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Bacterial production of free fatty acids from freshwater macroalgal cellulose

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Abstract

The predominant strategy for using algae to produce biofuels relies on the overproduction of lipids in microalgae with subsequent conversion to biodiesel (methyl-esters) or green diesel (alkanes). Conditions that both optimize algal growth and lipid accumulation rarely overlap, and differences in growth rates can lead to wild species outcompeting the desired lipid-rich strains. Here, we demonstrate an alternative strategy in which cellulose contained in the cell walls of multicellular algae is used as a feedstock for cultivating biofuel-producing micro-organisms. Cellulose was extracted from an environmental sample of *Cladophora glomerata*-dominated periphyton that was collected from Lake Mendota, WI, USA. The resulting cellulose cake was hydrolyzed by commercial enzymes to release fermentable glucose. The hydrolysis mixture was used to formulate an undefined medium that was able to support the growth, without supplementation, of a free fatty acid (FFA)-overproducing strain of *Escherichia coli* (Lennen et. al 2010). To maximize free fatty acid production from glucose, an isopropyl -D-1-thiogalactopyranoside (IPTG)-inducible vector was constructed to express the *Umbellularia californica* acyl–acyl carrier protein (ACP) thioesterase. Thioesterase expression was optimized by inducing cultures with 50 μ M IPTG. Cell density and FFA titers from cultures grown on algae-based media reached 50% of those (~90 μ g/mL FFA) cultures grown on rich Luria–Bertani broth supplemented with 0.2% glucose. In comparison, cultures grown in two media based on AFEX-pretreated corn stover generated tenfold less FFA than cultures grown in algae-based media. This study demonstrates that macroalgal cellulose is a potential carbon source for the production of biofuels or other microbially synthesized compounds.

Keywords

Biofuel; Algae; Fatty acid; *Escherichia coli*; Thioesterase

Introduction

Recently, oil price volatility, demand for national energy security, and interest in global sustainability have spurred the development of bioprocessing as an alternative to traditional petroleum processing. One route to sustainably synthesizing fuels and chemicals makes use of renewable carbon sources (e.g., biomass) instead of fossil fuels. The development of a robust, inexpensive, and environmentally sustainable biomass industry is central to this strategy, and efforts to capture existing biomass, to recycle waste materials, and to cultivate new biomass crops are well underway (Perlack et al. 2005). Accordingly, new biological and chemical processes are required to isolate useable substrates and to convert them into relevant compounds (Houghton et al. 2006).

The microbial conversion of sugars to small organic compounds is currently the most prevalent strategy for producing biofuels and chemicals. While research to develop microorganisms capable of converting cellulosic biomass to ethanol is ongoing (Vispute and Huber 2008), focus has shifted to the production of direct-replacement hydrocarbon fuels that are more compatible with existing engines and fueling infrastructure. Recent work has demonstrated metabolic engineering strategies for producing next-generation biofuels including free fatty acids (FFAs) (Lennen et al. 2010; Lu et al. 2008; Steen et al. 2010), long-chain fatty alcohols and esters (Steen et al. 2010), long-chain olefins (Beller et al. 2010; Rude et al. 2011), short-chain alcohols, e.g., butanol (Atsumi and Liao 2008; Inui et al. 2008; Steen et al. 2008) and isobutanol (Atsumi et al. 2008), and isoprenoids (Withers and Keasling 2007). Traditional strain optimization and modern functional genomics approaches are currently being used to identify metabolic and regulatory bottlenecks in an

effort to further improve yield, product tolerance, and the commercial viability of strains incorporating these strategies. The major challenge to using these strains to produce biofuels is identifying abundant sources of glucose that can be used to formulate growth media.

Cellulosic sources of carbon, including agricultural residues (e.g., corn stover (CS), wood chips), perennial grasses (e.g., switchgrass, *Miscanthus* sp.), and fast-growing trees (e.g., poplar), have been promoted as viable feedstocks for sustainable biofuel production systems (Hill 2009). All of these carbon sources are rich in cellulose, but each also contain lignin, a structural polymer which is both a barrier to accessing the sugars in the plant cell wall and a source of compounds that are toxic to industrial microorganisms (Anderson and Akin 2008). In contrast to terrestrial plants, cladophoralean macro-algae do not need lignin for support and contain a cell wall composed primarily of crystalline cellulose (reviewed by Mihranyan 2011), making them an attractive carbon source for biofuel production by microorganisms. Species of fast-growing freshwater macroalgae are currently an environmental problem in freshwater lakes, where blooms have been correlated with nitrogen and phosphorus runoff (Sfriso et al. 1987). While wild sources are not likely to provide an economically viable source of glucose, the cultivation of macroalgae in wastewater treatment facilities could simultaneously address two environmental challenges—supplying ample cellulosic biomass and removing residual nitrogen and phosphorus from wastewater.

