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Characterization of three *Chlorella sorokiniana* strains in anaerobic digested effluent from cattle manure ☆

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HIGHLIGHTS

- Chlorella strains were compared in cattle anaerobic digested effluent and Bold's Basel Media.
- Algae biomass production is dependent on nutrient provision and removal from growth media.
- Starch and protein production was greater than lipid accumulation in anaerobic digested effluent.
- Chlorella grown in anaerobic digested effluent may be more suitable for animal feed application.

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ABSTRACT

Chlorella sorokiniana CS-01, UTEX 1230 and UTEX 2714 were maintained in 10% anaerobic digester effluent (ADE) from cattle manure digestion and compared with algal cultivation in Bold's Basal Medium (BBM). Biomass of CS-01 and UTEX 1230 in ADE produced similar or greater than 280 mg/L after 21 days in BBM, however, UTEX 2714 growth in ADE was suppressed by more than 50% demonstrating a significant species bias to synthetic compared to organic waste-based media. The highest accumulation of protein and starch was exhibited in UTEX 1230 in ADE yielding 34% and 23% ash free dry weight (AFDW), respectively, though fatty acid methyl ester total lipid measured less than 12% AFDW. Results suggest that biomass from UTEX 1230 in ADE may serve as a candidate alga and growth system combination sustainable for animal feed production considering high yields of protein, starch and low lipid accumulation.

1. Introduction

Anaerobic digestion (AD) allows generation of bioenergy from organic wastes such as manure (Bohutskyi and Bouwer, 2013). The biogas product from AD contains methane which can be used as a bioenergy source as well as carbon dioxide which can serve as a carbon source for algae growth. AD also produces a digestate containing most the original nitrogen, phosphorus and micronutrients from the input material such as manure that can serve as an inexpensive, nutrient-rich, organic fertilizer (Field et al., 1984) and may serve as a nutrient source for algal cultivation (Singh et al., 2011). A major challenge in sustainably producing biofuels and feed from algae cultivation is the need to provide nitrogen and phosphate nutrients. To address this challenge the value of anaerobic digested effluent (ADE) as a low cost nutrient suppliement has been evaluated in a number of studies (Chinnasamy et al., 2010; Kebede-Westhead et al., 2006; Wilkie, 2002). Microalgae compare favorably to terrestrial plants as a renewable biomass feedstock for multiple reasons: (1) efficient productivity per unit area per unit time yielding up to 300 times more oil per acre per year than conventional crops such as rapeseed, palms, soybeans, or jatropha (Greenwell et al., 2009); (2) rapid growth cycle of 10–14 days permitting several harvests in a short time period and throughout the year; (3) ability to convert significant fraction of biomass to oil for biodiesel or biofuel (Dismukes et al., 2008); and (4) ability to readily utilize CO_2 generated from a point source such as flue gas; and (5) capable of utilizing wastewater by means of consuming the excess nutrients in natural environments such as ponds, rivers and oceans (Singh et al., 2011).

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Several algae species have the ability to produce energy dense biomass enabling liquid biofuels production. For example, Chlorella is a unicellular green microalga that produces substantial biomass rich in starch and lipids under minimum conditions requiring addition of only nitrogen, phosphorus and other minor nutrients in the presence of light and carbon dioxide. Nutrient uptake from different manure-based ADE products and industrial effluents have been evaluated for production of biomass, lipids, carbohydrates and proteins in Chlorella species. Effluent sources have (1) poultry litter (Singh et al., 2011); (2) citric acid effluent produced by fermentation (Li et al., 2013); (3) carpet mill industrial effluent (Chinnasamy et al., 2010) and (4) animal manure from different sources (Hu et al., 2012; Huo et al., 2012; Kebede-Westhead et al., 2004; Mulbry et al., 2008; Zhou et al., 2012).

The sources of nitrogen and carbon as well as availability affect algae growth and the amount of protein, carbohydrate and lipid composition of the algae biomass (Hu et al., 2008). Similar studies have identified gene expression patterns in algae that correspond to oil and carbohydrate accumulation during nitrogen deprivation in *Chamydomonas reinhardtii* and *Coccomyxa* sp. (Msanne et al., 2012). Lipid profiling has allowed assessment of algal oils from photoautotrophic and heterotrophic growth of *Chlorella zofingiensis* for biodiesel production (Liu et al., 2011). Recently four microalgae strains, including Chlorella species, demonstrated higher biomass production when cultured in poultry waste ADE. The harvested algae yielded high levels of carbohydrate (22%) and protein (39% w/w) (Singh et al., 2011). Additionally, another study showed higher biomass production and fatty acid contents (7.5–11% of dry weight) of *Chlorella* sp. grown in reduced total nitrogen and phosphorous effluent; however, fermented swine manure with different chemical additives served as the substrate (Hu et al., 2012).

The aim of this study was to investigate nutritional utilization and lipid production by algae grown in cattle manure ADE. *Chlorella sorokiniana* CS-01, *C. sorokiniana* UTEX 1230 and *C. sorokiniana* UTEX 2714 were selected based on their high biomass and lipid productivity. The objective was to monitor and compare production of algal biomass, protein, carbohydrate and lipid in two different media types: chemically formulated Bold's Basal Media (BBM) and cattle ADE in large-scale 80 L hanging-bag (HB) cultures. Nitrogen and phosphorus composition was evaluated during the course of algae growth. The behavior of algae in growth medium differing in nutrient composition will aid in identifying the resources imperative for healthy algae cultivation and the accumulation of lipid, carbohydrate and protein. The results obtained will be used to develop a more comprehensive understanding of nutritional utilization by algae regarding growth and production of lipid, protein and carbohydrate using cattle ADE to promote both sustainable animal and biofuel production.

2. Methods

2.1. Anaerobic digester effluent (ADE)

ADE was obtained from the Animal Science Department at the University of Nebraska–Lincoln. Cattle waste was collected from crossbred steers consuming a diet consisting of 47.5% dry rolled corn, 40% wet distillers grains plus soluble, 7.5% alfalfa hay, and 5% supplement (minerals, vitamins, and feed additives with fine ground corn as a carrier). Total manure (feces and urine mixture) was collected for a 5 day period from six steers fed this diet. The collected composite manure slurry was used to feed an anaerobic digester from which ADE was collected daily. ADE was pretreated prior to the introduction of alga by centrifugation at 3,500*g* for 5 min thereby removing sediment. 10% ADE was added with distilled water to total 80 L HBs to accommodate Chlorella cultivation. ADE was stored at 4 °C.

