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Co-culturing *Chlorella minutissima* with *Escherichia coli* can increase neutral lipid production and improve biodiesel quality

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Short Title: Bacteria can increase algal lipid production

Abstract

Lipid productivity and fatty acid composition are important metrics for the production of high quality biodiesel from algae. Our previous results showed that co-culturing the green alga *Chlorella minutissima* with *E. coli* under high-substrate mixotrophic conditions enhanced both culture growth and crude lipid content. To investigate further, we analyzed neutral lipid content and fatty acid content and composition of axenic cultures and co-cultures produced under autotrophic and mixotrophic conditions. We found that co-culturing *C. minutissima* with *E. coli* under high substrate conditions (10 g/L) increased neutral lipid content 1.9-3.1 fold and fatty acid content 1.5-2.6 fold compared to equivalent axenic *C. minutissima* cultures. These same co-cultures also exhibited a significant fatty acid shift away from trienoic and toward monoenoic fatty acids thereby improving the quality of the synthesized fatty acids for biodiesel production. Further investigation suggested that *E. coli* facilitates substrate uptake by the algae and that the resulting growth enhancement induces a nitrogen-limited condition. Enhanced carbon uptake coupled with nitrogen limitation is the likely cause of the observed neutral lipid accumulation and fatty acid profile changes.

Key Words: Co-culture, *Chlorella*, lipid, fatty acid, nitrogen limitation, carbon dioxide

Introduction

Fatty acids obtained from microalgal triacylglycerol (TAG) have attracted considerable attention as a feedstock for biodiesel production (Sheehan et al. 1998). There is also a large body of research on characterization of algal oils for the purpose of omega3 fatty acid production as a nutrition supplement (Ahlgren et al. 1992; Hu and Gao 2003). Many research efforts have focused on increasing the total quantity of TAG in microalgae (Griffiths and Harrison 2009) while somewhat less attention has been invested in the quality of the fatty acids for products and fuels such as biodiesel (Liu et al. 2011; Talebi et al. 2013). However, both metrics must be addressed in order to achieve commercially viable products from microalgae.

To date, most efforts for enhancing algal TAG production have utilized two main approaches: 1) supplementation of the culture with organic substrates (Miao and Wu 2006), and 2) limitation of non-carbon nutrients such as nitrogen, phosphorus, sulfur and silicon (Breuer et al. 2013; Griffiths and Harrison 2009; Li et al. 2013; Reitan et al. 1994). Both strategies have been used in combination and optimized to maximize lipid yields in certain axenic algal cultures (Li et al. 2011). We have investigated a third approach to enhance lipid production: co-culturing the microalgae *Chlorella minutissima* under mixotrophic conditions with the model bacterium *Escherichia coli.* For co-cultures produced under mixotrophic conditions we observed a 3.4-7.2 fold increase in biomass productivity, a 2.8-9.5 fold increase in substrate uptake, and a 3.1-8.3 fold increase in crude lipid productivity compared to axenic cultures (Higgins and VanderGheynst 2014). At the time, we used the sulfo-phospho-vanillin (SPV) assay to measure total crude lipid. This assay suggested that lipid content was enhanced in algal-bacterial co-cultures under high substrate loading but no further effort was made to quantify neutral lipids or

determine the fatty acid profiles. Such information is vital to assess the viability of co-culturing as an approach to enhance the quantity and quality of algal biodiesel.

The fatty acid profile in particular affects biodiesel properties including the cloud point, cetane number, iodine value, and viscosity (Talebi et al. 2013). Cetane number is a measure of diesel fuel's ignition delay with higher numbers corresponding to shorter delay. Both cetane number and fuel viscosity generally increase with chain length and level of saturation (Krisnangkura et al. 2006; Piloto-Rodríguez et al. 2013). Likewise, greater chain saturation results in greater oxidative stability as reflected by a higher iodine value (Talebi et al. 2013). In general, medium chain (14-18 carbon) fatty acids with few double bonds yield biodiesel with both good flow properties and high oxidative stability.

In this study we used gas chromatography with mass spectrometry (GC-MS), thin layer chromatography (TLC), and a neutral lipid assay (Higgins et al. 2014) to thoroughly describe the lipid content of axenic cultures and co-cultures under autotrophic and mixotrophic conditions. Our objective was to determine the quantity of lipids conducive to biodiesel production and make inferences about potential biodiesel quality based on fatty acid data. We also investigated potential mechanisms that could explain changes in the lipid profile under different culture conditions.