Periphyton is a natural aquatic community that consists of algae and bacteria and that grows on fixed surfaces at the bottoms or edges of water bodies. The branched, filamentous green alga *Cladophora glomerata* (*Cladophorales*, *Ulvophyceae*, *Chlorophyta*) often dominates the periphyton of eutrophic freshwaters, where it can reach several meters in length and can produce up to 600 g/m² of dry standing crop biomass (reviewed by Higgins et al. 2008). Often adapted to hypereutrophic conditions, *Cladophora* and the associated periphyton algae can tolerate high concentrations of phosphorus, nitrogen, and heavy metals (Entwistle 1989; Lamai et al. 2005). These organisms often grow well in wastewater effluents. *Cladophora*-dominated periphyton is also rich in extractives useful in the production of biofuels. *Cladophora* cell walls, for example, are dominated by cellulose allomorph I, which is more readily degraded by acid treatment and/or fungal cellulases than the allomorph II-dominated celluloses of terrestrial plants (Igarashi et al. 2007, 2006). The physical characteristics and molecular interactions of *Cladophora* cellulose are well understood (Akerholm et al. 2004) because this high-crystallinity algal cellulose has current pharmaceutical use as a binding agent for medications dispensed in pill form (Gustafsson et al. 2003; Mihranyan et al. 2007) and industrial (Mihranyan 2011) applications.

This manuscript describes our development of macro-algal cellulose as a carbon source that supports the growth of an engineered *Escherichia coli* strain (Lennen et al. 2010) capable of producing free fatty acids, a precursor to diesel hydrocarbons (Fig. 1). In this work, cellulose fibers from an environmental sample of *Cladophora*-dominated periphyton were converted to a glucose-based growth medium. Then, an induction system controlling thioesterase expression was optimized for the new medium. Finally, the algal cellulose medium was compared to other undefined media including those derived from ammonia fiber expansion (AFEX)-pretreated corn stover.

Materials and methods

Media, cloning enzymes, and strains

Chemicals and reagents were purchased from Fisher Scientific (Waltham, MA, USA) or Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. All enzymes for cloning were purchased from New England Biolabs (Ipswich, MA, USA). The bacterial strains and plasmids used in this study are summarized in Table 1.

Algal cellulose collection and extraction

Biomass dominated by the branched, filamentous green alga *C. glomerata* (Ulvophyceae) was collected from near-shore rocks in Lake Mendota, WI, USA. Cellulose was purified from approximately 400 g of wet biomass by the bleaching and base treatment described in Mitranyan et al. (2004), except that a final acid hydrolysis step was omitted.

Enzymatic hydrolysis

AFEX-pretreated corn stover (CS) and hydrolytic enzymes were provided as gifts from Bruce Dale (Michigan State University, East Lansing, MI, USA). Hydrolysis of feed-stock material was performed using protocols adapted from (Lau and Dale 2009). For corn stover-based hydrolysates, cellulose glucan loading was 3% by weight (i.e., 30 g/kg hydrolysate mixture). This level of cellulose loading corresponded to 8.3% (w/w) solids in the hydrolysis mixture. The approximate cellulose content of the CS was provided by Bruce Dale.

The cellulose content of the algae was assumed to be nearly 100% based on bright appearance in crossed polarizers (Fig. 1) and Calcofluor White analysis (Fig. 2). Calcofluor White reagent (Polysciences Inc., Warrington, PA, USA) is a fluorochrome that specifically binds to fibrillar β -glucans (Wood and Weisz 1984). It is widely used to quantify β -glucans by flow-injection analysis (Kim and Inglett 2006). Here, random samples of the dried algae cellulose cake were treated with freshly made Calcofluor White solution (0.01% in 0.1 M K_2HPO_4) and examined using bright-field and fluorescence microscopy (Zeiss Axioplan; exciter filter 365, chromatic beam splitter FT 395, barrier filter LP420).