2.2. Algae strains and growth conditions

Samples of *C. sorokiniana* UTEX 1230 and *C. sorokiniana* UTEX 2714 were obtained from the Culture Collection of Algae at the University of Texas at Austin. *C. sorokiniana* CS-01 was provided by Minxi Wan at Johns Hopkins University. All algae strains were transferred to Bold's Basal Media (Bold, 1949) (BBM) sterile agar plates containing 100 μg/mL tetracycline and 10 μg/mL ampicillin and grown at 25 °C under continuous illumination at 160 μmol m^{-2} s^{-1}. Liquid culture was initiated by inoculation of a single isolated colony into 5 mL of sterile BBM. The 5 mL cultures were shaken at 250 rpm for 7 days under continuous illumination (160 μmol m^{-2} s^{-1}) at 25 °C.

2.3. Hanging bag (HB) cultivation

Cultures used for HB inoculation included 3 L aerated photobioreactors set up according to Kobayashi et al. (2013). The 3 L cultures were divided equally in two 80 L polyethylene HBs (each 4×10^6 cell/mL), one containing BBM and another composed of 10% ADE. Air stones with tubing were inserted in adjacent columns providing vigorous and ascending aeration supplied from an external compressed air source (~30 L/min). Cell growth was measured by hemacytometer (Cole–Parmer, Neubauer improved bright-line 0.1 mm depth). Algae growth was monitored by collecting 1 L samples per species during three stages of the algal growth cycle in both BBM and ADE treatment: early and late logarithmic phases and stationary phase. Samples were harvested by centrifugation at 5,000*g* for 5 min. The supernatant was stored at 4 °C and used for nutrient analysis. The pellet was lyophilized overnight and used for the biomass analysis and the analysis of biochemical compositions. The biomass of dry weight and ash were determined gravimetrically and were subtracted as ash free dry weight (AFDW).

2.4. Nutrient analysis

Measurement of nutrient uptake in the media was performed by AQ1 Discrete Multi-Chemistry Analyzer (SEAL Analytical WI, USA). The AQ1 is a computer controlled multi-chemistry discrete wet chemistry analyzer. Algal nutrition consumption was measured corresponding to the uptake of ammonia, ortho-phosphate, nitrate+nitrite, total phosphorus and total nitrogen concentrations in BBM and 10% ADE media. Ammonia and total nitrogen were detected at 660 nm as the indophenol blue converted ammonia with two phenols under alkaline conditions. The AQ1 method number was EPA-129-B Rev. 0 with the detection limit of 0.05 mg N/L (range: 0.2 to 10 mg N/L). Ortho-phosphate and total phosphorus were detected at 660 nm as phosphomolybdenum blue converted ortho-phosphate to molybdate under acid conditions. The AQ1 method number was EPA-146-B Rev. 0 with the detection limit 0.007 mg P/L (range: 0.125 to 12.5 mg P/L). Nitrate+nitrite was detected at 520 nm as the colored azo compound reduced nitrate to nitrite via cadmium coil followed by a sulfanilamide reaction under catalyst conditions. The AQ1 method number was EPA-114-B Rev. 0 with the detection limit of 0.04 mg N/L (range: 0.25 to 15 mg N/L).

The supernatant sampled from BBM and 10% ADE media was diluted 25× and 50×, respectively for ammonium, ortho-phosphate and nitrate+nitrite measurements with deionized water into 2 mL sampling cups to improve measurement accuracy. All samples were centrifuged in 1.5 mL microtubes for 10 s. For the total phosphorus measurement the supernatant was digested with acid-persulfate following the USEPA method 365.1 and for total nitrogen copper(II) was used as a digestion catalyst following the USEPA methods 351.2 Rev. 2.0 in the AQ1 procedure. The ammonia, ortho-phosphate, nitrate+nitrite, digested total phosphorus

and nitrogen concentrations in the diluted supernatant samples were measured by AQ1 with the programming as referenced above and quantified based on the standard curves of ammonia chloride for ammonia, potassium dihydrogen orthophosphate for orthophosphate and sodium nitrate for nitate+nitrite by AQ 1 software (Seal Analytical).

2.5. Lipid extraction

Lipid extraction followed the procedure provided by Kobayashi et al. (2013). The dried algae pellets (100 mg) grown with either BBM or 10% ADE were homogenized using a mortar and pestle with liquid nitrogen. Lipid extraction was performed by a modification of the method described by Bligh and Dyer (1959). Two mL of chloroform:methanol (1:2, v:v) containing 0.01% butyl hydroxyl toluene was added to the ground cells and pooled in a glass tube. This step was repeated 3 times. 500 μg of tripentadecanoin (15:0 TAG, Nu-Check Prep, MN, USA) was added as an internal standard. Two mL of zirconia beads (0.7 mm, BioSpec Products, OK, USA) were added and the mixture was shaken by a vortex at room temperature for 30 min. Two mL of chloroform and 4 mL of water were added and vortexed. After centrifugation at 1,500*g* for 5 min, the chloroform phase was collected and the aqueous phase was re-extracted with 5 mL of chloroform. This step was repeated 5 times. The pooled chloroform was evaporated to dryness under a stream of nitrogen. Total lipid content was determined gravimetrically.

2.6. Chromatographic methods

Thin layer chromatography (TLC) for neutral lipid separation and trans-methylation to fatty acid methyl ester (FAME) were performed as previously described (Kobayashi et al., 2013). FAMEs were analyzed using an Agilent 6890 Series gas-chromatography (GC) System with Agilent 5973 Network Mass Selective Detector (Agilent Technologies, Delaware USA) and chromatography was carried out using a 200 m × 250 μm × 0.25 μm Varian GC Capillary column (Varian Inc., CA, USA). GC inlet was held at 270 °C and 1 μL of the sample was injected with splitless. The oven temperature was programmed from 130 °C (10 min hold) to 160 °C (7 min hold), from 160 °C to 190 °C (7 min hold), from 190 °C to 220 °C (22 min hold) and from 220 °C to 250 °C (17 min hold) at a rate of 10 °C/min for each step with helium as the carrier gas. The total analysis time was 75 min. The GC/MS was carried out using 70 eV EI and the data was evaluated with total ion count (TIC).

The separation of lipids by the system gold high performance liquid chromatography (HPLC Beckman Coulter, CA, USA) with evaporative light scattering detector (ELSD, Shimadzu, MD, USA) was performed (Kobayashi et al., 2013). The Luna 3 μm silica column (100 × 4.60 mm, Phenomenex, CA, USA) was connected with the guard column (silica 4 × 3.0 mm, Phenomenex), which was incubated at 50 °C in the column oven. ELSD was set to gain 5 at 30 °C under 350 kPa of nitrogen pressure. The flow rate was 1 mL/min and 20 μL of the samples were injected. The solvent system used hexane based solvent A (98.9% hexane, 1% isopropanol and 0.1% acetic acid, v/v/v) and isopropanol based solvent B (99.9% isopropanol and 0.1% acetic acid, v/v). After a 7 min isocratic run with solvent A for elution of neutral lipids, solvent B increased to 95% for 1 min wash and then the column was equilibrated by 100% of solvent A for 6 min. The trihexadecanoin (16:0 triacylglycerol) and triocadecadienoin (18:2 triacylglycerol) in hexane were measured using the range of 0 to 15 μg for the calibration curve. Hexane was added to the dried total lipid samples for HPLC injection. Samples were stored at −20 °C.