Materials and Methods

Algae cultivation and lipid extraction

Chlorella minutissima (UTEX 2341) and *Escherichia coli* (ATCC 25922) were cultivated in hybridization tubes filled to 200 ml with N8-NH_4 medium according to methods previously described (Higgins and VanderGheynst 2014), unless otherwise noted. Briefly, algae were pre-cultured for approximately 7 days and inoculated into hybridization tubes to achieve ~$1x10^7$

cells/ml starting density. Cultures were then grown for 5 days under 10,000 lux illumination provided by T5 growth lamps and supplied with 125 ml/min aeration as air without supplemental CO_2 unless noted. Cultures were harvested by centrifugation, washed with dionized water, and freeze dried. Lipids were extracted by the Folch method with modifications (Folch et al. 1957; Higgins and VanderGheynst 2014).

Transesterification and GC-MS analysis

Lipid extracts were transesterified with 1 M methanolic HCl and analyzed by gas chromatography and mass spectrometry (GC-MS) using methods previously described (Higgins et al. 2014). Briefly, internal standard (nonadecanoic acid) was added to lipid extracts that were then dried, re-suspended in hexane, and transesterified. The reaction was stopped by the addition of sodium carbonate solution and phase separation was achieved. The hexane layer was transferred to a GC vial and stored at -20 °C until injection on an HP 6890 GC coupled to an HP 5973 mass spectrometer. A DB-23 column (J&W Scientific, Folsom, CA) was used with helium as the carrier gas.

Peaks were integrated using the ChemStation software and concentrations of individual fatty acids were determined from the internal standard plus response factors obtained from an analytical corn oil standard and fatty acid methyl ester (FAME) standard (Sigma Aldrich, St. Louis, MO). Equation 1 was used to calculate unknown fatty acid concentrations in samples:

$$C_{FAi,sample} = \left(\frac{C_{FAi,std}}{A_{FAi,std}}\right)\left(\frac{A_{IS,std}}{A_{IS,sample}}\right)A_{FAi,sample} \quad (1)$$

where “A” is peak area and “C” is fatty acid concentration. The subscript “FAi” represents an arbitrary fatty acid “i”, “std” indicates quantity found in the external standard, and “IS” indicates internal standard.

Thin layer chromatography

Thin layer chromatography (TLC) was performed on silica gel plates with glass backing using 80:20:1 (v/v) hexane/diethyl ether/acetic acid as the solvent as described previously (Higgins et al. 2014). Primuline dye was used in conjunction with a UV imager to detect lipid bands. Select lipid bands were scraped from the TLC plate using a razor blade and subjected to the transesterification reaction previously described for analysis of fatty acid composition by GC-MS.

Neutral lipid assay

A microplate assay was used to measure neutral lipids in the cultures as described elsewhere (Higgins et al. 2014). This assay was shown to exhibit good sensitivity toward triacylglycerol but also moderate sensitivity to sterol lipids.

Measurement of ammonium concentration by cation exchange chromatography

A Prominance Liquid Chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a conductivity detector was used for cation exchange chromatography and detection. A CS14 cation exchange column protected by a CG14 guard column was used in conjunction with a CSRS300 ion suppression unit (Dionex). Methanesulfonic acid (10 mM) in Milli-Q water was used as mobile phase under isocratic conditions (1 ml/min). The column was maintained at 26 °C. Peak integration was performed using Shimadzu's LCSolutions software. Samples were prepared by centrifugation of culture samples followed by 0.2 μm filtration to remove cells (Titan2 PTFE, SUN-SRi, Rockwood, TN). The sample was then diluted 1:1 with 20 mM methanesulfonic acid in Milli-Q water.

Measurement of substrate uptake by HPLC

HPLC was used to measure concentrations of glucose, glycerol, and acetate in the growth medium as described previously (Higgins and VanderGheynst 2014). Briefly, an Aminex 87H column (Bio-Rad) was used in combination with refractive index and diode array detectors.

Nitrogen and protein content of biomass

Samples of 1.5-2 mg of freeze-dried algae were suspended in 1.5 ml dH_2O followed by addition of 0.5 ml of 0.5 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK). Samples were disrupted four times using a FastPrep FP120 (Savant Instruments, Holbrook, NY) for 20-second intervals at maximum speed followed by cooling on ice. An aliquot of the whole disrupted sample was used for the total nitrogen assay (TestNTube Total Nitrogen, HACH, Loveland, CO), which was performed according to the manufacturer's instructions. This assay oxidizes all nitrogen species to nitrate, which then reacts with chromotropic acid, forming a chromophore that was read at 420 nm. Ammonium chloride was used as a standard.