Each of the hydrolysis mixtures was prepared in a 2-L baffled shake flask containing 1 L of hydrolysis mixture (8.52 mL deionized water per gram of CS). Each hydrolysis mixture was autoclaved at 121 °C for 40 min. After autoclaving, 1 M phosphate buffer (pH 4.3) was added to the CS to produce a final mixture with 50 mM phosphate. That mixture is hereafter called “AFEX-P medium.” Alternately, a 50-mM citrate buffer was used in place of the phosphate buffer to generate “AFEX-C medium.” To hydrolyze each cellulose preparation, a hydrolytic enzyme mixture consisting of Accellerase 1000 (0.492 mL/g glucan), Multifect xylanase (0.009 mL/g glucan), and Multifect pectinase (0.006 mL/g glucan) was used according to hydrolysis protocols provided by Bruce Dale (Michigan State University, East Lansing, MI, USA). The components of the hydrolytic enzyme mixture are each commercial preparations available from Genencor, Inc. (Rochester, NY, USA). Each enzyme preparation was filter-sterilized prior to use. To initiate hydrolysis, one half of the required enzyme cocktail was added to the cellulose mixture, and the vessel was incubated at 50 °C for 3 h at 100 rpm in a shaking incubator. After 3 h, the remaining hydrolysis enzymes were added to the mixture, and the vessel was incubated for an additional 93 h. After incubation, the unpolished hydrolysate was centrifuged for 60 min at $8,200 \times g$ in a Beckman Avanti-J centrifuge (Beckman Coulter, Brea, CA, USA). The supernatant was rough-filtered through a 0.5- μ m filter. The filtrate was centrifuged at $8,200 \times g$ for 16 h to remove fine particulates, and the supernatant was sterile-filtered using a 0.2- μ m filter. After neutralization with solid sodium hydroxide, the polished hydrolysate was used in subsequent growth studies. Algal cellulose was similarly processed, except that neutralization with sodium hydroxide was unnecessary. One liter of culture medium was prepared using all of the cellulose isolated from the algae biomass. LB-glucose medium was prepared by supplementing the standard Luria-Bertani broth with 0.2% glucose.

Cloning and optimization of pTrc99A-BTE

An *E. coli* codon-optimized gene encoding the California bay tree (*Umbellularia californica*) acyl-ACP thioesterase (*BTE*) was described previously (Lennen et al. 2010). The thioesterase, encoded by *BTE*, cleaves C12 and C14 saturated and unsaturated acyl groups

from the corresponding acyl-ACPs to yield free fatty acids (Voelker and Davies 1994). To clone *BTE* into a glucose-compatible expression vector, the fragment between the *HindIII* and *XmaI* sites in pBAD34-BTE was cloned into the pTrc99A backbone under the control of the isopropyl -D-1-thiogalactopyranoside (IPTG)-inducible *trc* promoter (Amann et al. 1988). A gene encoding a non-functional mutant BTE (H204A) was similarly cloned from pBAD34-BTE-H204A into pTrc99A.

An induction study was completed for the biomass-derived media using a Nile red assay as a proxy for fatty acid titer. Liquid cultures of the bacteria were grown overnight in Luria-Bertani broth plus 100 g/mL ampicillin from single colonies of *E. coli* MG1655 *araBAD fadD* (Lennen et al. 2010) containing pTrc99A-BTE. Black, clear-bottomed 96-well microtiter plates were filled with 190 μ L of the media to be tested and 0.5 μ L of 0.25 mg/mL Nile red in dimethyl sulfoxide. Ten microliters of the starter culture was added to each well, and the culture was then induced with IPTG at concentrations ranging from 0 to 1 mM. Cultures were started in triplicate, and microtiter plates were placed in a humidified shaker at 30 °C overnight. A Tecan Infinite M1000 plate reader (Männedorf, Switzerland) was used to measure the fluorescence (550-nm excitation, 630-nm emission) and absorbance (OD₆₀₀) of each well after 24 h.

Shake flask experiments and sampling

A single colony of MG1655 *fadD araBAD* harboring the pTrc99A-BTE plasmid was inoculated into 5 mL of LB broth+100 μ g/mL ampicillin and incubated at 37°C overnight. Baffled 500-mL shake flasks containing 100 mL of an undefined media (algae-based, LB +glucose, AFEX-C, or AFEX-P), 100 μ g/mL ampicillin, and 50 μ M IPTG were inoculated with 200 μ L of the overnight culture. Samples were taken hourly from 0 to 6 h and at 12, 24, 48, and 72 h after inoculation to measure optical density at 600 nm (OD₆₀₀) using an Eppendorf biophotometer (Hamburg, Germany). Media samples were also taken at 0, 6, 12, 24, 48, and 72 h for sugar and fatty acid analysis (described below). Samples for nitrogen and phosphorous analysis were taken from the initial media and from the endpoint media at 72 h.

Fatty acid processing and analysis

Bacterial fatty acid profiles were measured as described previously (Lennen et al. 2010). Briefly, pentadecanoic acid and heptadecanoic acid internal standards (Sigma Aldrich, St. Louis, MO, USA) were added to 2.5 mL of culture taken from each medium and were acidified with 100 μ L of glacial acetic acid. These solutions were then extracted with 5 mL of 1:1 chloroform/methanol. After vortexing, samples were spun at $1,000 \times g$ for 10 min, and the aqueous layer was removed. The organic layer was stored at -80°C. The organic layer was evaporated under nitrogen gas and samples were lyophilized for 1 h to remove all water. To convert each fatty acid to the corresponding methyl esters, 500 μ L of 10% hydrochloric acid in methanol solution was added to each sample. The samples were then heated to 80 °C in a hot water bath for 1 h, cooled to room temperature, neutralized with 5 mL of 100 mg/mL sodium bicarbonate, and extracted twice with 500 μ L hexane. Samples were separated on an Agilent 7890 GC with a 30-m $\times$ 0.25-mm DB-5 capillary column and analyzed using a 5975 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) as described previously (Lennen et al. 2010). A standard curve was generated using the Supelco 18918 fatty acid methyl ester mixture with supplemented methyl pentadecanoate and methyl heptadecanoate.