2.7. Starch assay

Ten mg of dried algae were homogenized using a mortar and pestle with liquid nitrogen. Three mL of 90% ethanol was added and heated at 70 °C for 20 min. The ethanol mixture was cooled at room temperature and centrifuged at 2,000*g* for 5 min. The chlorophyll containing supernatant was discarded. Next, 0.5 mL of DMSO and 0.1 mL of 8 M HCl were added and heated at 60 °C for 15 min for starch solubilization and cooled at room temperature. One mL of deionized water was added to the mixture and then adjusted to pH 4–5 by 5 M NaOH in combination with 2 mL of deionized water. The mixture was centrifuged at 2,000*g* for 5 min and the supernatant was applied by a commercial starch assay kit (Starch Assay Kit SA-20; Sigma–Aldrich, Mi, USA) and total starch was quantified.

2.8. Protein determination

Three mL of 0.5 N NaOH was added to 10 mg of dry algae and homogenized using a mortar and pestle. The extracted samples were incubated at 80 °C for 10 min and mixed occasionally then allowed to cool to room temperature. The mixture was centrifuged at 2,000*g* for 5 min and the supernatant was transferred to a new tube and used for protein determination. The protein concentration was quantified by the Bradford assay (Bio-Rad, CA, USA) (Bradford, 1976).

3. Results and discussion

3.1. Optimization and characterization of ADE

Biochemical analysis of ADE from beef steers cattle waste revealed the presence of micronutrients suitable for supporting algal growth. The raw ADE collected from anaerobic digesters contained high concentration of total N (2313 mg/L), NH_3 (893 mg/L), P (1119 mg/L), K (1303 mg/L), Ca (2385 mg/L) and other micronutrients (Table 1). Additionally, regardless of diet fed to cattle and resulting manure, effluent dry matter composition contained approximately doubl in the inorganic mineral concentrations in comparison to input manure form due to the loss of water during anaerobic digestion and conversion of the organic carbon to methane. Multiple studies have demonstrated similar effluent:water ratios as what was chosen in this study for optimal growing conditions for alga. Microalga isolate *Chlorella vulgaris* YSW-04 grown on a 20% concentration of piggery wastewater effluent produced more biomass and lipid while increasing nutrient removal in comparison to culture conditions ranging from 20 to 80% effluent (Ji et al., 2012). Industry effluent with 10–15% sewage was the best growth medium for polybag cultivation of three mixotrophic algal strains *Chlamydomonas globosa*, *Chlorella minutissima* and *Scenedesmus bijuga* (Chinnasamy et al., 2010).

Due to the high nutrient concentration, the ADE needed to be diluted with deionized water to a working concentration of 10% ADE which adjusted the nitrogen and phosphorus contents to be in the same range as synthetic BBM ($NaNO_3$: 250 mg/L and KH_2PO_4/K_2HPO_4: 250 mg/L, Table 1). Cattle ADE supplied the native organic compounds nitrogen and phosphorus replacing $NaNO_3$ and K_2HPO_4/KH_2PO_4 found in the artificial components of BBM. Additionally, cattle ADE relies on high NH_3 and total nitrogen concentrations as a nitrogen source instead of its low sodium nitrate concentration. In contrast, sodium nitrate serves as the primary nitrogen source while the NH_3 concentration is low in BBM ($NaNO_3$ 250 mg/L vs. $(NH_4)6Mo_7O_{24}$ $4H_2O$ 1.74 mg/L).

Animal wastes such as cattle manure contain most required nutrients to accommodate productive algal growth while serving as low cost algae cultivation medium (Chinnasamy et al., 2010;

Table 1
Comparison of media components between ADE and BBM.

ADE components	mg/L
Total N	2313
NH_3	893
$NaNO_3$	0.01
P	1119
K	1303
S	395
Ca	2385
Mg	635
Na	321
Zn	36
Fe	324

BBM components	mg/L
$NaNO_3$	250
$CaCl_2{\cdot}2H_2O$	25
$MgSO_4{\cdot}7H_2O$	75
K_2HPO_4	75
KH_2PO_4	175
NaCl	25
KOH	31
Na_2EDTA	50
$FeSO_4{\cdot}7H_2O$	4.98
H_3BO_3	11.42
$ZnSO_4{\cdot}7H_2O$	17.64
$MnCl_2{\cdot}4H_2O$	2.88
$CuSO_4{\cdot}5H_2O$	3.14
$(NH_4)6Mo_7O_{24}{\cdot}4H_2O$	1.74
$CoCl_2{\cdot}6H_2O$	0.8

Kebede-Westhead et al., 2006; Wilkie, 2002). Considering the reproducible pattern of higher alga biomass production from the integration of anaerobic digestion and poultry manure, as well as aqueous extract of poultry litter (Bhatnagar et al., 2010; Mahadevaswamy and Venkataraman, 1986), exploration of organic media types like cattle ADE seems warranted and projects an optimistic platform for better accessing the balance of biomass, input nutrients and output production.

3.2. Biomass production and productivity of Chlorella strains

C. sorokiniana CS-01, *C. sorokiniana* UTEX 1230 and *C. sorokiniana* UTEX 2714 were grown in 80 L HBs in ADE and BBM for 24 days, in an open cultivation system (Fig. S1). Polyethylene bags were used as photobioreactors in this study to maximize alga cultivation considering their overall biomass productivity has been shown to exceed alternative vertical tank reactors and raceways (Chinnasamy et al., 2010). 8 L of 10% ADE and BBM were prepared in HBs and aerated constantly at ~30 L/min (Fig. S1). After applying four million cells/mL of each respective Chlorella strain into both media types, the three Chlorella strains were grown for 24 days. The Chlorella species in BBM were visibly pale green at day-1 and gradually became dark green by day-6. The same Chlorella species at day-6 in 10% ADE maintained the dark brown color of the native input ADE. The cloudy media masked the green alga therefore preventing any visible green color. However, Chlorella strains in both media types were dark green at day-10. Interestingly, BBM and ADE cultures had similar OD and were visually indistinguishable at day-21 (Fig. S1).

Biomass production was compared in the three Chlorella strains grown with BBM and 10% ADE conditions (Fig. 1). The maximum biomass production of *C. sorokiniana* was measured at 313–387 mg/L in poultry ADE (Singh et al., 2011) while various *C. vulgaris* strains yielded 0.52–0.765 g/L in citric acid effluent (Li et al., 2013). The biomass of *C. sorokiniana* CS-01 under both 10% ADE and BBM conditions reached 280 mg/L (cell density: 50–60 million cells/mL) at day-16 before decreasing in cell number. Both biomass of *C. sorokiniana* UTEX 1230 and UTEX 2714 increased to 360 mg/L (cell density: 80–90 million cells/mL) under BBM conditions. In comparison, the biomass of *C. sorokiniana* UTEX 1230 reached 280 mg/L under 10% ADE treatment (cell density: 60 million cells/mL), while the biomass of UTEX 2714 yielded 150 mg/mL (cell density: 25 million cells/mL), approximately half the biomass of CS-01 and UTEX 1230. Although under BBM conditions the growth of UTEX 2714 rivaled UTEX 1230, the biomass of UTEX 1230 and CS-01 showed greater production in 10% ADE.