Soluble protein content was measured using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. An aliquot of disrupted cell suspension (as prepared for the total nitrogen assay) was combined with 3x SDS extraction buffer (150 mM sodium phosphate buffer adjusted to pH 7, 3 mM disodium EDTA, 0.3% SDS, 0.3% Triton X-100) in a 2:1 ratio. This extraction buffer was based on that used by Jefferson (Jefferson 1987) with modifications. Bead beating was performed for an additional 30 seconds followed by centrifugation (13,500 g for 5 min). Supernatant was assayed in a microplate for soluble protein per the manufacturer's instructions.

DNA extraction and amplification

DNA was extracted using a FastDNA Spin Kit (MP Biomedicals, Solon, OH) and quantitative PCR (qPCR) was performed on a 110 base segment of *E. coli* 16S rDNA as described previously (Higgins and VanderGheynst 2014).

Data analysis

Statistical analysis was carried out using the JMP v.11 software package (SAS Institute, Cary, NC). Data were tested for heteroscedasticity using Levene's test and were log-transformed to achieve homogeneity of variance if needed. Thereafter, ANOVA and Tukey's HSD test were performed with significance assessed at the 0.05 level.

Results

Effect of mixotrophy on fatty acid content and composition and neutral lipid content

Mixotrophic cultivation significantly affected fatty acid composition in *C. minutissima*. In axenic cultures, total fatty acid content (as percent of dry weight) increased modestly under mixotrophic growth on glucose, glycerol, and acetate substrates (Table I). Provision of 10 g/L of each substrate yielded an 18-36% increase in total fatty acid content compared to autotrophic growth. Increased fatty acid saturation was observed as substrate concentration increased. Oleic (18:1 cis-9) acid content increased from roughly zero to 2-7% of total fatty acids and linoleic (18:2 cis-9,12) acid levels approximately doubled upon addition of 10 g/L of substrate compared to autotrophic controls. α-linolenic (18:3 cis-9,12,15) acid levels decreased with increasing substrate level, declining from about 70% of total fatty acid content for autotrophic cultures to approximately 40% for mixotrophic cultures with 10 g/L substrate.

Compared to axenic *C. minutissima*, provision of organic substrate had a dramatic effect on fatty acid content in co-cultures of *C. minutissima* and *E. coli*. This was particularly true at 10

g/L substrate where total fatty acid content was more than double that of the autotrophic controls (Table I). Fatty acid levels were also 1.5-2.6 fold greater than those found in axenic cultures provided with 10 g/L substrate. This large increase in total fatty acid content was driven primarily by increases in oleic acid and linoleic acid contents. As with axenic *C. minutissima*, increased substrate levels resulted in a decline in α-linolenic acid content. Two other fatty acids: 16:1 and 18:1,11 also appeared in much greater quantities in the co-cultures compared to the axenic cultures and likely derive from *E. coli* (Table II). Both 16:1 cis-9 (palmitoleoyl acid) and 18:1 cis-11 (vaccenic acid) are naturally found in *E. coli* (Cahoon et al. 1996).

Total fatty acid content in *E. coli* was 3.5-6.7% of total biomass when grown on varying glucose concentrations. Given that *E. coli* primarily use glycogen rather than lipids for energy storage (Jones et al. 2008), it can be inferred that these fatty acids likely derive from polar lipids associated with bacterial membrane and cellular functions. This was confirmed by the weak response in the neutral lipid assay (Table II) and no apparent TAG band on the TLC plate (Figure 1). Interestingly, *E. coli* fatty acid content was only marginally lower than autotrophic *C. minutissima* cultures grown on air, which were in the range of 7-9% of total biomass. Here too, lipids were predominantly polar (Figure 1).

Similar to the fatty acid data, the neutral lipid assay revealed a 1.9-3.1 fold increase in neutral lipid content of co-cultures compared to axenic *C. minutissima* supplied with 10 g/L substrate (Table I). Elevated neutral lipid content was also observed in axenic cultures supplemented with organic substrates although the effect was small. TLC revealed that these increases in neutral lipid content were largely the result of TAG accumulation (Figure 1).