Sugar processing and analysis

Two milliliters of culture was added to a microcentrifuge tube and heated in a boiling water bath for 15 min. The samples were centrifuged at $14,000 \times g$ for 5 min, and the supernatants

were passed through a 0.22- μ m syringe filter into a new microcentrifuge tube. The samples were frozen at -20°C until the analysis could be completed. After thawing, each sample was diluted at 1:20 (for environmental media) or 1:10 (for LB+0.2% glucose) in deionized water and put into Ultra Performance Liquid Chromatography (UPLC) vials. One microliter of each sample was separated using a Waters Acquity UPLC system equipped with a Waters BEH glycan 1.7- μ m column (Waters Corporation, Milford, MA, USA) and analyzed using an evaporative light-scattering detector. The mobile phase used for this analysis was 75% acetonitrile+25% deionized water+0.2% triethylamine at pH 9.1. Xylose, glucose, and cellobiose were used to generate a standard curve at concentrations of 0.5, 1.0, 2.0, 3.0, and 5.0 mg/mL in water. Data were collected in triplicate for each sample, and concentrations were calculated using the standard curve.

Nitrogen and phosphorus quantification

Soluble reactive phosphorus was measured in filtered media samples by the ascorbic acid method 4500 P E (Association 2005). Total phosphorus was measured as soluble reactive phosphorus in unfiltered, acidified samples following persulfate digestion in an autoclave for 1 h (APHA 2005). Nitrate and nitrite were measured in filtered samples with a Shimadzu high-performance liquid chromatograph (Kyoto, Japan) equipped with an Alltech Previal Organic Acid Column (Deerfield, IL, USA) and a photodiode array detector set at 210 and 214 nm. Total nitrogen was measured as nitrate after the persulfate digestion described above.

Results

Preparation of bacterial media from purified macroalgal biomass

Periphyton was collected from near-lakeshore rocks of Lake Mendota in Wisconsin, USA. Cellulose was extracted from dewatered biomass samples according to the protocol of Mhrranyan et al. as described above (Mhrranyan et al. 2004). Fluorescence microscopy was used to identify the source and purity of the extracted cellulose. The vast majority of treated *Cladophora* community biomass appeared to be the remains of *Cladophora* cell walls, indicated by the size and shape of blue–white fluorescent structures imaged with bright-field optics and UV excitation (Fig. 2). A few filaments of the unbranched chlorophycean green algal genus *Oedogonium* were observed, but remains of other organisms were not observed. All materials visible in the bright-field imagery were also bright in crossed polarizers and fluorescent during UV excitation and displayed the same dimensions as the cell walls of *C. glomerata* or of *Oedogonium* sp., which also featured distinctive wall rings as illustrated in Graham et al. (2009). No other objects were observed to occur in the samples of the extracted material. Chemical treatments and washings had apparently removed the cytoplasmic contents and associated organisms lacking cellulosic walls. Purified algal cellulose was hydrolyzed and the material was formulated into undefined media using a protocol adapted from Lau and Dale (2009), as described above. Similar methods were used to prepare undefined media from AFEX-pretreated corn stover.

Induction study to determine the optimal level of *BTE* expression from P_{trc}

An overexpression plasmid (pTrc99A–*BTE*) containing a codon-optimized gene (*BTE*) encoding the California bay tree (*U. californica*) thioesterase was constructed as described above. The *BTE* gene encoded a thioesterase that specifically hydrolyzes C12 and C14 acyl–ACPs, diverting a portion of fatty acid biosynthesis intermediates into a free fatty acid pool (Voelker and Davies 1994). In a prior work, we have shown that an intermediate level of *BTE* expression leads to maximal FFA production in *E. coli* (Lennen et al. 2010). In this study, thioesterase expression was optimized by varying the amount of IPTG used to induce the transcription of *BTE* from P_{trc} . Nile red fluorescence was measured as an indicator of