Alternative studies have demonstrated that biomass productivity values increase with the addition of cattle manure effluent to approximately 37–66 mg L^{-1} d^{-1} in *C. sorokiniana* compared to poultry ADE (Singh et al., 2011). Biomass productivities were compared in the three Chlorella strains under 10% ADE and BBM conditions in 80 L HBs (Table 2). The biomass productivities in all three Chlorella strains in BBM were 16–21 mg L^{-1} d^{-1}. Under ADE conditions, biomass productivity of UTEX 1230 and CS-01 were 13–17 mg L^{-1} d^{-1}, while UTEX 2714 was 9 mg L^{-1} d^{-1}. This observed relationship indicates that ADE marginally suppressed the growth of UTEX 1230 and CS-01; however, it inhibited growth of UTEX 2714. Propionic acid originating from ammonium proprionate in ADE may alleviate growth inhibition as previously described (Hu et al. 2012). It has been reported that some of *C. sorokiniana* cannot degrade propionic acid (Imase et al., 2008) and therefore the growth difference between UTEX 1230, CS-01 and UTEX 2714 under ADE treatment may be explained by the degradation of propionic acid to acetate.

3.3. Chlorella nutrient uptake in BBM and ADE

The nutrient uptake of total phosphorus (TP), ortho-phosphate (PO_4-P), total nitrogen (TN), nitrate/nitrite (NO_2/NO_3-N) and ammonium (NH_3-N) were measured in three Chlorella strains in comparison of 10% ADE and BBM conditions (Fig. 2). TP and PO_4-P remained constant at 70 mg/L during growth of all Chlorella strains in BBM condition, while in 10% ADE the contents of TP and PO_4-P decreased from 50 to 20 mg/L and from 30 to 18 mg/L, respectively by day-5 and remained at these levels during the rest of the growth. The difference of phosphorus removal between BBM and ADE is accounted for by the additional conversion step of phosphorus pentoxide to PO_4-P in ADE. While KH_2PO_4/K_2HPO_4 in BBM converted immediately to PO_4-P, the expense of the extra conversion step in ADE results in the retention of an amount of phosphorus pentoxide in the media that is not converted to PO_4-P. Phosphorus removal of TP and PO_4-P were 61–65% and 47–58%, respectively under 10% ADE and 7–13% and 13–24%, respectively, under BBM (Table 2). Phosphate removal in ADE was demonstrated by the decrease in biomass (53–75%) and PO_4-P (46–68%). In contrast, biomass phosphorus decrease in BBM was less significant measuring 2–3% TP and 0.2–5% PO_4-P (Table 2). Growth rates of many microalgae species are independent of phosphate concentrations when measured above 0.03 mg/L (Hu et al., 2012), however phosphorus pentoxide has been recognized as the effective fertilizer in plants. Therefore phosphorus pentoxide uptake in ADE may be more favorable than KH_2PO_4/K_2HPO_4 in BBM. TN decreased from 70–90 to 15 mg/L in 10% ADE, while the nitrogen level increased by 20 mg/L in the three Chlorella strains in BBM. Nitrogen content of NO_2/NO_3-N decreased from 55 to 10 mg/L under BBM while the nitrogen level was beneath detection in the three Chlorella strains in 10% ADE. Furthermore, the NH_4-N content decreased from 90 to 20 mg/L in 10% ADE but increased from 15 to 25 mg/L in BBM in all Chlorella strains. Nitrogen removal was compared between 10% ADE and BBM in all Chlorella strains (Table 2). Nitrogen removal of TN and NO_2/NO_3-N measured 85–89% from 10% ADE and 90–95% from BBM, respectively, in the three Chlorella strains. The decrease in biomass TN was