We previously reported that co-cultures had biomass productivities that were 3.5, 3.4, and 7.2 fold higher than the axenic cultures grown on 10 g/L glucose, glycerol, and acetate,

respectively (Higgins and VanderGheynst 2014). When combined with the present fatty acid data, these same co-cultures achieved total fatty acid productivities that were 9.1, 5.2, and 10.4-fold higher than equivalent axenic *C. minutissima* cultures, respectively. We previously reported that co-cultures provided with 10 g/L substrate consumed 2.8-9.5 times more substrate than equivalently prepared axenic cultures; and that co-cultures consumed more substrate than the sum of axenic *C. minutissima* and *E. coli* cultures (Table I). We hypothesize that increased substrate uptake can at least partially explain the high fatty acid and neutral lipid contents in co-cultures.

Effects of nitrogen and CO_2 levels on fatty acid content

Two other mechanisms may also lead to high fatty acid content in co-cultures: carbon dioxide utilization and nitrogen limitation. Carbon dioxide released by *E. coli* and subsequently used by *C. minutissima* could affect fatty acid content. Likewise, the rapid growth in co-cultures could have resulted in nitrogen deficient conditions, leading to neutral lipid accumulation by algae.

To test if the co-cultures were nitrogen limited, we initially utilized cation chromatography to measure ammonium levels in the media of co-cultures after growth on varying glucose levels. All cultures grown on 2 and 10 g/L glucose consumed all available nitrogen within 48 hours despite much higher biomass growth at 10 g/L substrate (Figure 2). This suggested the presence of a nitrogen uptake mechanism that is independent of growth. Hence, nitrogen depletion in the media could not be used as a direct indicator of intracellular nitrogen limitation. Using a total nitrogen kit, the nitrogen content of freeze-dried biomass was measured for all culture conditions. Nitrogen contents of the biomass in co-cultures at 10 g/L substrate were significantly lower than that of most other treatments providing evidence for the

nitrogen limitation hypothesis (Table III). In addition, total soluble protein content of the biomass at 10 g/L substrate levels was about half the protein content of other substrate treatments.

To test our hypothesis about nitrogen limitation and CO_2 supplementation associated with *E. coli* growth, we cultivated an additional set of co-cultures on 10 g/L glucose since this culture condition produced the most dramatic increases in neutral lipids. We perturbed the nitrogen and CO_2 levels by supplying either 200 or 400 mg/L nitrogen and 0.04% or 2% CO_2. The biomass of cultures receiving 200 mg/L nitrogen had approximately half the nitrogen content of biomass from cultures receiving 400 mg/L nitrogen (Table IV) regardless of the level of CO_2 supplied, suggesting more severe nitrogen limitation in the former condition. The higher nitrogen level resulted in moderately lower total fatty acid content and significantly lower neutral lipid content (Table IV). Moreover, the low nitrogen level had significantly higher oleic acid content ($p = 0.03$) suggesting that nitrogen limitation could lead to a shift in the fatty acid profile (Table S1). Interestingly, even cultures grown on 400 mg/L nitrogen exhibited fatty acid contents that were >15% suggesting that other factors besides nitrogen limitation may contribute to high fatty acid content in co-cultures.

Elevated CO_2 levels led to a decline in total fatty acid content of co-cultures but the effect was not statistically significant. Likewise, CO_2 supplementation also was found to halve neutral lipid content at the higher nitrogen level but had an insignificant effect at 200 mg/L nitrogen. These results suggest that carbon dioxide cycling within the co-culture cannot explain the dramatic increase in neutral lipid and total fatty acid content. The elevated CO_2 levels did lead to a 72% and 29% increase in the growth rates of the co-cultures supplied with 200 and 400 mg/L nitrogen, respectively, implying an increase in photosynthetic activity. Interestingly, elevated CO_2 levels significantly increased total glucose consumption by 11-35% and the cell yield

coefficient by approximately 16-35% although the latter differences were not statistically significant at the 0.05 level in Tukey's HSD test (Table IV). Supplemental CO_2 also appeared to significantly increase the linoleic fraction of total fatty acids from 24.6% to 29.4% ($p = 0.001$). More detailed fatty acid data for this experiment can be found in Table S1.