fatty acid production. Nile red fluorescence increases with the hydrophobicity of a solution and has been used as a proxy for lipid and polyhydroxyalkanoate titers (Spiekermann et al. 1999; Greenspan et al. 1985). Induction studies were carried out in four undefined media: AFEX-P and AFEX-C (CS-based, phosphate- and citrate-buffered, respectively), *Cladophora*-derived algal media, and LB+0.2% glucose. Each media was inoculated with *E. coli* strain RL08 (MG1655 *araBAD fadD*) harboring pTrc99A-BTE. Cultures of *E. coli* RL08 harboring pTrc99A-BTE-H204A, which contains a non-functional BTE (H204A) under P_{trc} control, had fluorescence levels that were indistinguishable from the background fluorescence for all IPTG concentrations tested (Fig. 3). Cultures grown in the biomass-derived media (i.e., AFEX-P (Fig. 3a), AFEX-C (Fig. 3b), and algal media (Fig. 3c)) all exhibited maximal or near-maximal fluorescence when induced with 50 μ M IPTG. Cultures grown in LB-glucose showed strong fluorescence when induced with 0–100 μ M. Cultures grown in the presence of higher concentrations of IPTG resulted in lower fluorescence in all media. The trend in fluorescence with increasing IPTG concentration (i.e., BTE expression) is consistent with previous studies where the DNA copy number was varied (Lennen et al. 2010). In each case, the maximum fluorescence was obtained with an intermediate level of thioesterase expression. Based on these data, 50 μ M IPTG was used to induce all cultures in subsequent experiments.

Comparison of *E. coli* growth in algae-based media to those grown in undefined laboratory media and media derived from terrestrial biomass

E. coli RL08 harboring pTrc99A-BTE was grown in baffled shake flasks at 37°C to compare the growth rates and fatty acid titers over 72 h. As shown in Fig. 4a, all media showed a lag of approximately 6 h. Cultures grown in LB+0.2% glucose showed the fastest growth, reaching a maximum density at 12 h before declining between 12 and 72 h. Cultures grown in AFEX-P and AFEX-C media both grew at a slower rate than the cultures grown in LB+0.2% glucose, but between 12 and 72 h the cultures did not show the same decline in OD₆₀₀. Ultimately, LB+0.2% glucose, AFEX-P, and AFEX-C reached similar maximum OD₆₀₀ values. Cultures grown in algae-based media reached a stationary phase at 12 h and maintained a constant cell density between 12 and 72 h, albeit at about half of the cell density reached by cultures grown in other media. These data indicate that *Cladophora*-derived media, without any supplementation, are capable of supporting the growth of an industrially relevant strain of *E. coli*.

Free fatty acid production from cultures of *E. coli* grown in biomass-derived media

FFA titers were determined over a period of 72 h from cultures of *E. coli* RL08 harboring pTrc99A-BTE and grown on each medium. Lipid samples were taken by extracting culture samples in chloroform/methanol. Fatty acids present in lipid extracts were converted to methyl esters for analysis and quantification by GC/MS. Figure 4b and Table 2 (titers of fatty acid species at 72 h) show the production of C12 and C14 FFA generated by the BTE-induced cleavage of the corresponding acyl-ACPs. In LB+0.2% glucose, FFA titers reached 174.0 μ g/mL by 12 h and remained relatively constant thereafter. FFA titers in cultures grown on algae-based media followed a biphasic curve consisting of a rapid increase to 66.0 μ g/mL followed by a more gradual increase in FFA up to a titer of 90.1 μ g/mL at 72 h. FFA production from cultures grown in AFEX-P and AFEX-C was delayed until 12 to 24 h and reached final titers that were more than tenfold less than the cultures grown in either algae-based media or LB+0.2% glucose. As expected, increases in C16 fatty acids (Fig. 4c), which are the major component of bacterial cell membranes, correspond tightly with the growth curves observed in Fig. 4a. Cultures grown in algae-based media contained 50% of the total fatty acids observed in cultures grown in LB+0.2% glucose, but they had a slightly higher percentage of C12 FFAs. Considering that the final OD₆₀₀ reached in algae-based media was also 50% of the value obtained in LB+0.2% glucose, the algae-based media was capable

of supporting robust FFA production. The specific productivity (FFA titer/maximum OD600) was highest for *E. coli* grown in the algae-based media and nearly twofold higher than the LB+glucose media. In contrast, cultures grown in AFEX-P and AFEX-C media showed poor C12 and C14 FFA production. In these cultures, C16 fatty acids accounted for the majority (> 80%) of the fatty acids detected. Considering that the C16 titers were similar to cultures grown in LB+0.2% glucose, our data suggest that *BTE* expression and FFA production are less effective in cultures grown in media based on AFEX-pretreated corn stover.

Carbon, nitrogen, and phosphorus consumption analysis for cultures grown in biomass-based media

A comparison of the nutrient levels in each media was performed to further examine their effect on growth and FFA production. Glucose levels were measured in each culture at 0, 6, 12, 24, 48, and 72 h via UPLC analysis, as shown in Table 3. At 0 h, both CS-derived media contained approximately 25.0 g/L of glucose, which was more than twice as much as the algae-based medium and more than ten times greater than LB+0.2% glucose. The glucose concentration decreased with time during the exponential growth phase of the respective cultures. At 72 h, significant concentrations of glucose remained in each medium, but different amounts of glucose were consumed in each. Table 3 presents these data in g/L of glucose consumed. As expected, xylose levels remained unchanged in AFEX-P, AFEX-C, and algae-based media (data not shown) because *E. coli* preferentially uses C6 sugars, and glucose was not completely exhausted after 72 h.