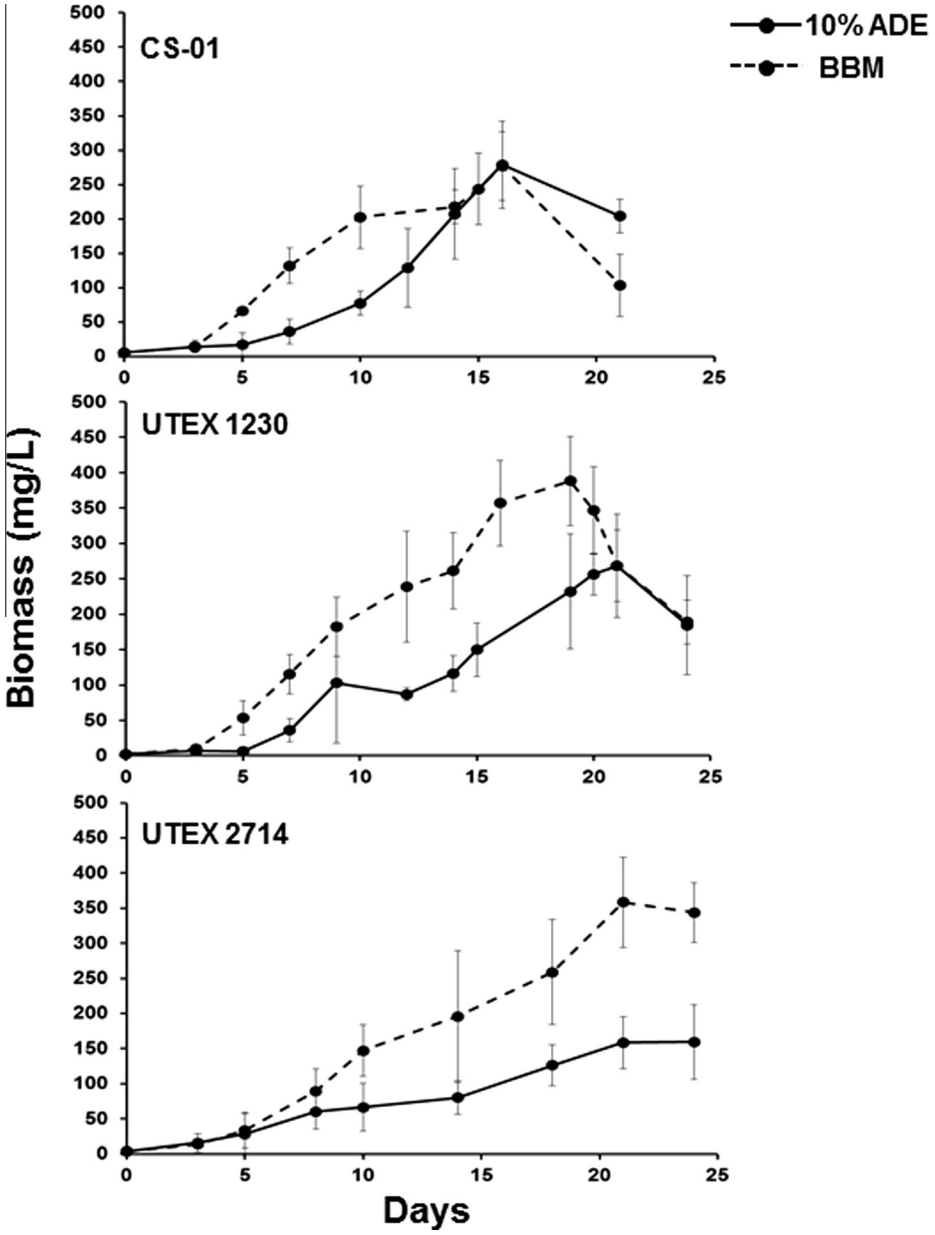

Fig. 1. Growth curves of *C. sorokiniana* CS-01, *C. sorokiniana* UTEX 1230 and *C. sorokiniana* UTEX 2714. Dotted line: BBM; Solid line: 10% ADE. Biomass was measured as ash free dry weight. Error bars; average of three biological replicates ± standard deviation.

Table 2
Comparison of biomass productivity and nutrient removal (%) from the media of *C. sorokiniana* CS-01, UTEX 1230 and 2714 (% of AFDW biomass).

Samples	Biomass Productivity (mg/L/d)	PO_4-P (%)	TP (%)	NH_3-N (%)	NO_2/NO_3-N (%)	TN (%)
C. sorokiniana CS-01						
BBM	16.31	23.52 (2.58)	7.33 (0.16)	n.d.	90.24 (81.75)	n.d.
10% ADE	16.40	47.03 (60.36)	61.31 (57.53)	74.70 (74.60)	n.d.	85.46 (69.38)
C. sorokiniana UTEX 1230						
BBM	21.00	13.34 (1.49)	12.98 (2.55)	n.d.	89.99 (89.57)	n.d.
10% ADE	12.77	55.82 (68.07)	64.98 (74.61)	64.97 (79.10)	n.d.	88.65 (71.15)
C. sorokiniana UTEX 2714						
BBM	17.53	19.19 (3.34)	10.45 (4.77)	n.d.	95.41 (87.75)	n.d.
10% ADE	9.37	57.70 (46.39)	64.10 (52.66)	72.17 (58.23)	n.d.	87.35 (60.55)

n.d.: not detectable.

20% lower (61–71%) while similarly NO_2/NO_3-N decreased 82–90%. The significant NO_2/NO_3-N removal from BBM and decrease in biomass are accredited with the larger final biomass production and cell counts. The NH_4-N removal from 10% ADE was 65–75% while the amount of NH_4-N in biomass was reduced 58–75%, which strongly indicates that NH_4-N was most likely utilized as a nitrogen

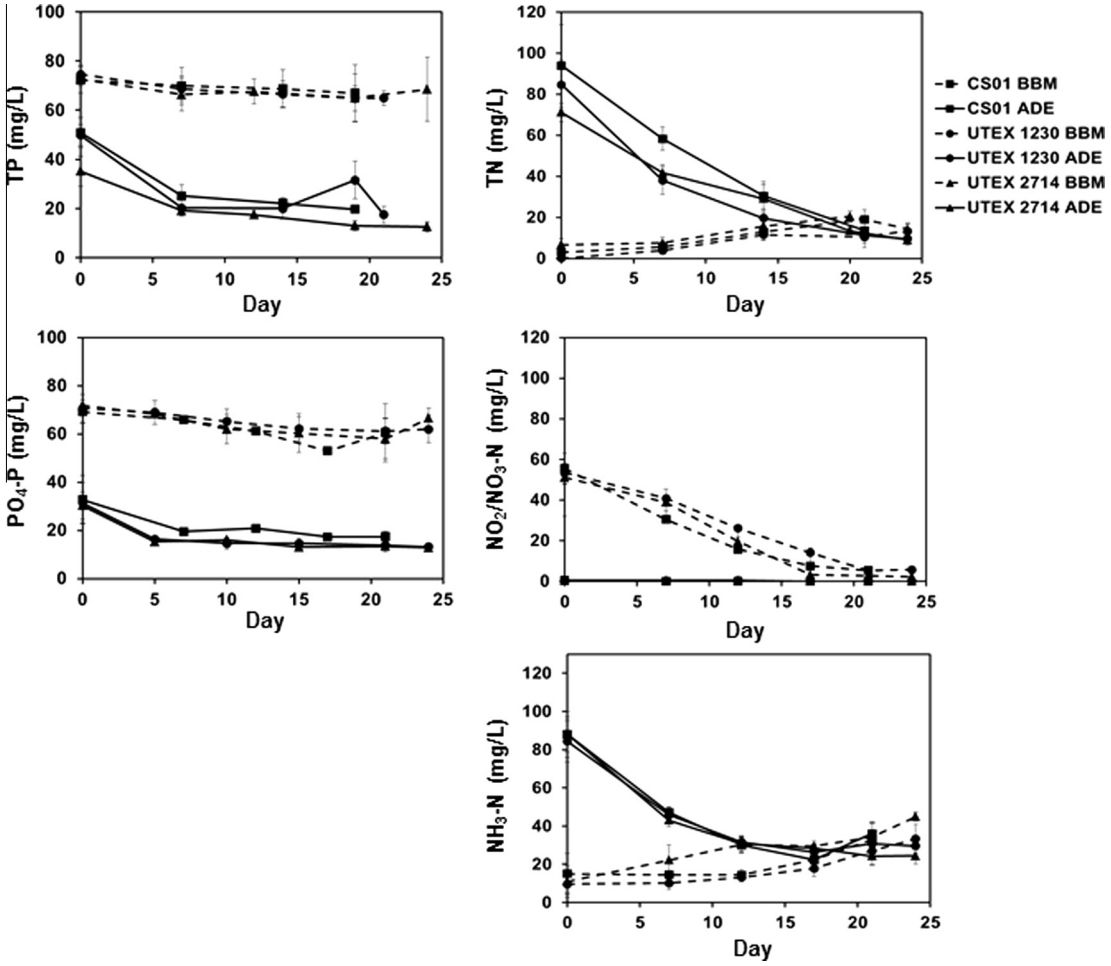

Fig. 2. Uptake of total phosphorus (TP), total nitrogen (TN), ortho-phosphorus (PO_4-P), nitrate/nitrite (NO_2/NO_3-N) and ammonium (NH_3-N) in *C. sorokiniana* CS-01, UTEX 1230 and UTEX 2714 culture during growth. Dotted line: BBM; Solid line: 10% ADE. Error bars; average of three biological replicates ± standard deviation.