Nitrogen limitation and CO_2 supplementation in autotrophic cultures

To clarify the effects of nitrogen limitation independent of mixotrophy and *E. coli*, autotrophic *C. minutissima* cultures were grown with 200 mg/L nitrogen as ammonium, then a portion of cells were transferred into nitrogen-free medium and cultivated for an additional four days. The result was an 85% increase in total fatty acid content and a three-fold increase in neutral lipid content over samples that received 200 mg/L nitrogen supplementation (Table V). TLC of select samples indicated that these increases in neutral lipid content were almost exclusively driven by an accumulation of TAG (Figure 1). As with the high-substrate co-cultures, the nitrogen-limited cultures exhibited a dramatic increase in the fraction of oleic acid coupled with a decline in α-linolenic acid. Interestingly, nitrogen limitation did not appear to affect the linoleic acid fraction.

The effect of CO_2 on fatty acid content was also investigated in autotrophic cultures (Table V) but similar to co-cultures, elevated CO_2 did not significantly change the total fatty acid content. It did increase the linoleic acid fraction from 11% to 43% of total fatty acids, however, compared to cultures supplied with air. Elevated CO_2 also increased the oleic acid fraction from 0.2% to 7.1% of total fatty acids while decreasing the share of α-linolenic acid. The effects of CO_2 on fatty acid composition appeared to be very similar to those associated with organic substrate uptake in axenic *C. minutissima* cultures.

Analysis of lipid classes by TLC

We observed a strong positive correlation between neutral lipid content and monounsaturated fatty acid content in algae (R = 0.97) and a negative correlation with trienoic fatty acids (R = -0.81). In order to determine if the increase in TAG was directly responsible for the shift in fatty acids, we analyzed the fatty acid contents of the polar and TAG fractions obtained from the TLC plate. Indeed, we found that polar lipids from autotrophic algae were rich in α-linolenic acid and contained no detectable oleic acid (Table VI). Likewise, TAG obtained from mixotrophic co-cultures and nitrogen-limited cultures were rich in oleic and linoleic acid. However, we also observed that polar lipids in mixotrophic co-cultures exhibited elevated levels of oleic and linoleic fatty acid compared to autotrophic conditions. This result suggests that elevated TAG levels are not the sole cause of the fatty acid shift toward lower saturation despite the apparent correlation.

E. coli quantitation from fatty acid content

E. coli content in co-cultures was quantitated using qPCR, as we have previously reported (Higgins and VanderGheynst 2014). Given the differences in fatty acids produced by *E. coli* and *C. minutissima*, we hypothesized that *E. coli* content could be calculated directly from fatty acid measurements. This strategy could prove valuable for detection and quantitation of non-algal contaminants in algae cultures since fatty acid profiling is already frequently used for lipid analysis.

Vaccenic acid (C18:1 cis-11) content was calculated in pure *E. coli* cultures grown on glucose and the resulting data were used to calculate *E. coli* content in all co-cultures. The results were compared to those previously obtained by 16S rDNA qPCR and a strong correlation was

found ($R^2 = 0.88$). The fit line's slope was close to unity (Figure 3) suggesting that this could be a viable tool for culture community composition analysis in simple mixtures such as co-cultures.

Discussion

Mixotrophic growth resulted in a modest, but significant, shift in the fatty acid profile of axenic *C. minutissima*. Provision of organic substrates generally increased the saturation level of acyl chains, which is consistent with findings from other researchers (Cerón García et al. 2000; Tan and Johns 1996). Similar to our findings, Chen and Johns observed elevated levels of oleic and linoleic acids coupled to a decline in α-linolenic acid when *Chlorella sorokiniana* was grown on increasing levels of glucose (Chen and Johns 1991).

Our previous work demonstrated that *E. coli* accounted for a relatively small fraction (5-10%) of total biomass in co-cultures supplied with 10 g/L substrate (Higgins and VanderGheynst 2014). All else held constant, this led us to hypothesize that there would be comparable total fatty acid contents between axenic and co-cultures, yet fatty acid content in the co-culture was nearly double that of the axenic culture. The very high fatty acid content observed in high-substrate mixotrophic co-cultures can likely be explained by two mechanisms: high levels of carbon substrate uptake coupled with nitrogen limitation. Several researchers have hypothesized that algae store excess carbon substrate in the form of neutral lipids (Cerón García et al. 2000; Chen and Johns 1991; Tan and Johns 1996). Utilization of a carbon source (either CO_2 or organic), significantly increased linoleic acid content in axenic mixotrophic cultures with smaller but still significant increases in oleic acid content. Hence, the significant increase in linoleic acid observed in the 10 g/L co-cultures was likely due in part to the very high organic substrate uptake observed in these cultures. Carbon dioxide produced by *E. coli* metabolism may also have

increased linoleic acid levels in co-cultures. The mechanisms accounting for enhanced substrate uptake in the presence of *E. coli* are subject to ongoing investigation.