Total phosphorus (TP; Table 4) and soluble reactive phosphorus (data not shown) in each of the four media was measured at 0 h as described above. All media samples contained phosphorus concentrations above 4 mM. This finding was expected for the algal and AFEX-P media which were phosphate-buffered. At 0 h, the AFEX-C medium had the highest C/TP ratio (150 mol carbon from glucose per mole total phosphorus; Table 4), which is above the C/P ratio (approximately 45:1) of growing *E. coli* cells (Cotner et al. 2006). The remaining media had C/TP ratios in the range of 11–18. These data suggests that of the four media only cultures grown in AFEX-C may be phosphorus-limited.

Digestible total nitrogen in each medium at 0 h was measured as described above (Table 4). Prior to inoculation, LB+glucose medium contained approximately 1.8 mg/mL of digestible nitrogen, which was consistent with the expected contributions of the roughly 10–12 wt.% N found in both tryptone (10 mg/mL in LB) and yeast extract (5 mg/mL in LB). The AFEX-C and AFEX-P media contained slightly less nitrogen, but each had digestible N in concentrations greater than 1 mg/mL. The algal media, however, contained a much lower level of digestible N (0.12 mg/mL). The C/N ratio (moles of carbon from glucose to moles of digestible total nitrogen; Table 4) for the three biomass-based media ranged between 8 and 33, which is above the common value of 5:1 associated with bacterial media (Cotner et al. 2006). Our data suggest that nitrogen is deficient in each of these media and may be the cause of the lower final cell densities observed in cultures grown in the algae-based medium. Considering the large amount of glucose remaining in each medium at 72 h, further optimization is needed to maximize the production of fatty acids from the four media.

Discussion

The demand for sustainable fuels has driven the growth of biofuels research programs. Biofuel production strategies center on harnessing photosynthetic organisms, either terrestrial crops or aquatic algae, to convert carbon dioxide to higher molecular weight organic compounds. The strategies rely on one of two paradigms. Either high-yielding crops are used to produce natural intermediates that can be converted to desired fuel compounds or

species are genetically engineered to convert sunlight and carbon dioxide directly to fuels. For terrestrial plants, carbon dioxide is converted to sugar polymers that make up the cell wall. Once harvested, the sugar polymers are broken down and converted by biological or chemical methods to compounds with good fuel properties (e.g., energy density, infrastructure compatibility, etc.). The major barrier to this strategy is the extraction and breakdown of lignocellulosic biomass into fermentable substrates. As shown here, cultures of biofuel-producing strains may experience reduced yields when grown on lignocellulosic hydrolysates. Significant research is needed to identify tolerant micro-organisms or to engineer mechanisms to circumvent toxicity when grown in a bioreactor setting.

Conversely, algae-based biofuel research has focused on bypassing the lignocellulosic intermediate by harvesting oils from lipid-rich microalgae. Once harvested, algal oils can be converted to current biodiesel via transesterification or to green diesel via chemical processing (Kalnes et al. 2007; Chisti 2007). Wijffels and Barbosa identified several issues that need to be addressed for microalgae to become a cost-effective source of biofuels over the next 10 to 15 years (Wijffels and Barbosa 2010). Many of these limitations could be alleviated by using macroalgae as a carbon source for engineered microorganisms that are already genetically tractable and amenable to large-scale fermentation.

For algal biofuels to become economically viable, new strains capable of producing high titers of biofuels must be isolated or engineered. Natural strains infrequently possess the ability to grow rapidly and accumulate high oil titers under the same nutrient conditions (Hu et al. 2008; Sheehan et al. 1998). These properties will be difficult to manipulate via metabolic engineering. Currently, only ten species of algae can be transformed with DNA, and even fewer have genetic tools available for performing advanced metabolic engineering (Walker et al. 2005). Alternatively, many strains of bacteria and yeast have been engineered to produce biofuel compounds at high conversions and titer. In addition, the systems and synthetic biology tools needed to make further improvements are more available for these strains than for microalgae. In a prior work, we demonstrated a metabolic engineering strategy for overproducing fatty acids by disrupting β -oxidation (*fadD*) and optimizing the expression of an acyl–acyl-carrier protein thioesterase in *E. coli* K-12 MG1655 (Table 1). The work presented here demonstrates that this metabolically engineered strain of *E. coli* is capable of both growth in and FFA production from a medium derived from macroalgal cellulose. Because algal cellulose is an essential component of the cell wall, algal strains capable of quickly reaching high cell densities will, in the process, produce elevated levels of cellulose that can be converted to biofuels.