source from ADE while the trace amounts of nitrogen in ADE suggest less nutrient dependence. Comparison of the three Chlorella strains in ADE showed UTEX 2714 having the lowest biomass, which was taken up PO_4-P, TP and NH_4-N 10–20% lower than other two strains. This further suggests its inability to effectively utilize the available nutrients and might also effect to the less growth under ADE condition. The ammonia production from cell death or degradation may be responsible for the TN and NH_4-N levels following day-15 in BBM. Evidence suggests that the degree of nutrient removal may be influenced by effluent types and Chlorella strains. For example, rates of nitrogen and phosphorus removal of *C. sorokiniana* were 50–68 and 1.4–2.6 mg/L respectively, from poultry manure ADE (Singh et al., 2011). Removal of TN, TP and NH_4-N of *Chlorella vulgaris* were 99%, 96% and 100% from 10% citric acid effluent (Li et al., 2013). TN, TP and NH_4-N removals of *Chlorella* sp. were 13%, 80% and 27%, respectively, from fermented swine manure (Hu et al., 2012). Additionally, it has been reported that microalgae growth could be inhibited by highly concentrated ammonium ion and nitrite of which either could be assimilated even at very low concentrations (de Godos et al., 2010). These results identify nitrogen sources as the limiting reagent for biomass and biochemical production in alga cultivation.

3.4. Comparison of lipid, protein and starch contents in BBM and ADE

In order to access the output biochemical products under BBM and ADE conditions in three Chlorella strains, the contents of lipid, protein and starch were compared during cultivation (Fig. 3). Contents were measured as ash free dry weight (AFDW) of algae following the removal of the ADE residual contamination from the collected algae. The total lipid contents in the three Chlorella strains were 25–35% of AFDW under both BBM and 10% ADE conditions (Fig. 3a). The lipid levels of the three Chlorella strains are equal to the reported 30% DW of *Chlorella* sp. in fermented swine manure (Hu et al., 2012). The protein level in the BBM group reached 50–60% AFDW by day-21 and decreased to 30–40% AFDW by day-24, while the protein content in 10% ADE decreased from 40 to 30–35% AFDW during growth of the three Chlorella strains (Fig. 3b). In another study, the protein levels in different species of marine microalgae ranged between 50–60% DW when grown in chemically formulated F2 medium (Renaud et al., 2002), which were congruent with the protein results in the Chlorella strains under BBM conditions. In contrast, starch levels under 10% ADE increased from 2–7% to 20–25% over the course of growth but remained at 10–15% in all Chlorella strains grown in BBM cultivation (Fig. 3c). These changes in composition demonstrated that under ADE conditions the starch production was more efficient. These results indicate that algae metabolism is dedicated to storing intracellular starch in ADE supplimented media and may represent the conversion of some of the organic carbon sources in ADE to starch or that the Chlorella strains utilize ammonia as a nitrogen source more efficiently that the nitrate in BBM and thus are in a greater starch producing "nitrogen replete" mode of growth.

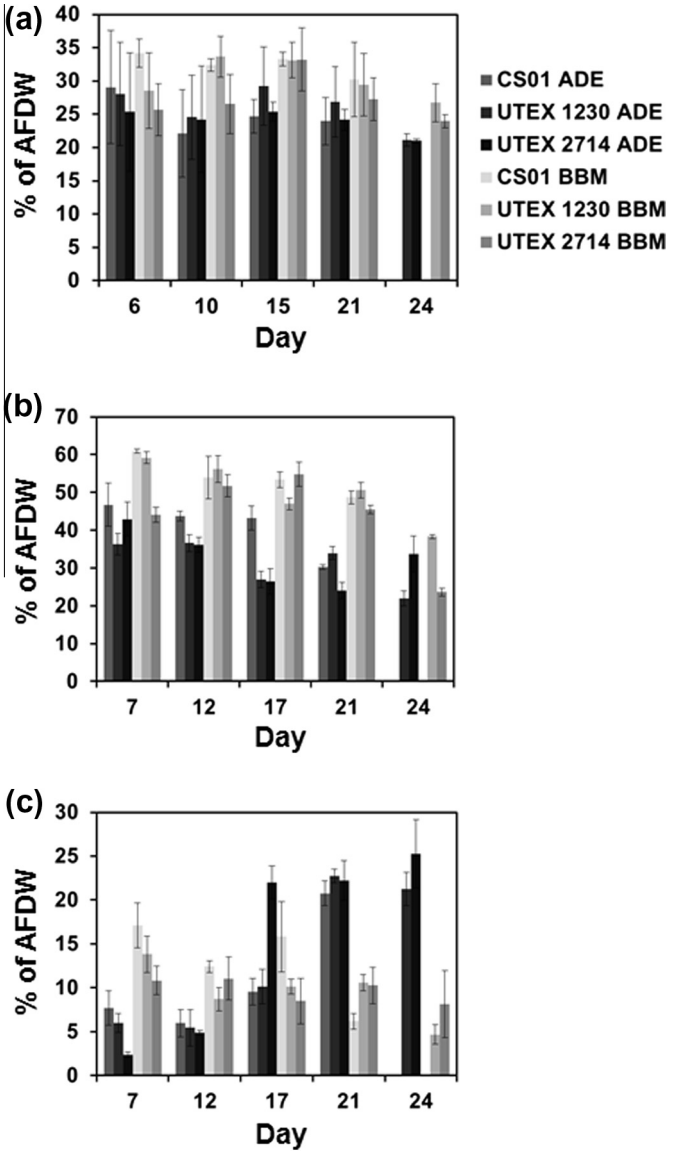

Fig. 3. Comparison of protein (a), lipid (b) and starch (c) in *C. sorokiniana* CS-01, UTEX 1230 and 2714 under BBM (light colored bars) and 10% ADE (dark colored bars) conditions during growth. Contents were expressed as percentage of ash free dry weight. Error bars; average of three biological replicates ± standard deviation.

3.5. Lipid analyses in BBM and ADE

Total lipid contents (25–35% AFDW) were similar in the three Chlorella strains grown in either BBM and 10% ADE conditions (Fig. 3a). Closer examination of the contents of FAME total lipid and TAG contents in the lipids of the three Chlorella strains was pursued to determine if the BBM and ADE media had an effect on lipid composition (Fig. 4). The contents of FAME total lipid reached 15% AFDW by day-15 in both CS-01 and UTEX 1230, and in UTEX 2714 at day-20. FAME total lipid then decreased to 4–9% AFDW during the final days of BBM cultivation. However, under 10% ADE conditions the contents of FAME total lipid increased from 3 to 8–12% AFDW and decreased to 8–10% AFDW during the final days of cultivation in the three Chlorella strains (Fig. 4a). Furthermore, the contents of FAME TAG of CS-01 and UTEX 1230 were either under the detection limit or less than 2% AFDW in both BBM and 10% ADE conditions, respectively. The FAME TAG contents of UTEX 2714 were under 2% AFDW in BBM but increased to 6% AFDW in 10% ADE (Fig. 4b). Considering the low ratio of TAG in FAME total lipid contents, this may suggest that more membrane lipids than neutral lipids were produced.

