculated with TC60 unless otherwise noted. *C* cellulose, *Ch C. vulgaris, D D. tertiolecta*

The C content in the solid fraction decreased after incubation in samples with a combination of *D. tertiolecta* and cellulose (Fig. 4). When TC60 was fed *D. tertiolecta* only, the C content did not change after 10 days of incubation, indicating negligible digestion of the insoluble biomass. These results suggest that the soluble compounds rather than the solid fraction of the *D. tertiolecta* biomass were the source for increased gas production by TC60. The level of C in solids did not change after 10 days of incubation in samples containing *C. vulgaris* (Fig. 4). *C. vulgaris* biomass appeared to suppress cellulose

utilization by TC60, consistent with the low production of gas and other indicators of poor growth.

After incubation, the N content of solids increased from 0.7 to 6.2 mmol/g dry wt and 1.3 to 3.6 mmol/g dry wt for 1:2 and 2:1 *D. tertiolecta* to cellulose, respectively (Fig. 4). This trend was consistent with the utilization of cellulose for biomass synthesis by TC60 during anaerobic digestion. When only *D. tertiolecta* was fed to TC60, the N content in the solids did not increase, again indicating that TC60 only used cellulose and soluble microalgal nutrients as substrates. When TC60 was grown in the presence of *C. vulgaris* biomass, the level of N was relatively unaffected (Fig. 4), consistent with the apparent recalcitrance of *Chlorella* biomass to anaerobic digestion.

When TC60 was grown on cellulose, decreases in the C:N ratio of solids were attributed to biomass growth. C content stayed constant whereas the N levels in solids increased by day 10. Similarly, decreases in the C:N ratios were also seen in solids from TC60 cultures fed 1:2 and 2:1 of *D. tertiolecta* to cellulose (Fig. 4). Ratios did not change in TC60 fed *D. tertiolecta* only or in uninoculated *D. tertiolecta*, agreeing with the lack of metabolic activity in these samples. In the case of *Chlorella*, the C:N ratios of solids showed relatively little change from day 0 to 10. These data suggested that growth of TC60 and satellite heterotrophs took place at the expense of soluble substrates. Metabolic data indicated that it was the satellite heterotrophs, not TC60, that were the active microorganisms in *C. vulgaris*-fed samples. Microscopic examination showed that the *C. vulgaris* biomass remained intact and accounts for a large percentage of the dry weight. Therefore, the microalgal biomass would mask the relatively minor increase of N due to satellite heterotrophic growth.

Conclusions

This study focused on H_2 generation through anaerobic degradative metabolism and dark fermentative pathways. The overall energy balance of the bioprocess was not compiled because this was an initial feasibility study with no optimization. The calorific yields calculated from the maximum H_2 yields were equal to 1.86 and 1.01 kJ/g VS for the 1:2 *D. tertiolecta* to cellulose and 4 g/l *C. vulgaris*, respectively. These yields indicate major differences in the biodegradability of the two algal biomass substrates. The marine algae, *Dunaliella tertiolecta*, lysed readily and thereby provided additional nutrients for cellulolytic activity and H_2 accumulation by TC60. Heterotrophs associated with the marine species were deemed to have a negligible effect on the digestion. In contrast, the freshwater *Chlorella vulgaris* biomass remained recalcitrant and suppressed TC60 activity. In spite of the thermophilic conditions, heterotrophs associated with *Chlorella* biomass produced H_2 yields similar to those obtained with TC60. Hydrolytic pretreatment of microalgal slurries was not tested for *Chlorella* biomass. The yields obtained in this study indicate the need for improvement of H_2 yields through biomass pretreatment and process optimization.

Acknowledgments Partial funding for this study was received from the Finland Distinguished Professorship Program (402/2006) of the Finnish Agency for Technology and Innovation (S.M. Carver and O.H. Tuovinen), USDA National Needs Graduate Fellowship Program (S.M. Carter), Academy of Finland (D.N. Thomas), and Engineering and Physical Sciences Research Council Studentship in conjunction with RWE Power (U.K.) and Varicon Aqua Ltd. (C.J. Hulatt and D.N. Thomas).