Second, the tension between algal growth and lipid production can lead to culture instability at industrial scale. Lipid-rich species, especially those that have been genetically altered, grow more slowly and can be outcompeted by wild, lipid-lean species. This competition leads to poor stability in large-scale, open-cultivation systems such as raceway ponds. In contrast, the periphyton used to formulate algae-based media in this study is a naturally occurring community and is therefore less likely to be outcompeted by invasive species. In fact, smaller algae grow abundantly either on *Cladophora* surfaces or entangled with it. In shallow freshwaters, the productivity of this complex community can be higher than that of the suspended microalgae (phytoplankton) (Burkholder and Wetzel 1989; Wetzel and Sondergaard 1998).

Nutrient supplementation is a third critical area mentioned by Wijffels and Barbosa. Providing the nitrogen and phosphorus needed for large-scale cultivation of microalgae would require the synthesis of more fertilizer than is currently produced in Europe for farming (Wijffels and Barbosa 2010). Alternatively, macroalgae could be grown in wastewater effluents from sewage treatment plants, which are already rich in nitrogen and

phosphorus. A secondary benefit is that *Cladophora*, *Oedogonium*, and other filamentous periphytic algae are common components of turf-scrubber technology systems used to improve the quality of wastewater by removing pollutants (Davis et al. 1990a, b). Thus, excess nitrogen and phosphorus that are not removed by current treatment plants could be used to increase the yield of biofuels while simultaneously removing pollutants from the environment.

Lastly, cost-effective harvesting of microalgae is challenging because the cells are small (< 20 μm) and negatively charged and have a similar density to water (Lavoie and de la Noüe 1987; Moraine et al. 1979; Wijffels and Barbosa 2010). Current harvesting strategies focus on large-scale centrifugation, flocculation, or gravity-based settling (Wijffels and Barbosa 2010; Park et al. 2011), which lead to high purification costs. Additionally, micro-algae have thick, recalcitrant cell walls that require vigorous mechanical or chemical treatments to extract valuable oils from inside the cell. Alternatively, the large macroalgae strands that we describe here could be harvested by energy-efficient scraping or screen filtration. The subsequent extraction of cellulose from macroalgae is performed using simple acids and bases. The resulting cellulose cake can be enzymatically hydrolyzed with commercial preparations and used to formulate bacterial growth media. While currently expensive, the cost of hydrolytic enzymes is expected to decrease with continued research and development. Some researchers are currently pursuing the construction of consolidated bioprocessing microorganisms engineered both to secrete hydrolytic enzymes and to convert the released sugars into biofuels (Xu et al. 2009).

The data presented here demonstrate that engineered bacteria can grow in simple preparations of algal cellulose without the need for nutritional supplements. From a small collection (approximately 1 L) of wild periphyton (approximately 400 g of wet cell mass), a cake of nearly pure cellulose was isolated by a simple chemical treatment. The extracted cellulose represented approximately 6% (25.6 g) of the wet biomass. From this material, 1 L of 3.5 g/L glucose-based growth medium was formulated. When inoculated into this medium, cultures of engineered *E. coli* grew to a moderate cell density ($\text{OD}_{600}=2.0$) and produced approximately 90 mg/L of FFA in these non-optimized batch cultivations. In comparison, cells grown on LB+ 0.2% glucose used 1.0 g/L of glucose, achieved twice the cell density ($\text{OD}_{600}=4$), and produced 180 mg/L of FFA. Cells grown on corn stover-based media grew to moderate densities ($\text{OD}_{600}=4$) but produced tenfold less FFA (approximately 15 mg/L). The high specific productivity of strains grown in algae-based medium may be a result of the severely limited nitrogen concentrations, which would discourage the production of bacterial biomass but may not necessarily inhibit fatty acid production in existing cells. These results show that algae-based media are a strong candidate for producing biofuels, and our results leave significant room for process improvement. Future work will focus on periphyton cultivation, cellulose isolation, media optimization, and metabolic engineering of *E. coli* to increase FFA yields per cell. We postulate that the approach outlined in Fig. 1 may be optimized more quickly than microalgae cultivation and could provide an interim solution until microalgae fuel production is commercially viable.

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Fig. 1.

Overview of free fatty acid production from macroalgal biomass. *C. glomerata*-dominated periphyton was harvested from a freshwater lake and processed to yield a cake of cellulose that was then enzymatically hydrolyzed and used to formulate a glucose-based medium. This medium supported the growth of a bacterium engineered to overproduce fatty acids. The engineered *E. coli* strain converted a portion of the glucose into C12 and C14 free fatty acids (FFA) via a fatty acid biosynthesis pathway modified by the heterologous expression of a plant acyl-ACP thioesterase (*BTE*) that targets C12 and C14 acyl-ACPs. FFA can be converted into diesel fuel compounds enzymatically or catalytically (steps not shown)

Fig. 2. Algal cellulose is predominantly derived from *Cladophora* biomass. The purity of cellulose extracted from *C. glomerata*-dominated periphyton biomass was analyzed by staining the samples with Calcofluor White and visualizing them by bright field (**a, b**) and fluorescence during UV excitation (**c, d**). The samples taken from the cellulose cake included the cell wall remains of *C. glomerata* (**a, c**) and *Oedogonium* sp. (**b, d**). Scale bars represent 50 μm

Fig. 3.