The lipid compositions of the three Chlorella strains were compared under BBM and 10% ADE conditions during cultivation (Fig. 4c). The fatty acids were distributed in 16:0–16:3 and 18:1–18:3 fashion (carbon number: double bond number) in the three Chlorella strains. The main fatty acids detected were 16:0, 16:3, 18:2 and 18:3 in the three Chlorella strains (Hu et al., 2008; Vigeolas et al., 2012). The levels of the primary fatty acids, 16:0, 18:1 and 18:2 increased both under BBM and 10% ADE while the levels of fatty acid groups 16:3 and 18:3 decreased in all Chlorella under 10% ADE and BBM conditions during growth. The main fatty acid compositions in the *Chlorella* sp. were 16:0, 18:0, 18:2 and 18:3 at 21%, 28%, 16% and 16%, respectively, in total fatty acids under fermented swine manure (Hu et al., 2012). The fatty acid compositions of *Chlorella* sp. under citric acid were 16:0, 20:5 and 22:5 at 18%, 25% and 17%, respectively, of total fatty acids (Li et al., 2013). The predominant fatty acids composed of C16 and C18 chain length present in the Chlorella strains are appropriate for the biofuel and biodiesel application. Most conventional fuels including petroleum and diesel contain aliphatic hydrocarbons that are chemically similar to the fatty acid components of TAG (Durrett et al., 2008). The presence of polyunsaturated fatty acids in *Chlorella sorokiniana* strains may also provide for superior nutritional values in food applications.

The neutral lipid classes in the three Chlorella strains grown in 10% ADE, were also monitored by HPLC-ELSD (Fig. 5). TAG quantification was determined by scraping the TAG band on the developed thin layer chromatography in the GC/MS methods. The HPLC–ELSD method was established for rapid neutral lipid measurement and is able to detect different types of neutral lipids such as triacylglycerol (TAG), diacylglycerol (DAG), free fatty acids and also phospholipid (PC) and glycolipid (MGDG and DGDG) during the run (Kobayashi et al., 2013). In Chlorella strains such as *C. kessleri*, DAG accumulated as the main neutral lipids while there were low levels of TAG accumulation (Kobayashi et al., 2013). Contrary to *C. kessleri*, TAG was the main lipid class detected in *C. sorokiniana* strains (Fig. 5). The ability of high TAG production makes *C. sorokiniana* strains a desirable candidate for efficient lipid production and therefore biofuel application.

3.6. Characterization of Chlorella strains in BBM and ADE

The contents of biomass, protein, starch, lipid, FAME total lipid and TAG were compared in all three Chlorella strains under BBM and ADE conditions (Table 3). Although the lipid contents ranged from 24–30% AFDW in all Chlorella strains in both media types, the levels of FAME total lipid were approximately 10% AFDW in all Chlorella strains under both ADE and BBM conditions except UTEX 2714, which was 18% AFDW under BBM conditions. Total fatty acid contents were measured in another experiment at 7.5–11% AFDW in *Chlorella* sp. in a different manure-based effluent (Hu et al., 2012). Another study measured total fatty acid contents at 8.2–12.4% AFDW in *C. sorokiniana* under different poultry ADE (Singh et al., 2011). The difference between the contents of lipids measured gravimetrically and FAME total lipid may be explained by the high production of non-fatty acid types of lipids such as chlorophyll, carotenoids or others during growth. In contrast to the low FAME total lipid levels, the contents of protein were greater than 24% AFDW under both BBM and 10% ADE conditions in all three Chlorella strains. The starch contents were approximately 20% AFDW under 10% ADE conditions in contrast to less than 10% AFDW in BBM in all three Chlorella strains. The carbohydrate levels of *C. sorokiniana* were measured at 18–22% DW under poultry ADE, although those from the different marine microalgae showed 4–14% DW in chemically formulated F2 medium (Renaud