Research by others has shown that protein content of algal biomass can decline significantly under nitrogen limitation (Sialve et al. 2009) and that nitrogen limitation reduces protein synthesis and channels carbon metabolism toward storage molecules including TAG and starch (Scott et al. 2010). We observed all of these effects in co-cultures under high-substrate conditions. Our attempts to specifically induce nitrogen limitation in *C. minutissima* resulted in TAG accumulation and increased oleic acid levels but had little effect on linoleic acid as a fraction of total fatty acids. Zhila et al. showed that nitrogen limitation increased oleic acid content at the expense of trienoic fatty acids in the green alga *Botryococcus braunii* (Zhila et al. 2005). Thus nitrogen limitation is probably the most significant factor accounting for the very high oleic acid levels observed in high-substrate co-cultures.

One of the drawbacks of using nitrogen limitation to induce TAG accumulation is the concomitant decline in algae growth (Brennan and Owende 2009). As a result, some researchers have proposed using two-stage cultivation systems in which algae are first cultivated with sufficient nitrogen to achieve rapid growth and then transferred to a nitrogen-limited medium (Huntley and Redalje 2007; Su et al. 2011). Such an approach would likely increase capital and operating costs compared to a single-stage culture. Our results are particularly significant because the co-culture system could achieve a nitrogen-limited condition and lipid accumulation with no apparent tradeoff in growth rates. Upon doubling the initial nitrogen concentration in co-cultures, we saw no corresponding growth enhancement as shown in Table IV suggesting that the nitrogen level was not limiting algae growth.

The positive correlation between neutral lipid and monounsaturated fatty acid content led us to hypothesize that increased TAG directly caused changes in the fatty acid profile. Polyunsaturated fatty acids in algae have been associated with polar membrane lipids whereas monounsaturated fats have been associated with neutral storage lipids (Cerón García et al. 2000). Indeed, our results are consistent with these findings but we also observed a general shift toward unsaturation in both polar and non-polar lipid fractions under co-culture mixotrophy. Our finding is corroborated by Liu et al. who compared fatty acid levels in *Chlorella zofingiensis* under heterotrophic (30 g/L glucose) and autotrophic conditions (Liu et al. 2011). They observed elevated TAG levels under heterotrophic growth along with a large increase in oleic acid content, however, their GC results also showed an increase in oleic acid content in polar lipids. Liu et al. concluded that high TAG levels were the result of heterotrophic growth but their data showed that heterotrophic cultures also depleted media nitrogen halfway into the culture period suggesting that cultures were also nitrogen limited.

Carbon dioxide levels have been shown by other researchers to affect lipid composition in algae. Hu et al. showed that increased CO_2 can increase lipid content in algae under both autotrophic and mixotrophic conditions (Hu and Gao 2003). Likewise, Tsuzuki et al. (1990) showed that CO_2 supplementation enhanced the fraction of linoleic acid at the expense of α-linolenic acid in *Chlorella vulgaris*, consistent with our findings for *C. minutissima.* They hypothesized that these fatty acid changes were occurring in thylakoid membranes of chloroplasts.

Our findings showed that autotrophic cultures supplemented with 2% CO_2 led to fatty acid composition changes similar to those observed upon supplementation with organic carbon substrates. When mixotrophic cultures were supplemented with CO_2, biomass growth increased

but total fatty acid content remained largely unchanged and neutral lipid levels declined modestly. These findings suggest that photosynthetic products derived from CO_2 compete with exogenous organic substrates for metabolic pathways in *C. minutissima*. Sforza et al. showed that addition of excess CO_2 can inhibit uptake of organic carbon sources in mixotrophic cultures of *Chlorella protothecoides* and *Nannochloropsis salina* (Sforza et al. 2012). In contrast, our results showed that cultures supplied with 2% CO_2 actually utilized 11-35% more total glucose than those supplied with air. Supplemental CO_2 also enhanced culture growth by 30-80%, boosting the nominal cell yield coefficients slightly and suggesting that photosynthesis was contributing more toward growth. This result is noteworthy because it suggests a compounding effect of CO_2 on organic uptake as opposed to additive or inhibitive effects which have been proposed previously (Ogawa and Aiba 1981).