References

Ben-Amotz A, Polle JEW, Rao DVS (2010) The alga *Dunaliella*: biodiversity, physiology, genomics and biotechnology. Science, Enfield

Benemann JR (2000) Hydrogen production by microalgae. J Appl Phycol 12:291–300

Cordóba LT, Bocanegra ARD, Llorente BR, Hernández ES, Echegoyen FB, Borja R, Bejines FR, Morcillo MFC (2008) Batch culture growth of *Chlorella zofingiensis* on effluent derived from two-stage anaerobic digestion of two-phase olive mill solid waste. Electron J Biotechnol. doi:10.2225/vol11-issue2-fulltext-1 11(2):1–8

Department of Energy (2007) Roadmap for bioenergy and biobased products in the U.S. Biomass Research and Development, Technical Advisory Committee, Washington DC ADA494661

Eaton AD, Clesceri LS, Rice EW, Greenberg AE, Franson MAH (2005) Standard methods for the examination of water and wastewater. American Public Health Association, New York

Ghirardi ML, Dubini A, Yu J, Maness PC (2009) Photobiological hydrogen-producing systems. Chem Soc Rev 38:52–61

Golueke CG, Oswald WJ, Gotaas HB (1957) Anaerobic digestion of algae. Appl Microbiol 5:47–55

Hernández EPS, Córdoba LT (1993) Anaerobic digestion of *Chlorella vulgaris* for energy production. Resour Conserv Recycl 9:127–132

Hodaifa G, Martínez ME, Sánchez S (2008) Use of industrial wastewater from olive-oil extraction for biomass production of *Scenedesmus obliquus*. Bioresour Technol 99:1111–1117

Holmes RM, Aminot A, Kérouel R, Hooker BA, Peterson BJ (1999) A simple and precise method for measuring ammonium in marine and freshwater ecosystems. Can J Fish Aquat Sci 56:1801–1808

Horiuchi J, Ohba I, Tada K, Kobayashi M, Kanno T, Kishimoto M (2003) Effective cell harvesting of the halotolerant microalga *Dunaliella tertiolecta* with pH control. J Biosci Bioeng 95:412–415

Hulatt CJ, Thomas DN (2010) Dissolved organic matter (DOM) in microalgal photobioreactors: a potential loss in solar energy conversion? Bioresour Technol 101:8690–8697

Ike A, Saimura C, Hirata K, Miyamoto K (1996) Environmentally friendly production of H_2 incorporating microalgal CO_2 fixation. J Mar Biotechnol 4:47–51

Ike A, Toda N, Hirata K, Miyamoto K (1997) Hydrogen photoproduction from CO_2-fixing microalgal biomass: application of lactic acid fermentation by *Lactobacillus amylovorus*. J Ferment Bioeng 84:428–433

Kawaguchi H, Hashimoto K, Hirata K, Miyamoto K (2001) H_2 production from algal biomass by a mixed culture of *Rhodobium marinum* A-501 and *Lactobacillus amylovorus*. J Biosci Bioeng 91:277–282

Kojima H, Lee YK (2001) Photosynthetic microorganisms in environmental biotechnology. Springer, Hong Kong

Lardon L, Hélias A, Sialve B, Steyer J-P, Bernard O (2009) Life-cycle assessment of biodiesel production from microalgae. Environ Sci Technol 43:6475–6481

Levin DB, Pitt L, Love M (2004) Biohydrogen production: prospects and limitations to practical application. Int J Hydrogen Energy 29:173–185

Mata TM, Martins AA, Caetano NS (2010) Microalgae for biodiesel production and other applications: a review. Renew Sustain Energy Rev 14:217–232

Melis A, Happe T (2001) Hydrogen production. Green algae as a source of energy. Plant Physiol 127:740–748

Oswald WJ, Golueke CG (1960) Biological transformation of solar energy. Adv Appl Microbiol 2:223–262

Parkin GF, Owen WF (1986) Fundamentals of anaerobic digestion of wastewater sludge. J Environ Eng 112: 867–920

Perlack RD, Wright LL, Turhollow AF, Graham RL, Stokes BJ, Erbach DC (2005) Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of a billion-ton annual supply. U.S. Department of Energy DOE/GO-102005-2135

Peu P, Béline F, Martinez J (2004) Volatile fatty acids analysis from pig slurry using high-performance liquid chromatography. Int J Environ Anal Chem 84:1017–1022

Ren N, Wang A, Cao G, Xu J, Gao L (2009) Bioconversion of lignocellulosic biomass to hydrogen: potential and challenges. Biotechnol Adv 27:1051–1060

Sialve B, Bernet N, Bernard O (2009) Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnol Adv 27:409–416

Tam NFY, Wong YS (1996) Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media. Bioresour Technol 57:45–50

Yen HW, Brune DE (2007) Anaerobic co-digestion of algal sludge and waste paper to produce methane. Bioresour Technol 98:130–134