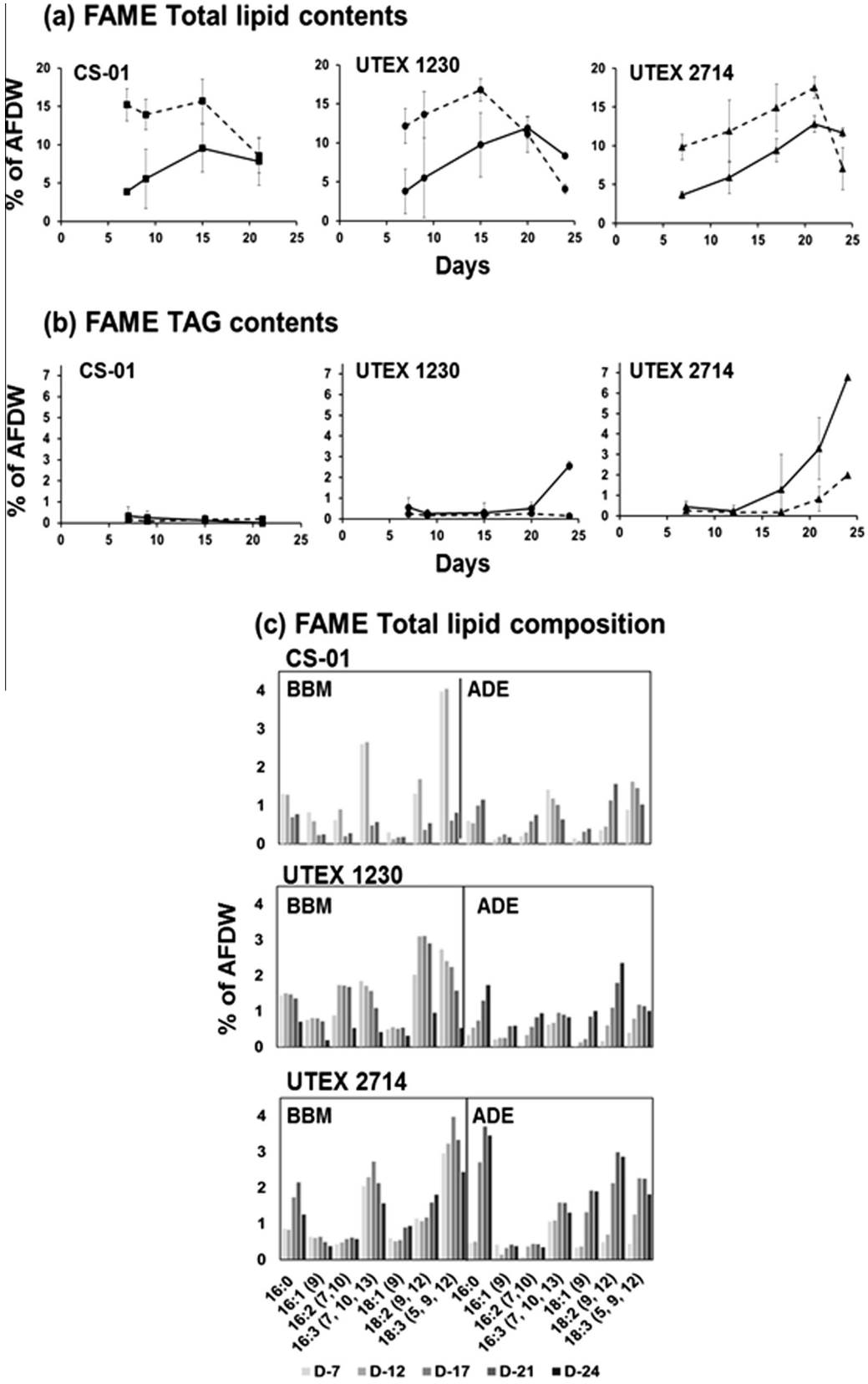

Fig. 4. Accumulation of FAME total lipid (a) and TAG (b) in *C. sorokiniana* CS-01, UTEX 1230 and UTEX 2714 during growth. Dotted line: BBM; Solid line: 10% ADE. Contents were expressed as percent AFDW. Error bars; average of three biological replicates ± standard deviation. Comparison of FAME total lipid composition in *C. sorokiniana* CS-01, UTEX 1230 and 2714 under BBM and 10% ADE conditions during growth (c). Contents were expressed as percent total fatty acids.

et al., 2002). *C. sorokiniana* and *C. minutissima* grown with poultry waste ADE yielded starch and protein contents as high as 21–26% and 37–30% AFDW, respectively while the levels of lipid were lower at 4–6% (Singh et al., 2011). In contrast, other reports have

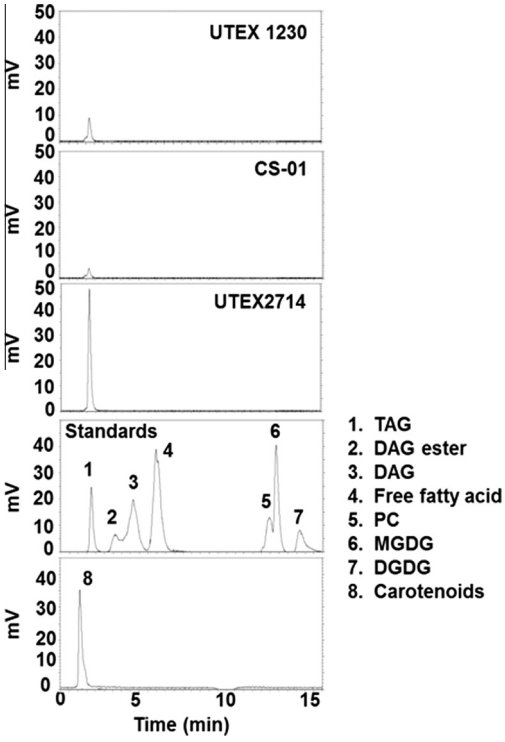

Fig. 5. Separation of lipid classes in *C. sorokiniana* CS-01, UTEX 1230 and UTEX 2714 and comparison with authentic standards by HPLC-ELSD. (1) Triacylglycerols (TAG, 1 μg); (2) diacylglycerol (DAG) ester; (3) diacylglycerol (DAG, 4 μg) (4) free fatty acids (palmitic acid 2 μg) (5) phosphaphatidylcholine (PC, 20 μg) (6) monogalactosyldiacylglycerol (MGDG, 20 μg); (7) digalactosyldiacylglycerol (DGDG, 40 μg); (8) carotenoids extracted from *Chlamydomonas reinhardtii*.

shown high accumulation of starch and FAME TAG but low levels of protein under nitrogen deprivation in *Chlamydomnous reinhardtii* (Msanne et al., 2012). This fluctuation of starch and protein content is dependent on nitrogen availability. These macromolecules are first synthesized and accumulated as stored energy followed by lipid production which may be promoted by nitrogen deprivation. Therefore in order to obtain optimal levels of FAME TAG for biofuel application, modification of media nutrients must be considered. The biomass production of UTEX 1230 and CS-01 were approximately 280 mg/L under 10% ADE conditions, while the biomass production of UTEX 2714 in 10% ADE yielded half that cell density. Considering the ability to exploit the nutrients offered by cattle ADE, *C. sorokiniana* UTEX 1230 is an optimal candidate for animal feed rather than bioenergy due to the higher production of biomass, protein and starch while exhibiting reduced lipid synthesis.

4. Conclusion

Three *C. sorokiniana* strains were compared in 10% ADE from cattle manure and synthetic BBM. Biomass was produced at 270 mg/L in UTEX 1230 and CS-01 however suppressed growth of UTEX 2714 more than 50% in ADE. Starch contents were 22% in ADE but less than 11% AFDW under BBM. Under both growth conditions in the three Chlorella strains, protein content measured greater than 24% while FAME total lipids were 12% AFDW. The Chlorella biomass under ADE was rich in starch and protein but lower in lipids and therefore may be more suitable for animal feed application.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.10.032.

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Table 3
Comparison of biomass production and contents of protein, starch, lipid, FAME total lipid and TAG in *C. sorokiniana* CS-01, UTEX 1230 and 2714 at day-21 under BBM and 10% ADE conditions (Ave. of % AFDW ± standard deviation).

Samples	Biomass (mg/L)	Lipid (%)	FAME total lipid (%)	FAME TAG (%)	Protein (%)	Starch (%)
C. sorokiniana CS-01						
BBM	277.23 ± 50.01	30.25 ± 5.58	8.57 ± 2.26	0.18 ± 0.08	48.70 ± 1.70	6.20 ± 0.89
10% ADE	278.84 ± 63.48	23.95 ± 3.58	7.83 ± 3.15	1.48 ± 0.17	30.23 ± 0.57	20.77 ± 1.41
C. sorokiniana UTEX 1230						
BBM	356.91 ± 60.33	29.43 ± 4.68	11.12 ± 2.31	0.25 ± 0.11	50.59 ± 2.10	10.58 ± 0.92
10% ADE	268.25 ± 73.21	26.83 ± 5.28	11.92 ± 1.39	0.50 ± 0.30	33.94 ± 1.67	22.76 ± 0.78
C. sorokiniana UTEX 2714						
BBM	368.03 ± 64.26	27.96 ± 1.01	17.50 ± 1.39	0.82 ± 0.60	45.41 ± 1.15	10.28 ± 2.08
10% ADE	152.55 ± 37.42	24.17 ± 0.30	12.80 ± 1.06	3.29 ± 1.59	24.03 ± 2.10	22.22 ± 2.23

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