E. coli appeared to contribute only a small portion of total fatty acids in co-cultures, particularly at zero and 10 g/L substrate. Although its direct contribution to fatty acids was minimal, the presence of *E. coli* in the culture appeared to facilitate both substrate uptake by algae and more robust algal growth compared to axenic *C. minutissima* cultures. The indirect effect of these developments was a nitrogen limited condition in high-substrate cultures which likely increased fatty acid and TAG content. Moreover, the fatty acid composition shifted toward greater monounsaturation which should improve biodiesel oxidative stability and increase its cetane number (Talebi et al. 2013).

These findings suggest the need for further research into co-culturing as a strategy for enhancing biodiesel production from algae. This area is not well studied but some research suggests that other algal-bacterial systems can increase algal lipid content and the diversity of fatty acids synthesized by algae (de-Bashan et al. 2002). The molecular mechanisms of *C.*

minutissima-E. coli symbiosis is the subject of ongoing investigation. Such knowledge could allow for the selection of organisms to further test this strategy.

Conclusion

The addition of *E. coli* was found to facilitate very high fatty acid and TAG levels in mixotrophic co-cultures with *C. minutissima*. Elevated organic substrate uptake in conjunction with nitrogen limitation likely contributed to these developments. Furthermore, the increase in oleic and linoleic acid combined with a decrease in α-linolenic acid should result in a higher quality biodiesel product. The results demonstrate the opportunity to exploit co-culture systems to improve lipid productivity by microalgae. Further research is needed to understand the mechanisms of co-culture symbiosis in order to select and test other organisms that are likely to improve lipid production.

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Table I: Neutral lipid content, fatty acid content and composition in axenic *C. minutissima* cultures and co-cultures with *E. coli.*

					Composition as % of total fatty acid						
Culture conditions	Productivity (mg/L/d)	Substrate consumed (g/L)	Neutral lipid content (%)	FA content (%)	C16:0	C16:1	C18:0	C18:1,9	C18:1,11	C18:2	C18:3
Axenic *C. minutissima*											
Glucose											
0 g/L	8.4 c		6.8 bc	7.6 a	13.6 a	0.0 a	1.1 a	0.0 b	0.0 a	10.6 c	74.7 a
0.5 g/L	38.0 b	0.40 b	5.3 c	6.4 a	16.2 a	0.0 a	1.1 a	0.3 b	0.0 a	17.2 b	65.3 a
2 g/L	98.8 a	1.70 a	9.7 ab	9.1 a	18.5 a	0.4 a	1.7 a	5.4 a	0.0 a	31.1 a	43.0 b
10 g/L	98.7 a	1.26 a	10.4 a	9.1 a	19.4 a	0.4 a	1.9 a	5.4 a	0.0 a	31.0 a	41.9 b
Glycerol											
0 g/L	13.5 d		6.8 bc	8.2 b	17.3 a	1.0 a	2.5 ab	0.3 b	0.0 a	13.9 b	65.0 a
0.5 g/L	42.3 c	0.47 b	6.5 c	8.5 ab	17.7 a	0.0 a	2.1 b	0.6 b	0.0 a	14.8 b	64.8 a
2 g/L	111.0 b	1.88 a	8.2 b	9.4 ab	28.5 a	0.0 a	2.7 ab	1.2 ab	0.0 a	19.6 a	48.0 b
10 g/L	123.4 a	2.07 a	11.1 a	10.8 a	33.0 a	0.8 a	2.9 a	1.9 a	0.0 a	22.3 a	39.1 b
Acetate											
0 g/L	19.9 d		7.6 b	8.6 b	11.3 a	0.0 a	1.4 b	0.1 c	0.0 a	21.6 c	65.6 a
0.5 g/L	48.3 c	0.46 c	7.7 b	10.1 ab	10.9 a	0.0 a	1.6 ab	0.7 c	0.0 a	22.9 c	64.0 a
2 g/L	125.8 a	1.86 a	11.0 a	12.0 a	15.4 a	0.4 a	1.9 ab	9.4 a	0.0 a	35.9 b	37.0 b
10 g/L	59.6 b	0.96 b	11.4 a	11.7 ab	15.4 a	0.0 a	2.2 a	7.3 b	0.0 a	40.7 a	34.5 b
Co-culture											
Glucose											
0 g/L	10.5 d		7.8 b	11.3 b	12.0 a	0.0 c	1.4 ab	0.1 b	0.0 c	9.3 c	77.1 a
0.5 g/L	85.0 c	0.47 c	6.1 c	10.1 b	12.9 a	4.9 b	1.3 ab	1.0 b	1.4 b	15.8 b	62.6 b
2 g/L	190.8 b	1.94 b	4.5 d	8.3 b	20.7 a	9.0 a	0.8 b	0.6 b	6.9 a	13.6 b	48.5 c
10 g/L	342.8 a	6.09 a	32.4 a	23.6 a	17.4 a	0.8 a	2.0 a	46.0 a	0.1 c	23.4 a	10.4 d
Glycerol											
0 g/L	12.9 d		5.9 b	7.0 b	17.2 a	0.0 c	2.7 a	1.7 b	0.0 d	16.7 b	61.7 a
0.5 g/L	74.7 c	0.50 c	5.5 b	8.2 b	18.5 a	4.5 b	2.7 a	1.5 b	2.9 b	19.6 b	50.2 ab
2 g/L	172.4 b	2.05 b	6.5 b	6.5 b	22.2 a	8.6 a	3.0 a	1.2 b	5.3 a	21.4 ab	38.3 b
10 g/L	419.8 a	6.26 a	21.7 a	16.4 a	25.5 a	3.8 bc	2.6 a	27.1 a	0.8 c	27.2 a	12.9 c
Acetate											
0 g/L	34.6 d		5.5 bc	8.2 b	18.9 a	0.0 a	2.4 a	3.4 b	0.0 c	20.4 b	54.8 a
0.5 g/L	87.5 c	0.45 c	6.5 b	8.4 b	16.2 a	0.0 a	2.4 a	2.7 b	0.0 c	21.2 b	57.4 a
2 g/L	207.7 b	1.91 b	4.4 c	9.0 b	17.4 a	5.4 b	2.4 a	2.5 b	2.4 a	24.3 b	45.7 a
10 g/L	428.9 a	7.89 a	22.9 a	17.0 a	21.7 a	6.9 b	2.3 a	27.0 a	1.3 b	32.6 a	8.2 b