Optimization of *BTE* expression for fatty acid production in glucose-based media. Cultures of *E. coli* *RL08* harboring pTrc99A-BTE (open bars) or pTrc99A-BTE-*H204A* (not shown) were grown in 96-well plates of various media: **a** AFEX-P, **b** AFEX-C, **c** algae-based, **d** LB +0.2% glucose in the presence of a range of IPTG concentrations. Nile red was added to each medium and fluorescence, which corresponds to the hydrophobicity of the medium, was measured as a marker of free fatty acid titer

Fig. 4.

Growth and fatty acid production on various undefined media. AFEX-P—squares; AFEX-C—triangles; algal media—cross symbols, LB+0.2% glucose—circles. **a** Growth of RL08 harboring pTrc99A-BTE in each medium over the course of the experiment as measured by optical density readings at 600 nm. **b** Free fatty acid (C12, C12:1, C14, C14:1) titers were measured by extraction and methylation followed by GC/MS analysis at 0, 6, 12, 24, 48, and 72 h. **c** Total C16 fatty acid concentrations were also measured as described in **b**

Table 1

Bacterial strains and plasmids

Strain or plasmid	Description	Source
Strain		
<i>E. coli</i> RL08	K-12 MG1655 <i>araBAD fadD</i>	Lennen et al. 2010
Plasmid		
pBAD34-BTE	pUC19 containing BTE under control of P _{BAD} , AMP ^R	Lennen et al. 2010
pBAD34-BTE-H204A	pUC19 containing BTE(H204A) under control of P _{BAD} , AMP ^R	Lennen et al. 2010
pTrc99A	pBR322 plasmid with P _{trc} , Amp ^R	Amann et al. 1988
pTrc99A-BTE	pTrc99A with BTE under control of P _{TRC} , Amp ^R	This report
pTrc99A-BTE-H204A	pTrc99A with BTE(H204A) under control of P _{TRC} , Amp ^R	This report

Table 2

Fatty acid species as a percent of total fatty acids at 72 h

Media	Percent of total fatty acids					C16:0+C16:1	Total free fatty acid (µg/mL)	Total fatty acid (µg/mL)	Specific FFA productivity (µg/mL/OD ₆₀₀ ^{Max})
	C12:1	C12:0	C14:1	C14:0	C16:0				
AFEX-P	ND	13.4	ND	3.3	83.3		7.9±0.5	47.4±4.2	2
AFEX-C	ND	12.0	ND	3.1	84.9		6.9±0.7	45.8±3.7	2
Algal	4.5	64.4	2.2	11.9	16.9		90.1±3.6	108.5±4.3	50
LB+0.2% glucose	5.4	60.6	4.8	12.4	16.9		180.8±24.5	217.5±30	29

Free fatty acids include C12, C12:1, C14:0, and C14:1

ND not detected

Table 3

Glucose concentration (mg/mL) in growth media over time

Media	Time (h)					Total sugar used	
	0	6	12	24	48	72	
AFEX-P	23.9±1.0	24.5±1.1	23.6±1.0	18.4±0.3	15.4±1.7	15.8±0.8	8.1±1.2
AFEX-C	23.4±1.2	22.6±1.5	23.3±0.6	19.7±1.0	14.9±0.6	14.8±0.3	8.6±1.0
Algal	8.3±0.2	8.4±0.2	7.7±0.2	7.0±0.1	5.7±0.2	4.7±0.0	3.6±0.1
LB+0.2% glucose	2.3±0.1	2.3±0.1	<1.4	<1.4	<1.4	<1.4	1.0±0.0

Table 4

Total phosphorus and digestible nitrogen in each medium at 0 h

Medium	TP $\mu\text{g/mL}$	TN $\mu\text{g/mL}$	C/TP ratio	C/TN ratio
AFEX-P	1,596 $\pm$ 16	1,260 $\pm$ 240	16:1	9:1
AFEX-C	148 $\pm$ 2	1,430 $\pm$ 330	150:1	8:1
Algal	754 $\pm$ 8	120 $\pm$ 40	11:1	33:1
LB+0.2% glucose	131 $\pm$ 1	1,780 $\pm$ 170	18:1	0.6:1

TP total phosphorus, *TN* total digestible nitrogen, *C/TP* ratio of the moles of carbon from measured glucose concentrations to the moles of total phosphorus, *C/TN* ratio of the moles of carbon from measured glucose concentrations to the moles of total nitrogen