Biomass productivity and substrate consumption over five days were obtained from data presented previously (Higgins and VanderGheynst 2014). All cultures were aerated with air only. Lipid and fatty acid (FA) contents are given as a percentage of dry culture weight, and fatty acid composition as a percentage of total fatty acids (mass

basis). Three biological replicates were used in all cases except for co-cultures grown on 10 g/L glycerol where two replicates were used. Within a substrate group, values followed by the same letter are not significantly different at the 0.05 level.

Table II: Neutral lipid and fatty acid content and composition for axenic *E. coli* grown on glucose

			Composition as % of total fatty acid						
Culture condition	Neutral lipid content (%)	FA content (%)	C16:0	C16:1	C18:0	C18:1,9	C18:1,11	C18:2	C18:3
Glucose									
0.5 g/L	2.6	3.52	40.7	26.2	3.2	0.0	29.9	0.0	0.0
2 g/L	2.5	5.73	48.4	25.8	0.0	0.0	25.8	0.0	0.0
10 g/L	3.2	6.67	51.7	24.6	1.4	0.0	22.3	0.0	0.0

Lipid and fatty acid (FA) content are given as a percent of dry culture weight, and fatty acid composition as a percent of total fatty acids (mass basis). Three biological replicates were used for neutral lipid determination; one biological replicate was used for fatty acid analysis.

Table III: Nitrogen and soluble protein content of co-culture biomass

Condition	Nitrogen content (%)	Soluble protein content (%)
Glucose		
0 g/L	6.6 b	32.6 a
0.5 g/L	6.9 b	26.2 b
2 g/L	8.3 a	25.1 b
10 g/L	3.8 c	13.0 c
Glycerol		
0 g/L	6.5 b	21.3 a
0.5 g/L	7.8 a	23.1 a
2 g/L	8.6 a	23.3 a
10 g/L	6.3 b	10.8 b
Acetate		
0 g/L	6.5 c	22.7 b
0.5 g/L	6.8 b	26.5 a
2 g/L	7.5 a	27.4 a
10 g/L	5.9 d	11.2 c

Total nitrogen content (mass basis) was measured by a Hach assay kit and soluble protein by BCA assay. Within a substrate group, values followed by the same letter are not significantly different. Each value is the average of 3 biological replicates.

Table IV: Effects of nitrogen and CO_2 supplementation on mixotrophic co-cultures

Nitrogen level as N (mg/L)	CO_2 level (% of airflow)	Neutral Lipid content (%)	Total FA content (%)	Total N content (%)	Biomass productivity (mg/L/d)	Glucose consumed (g/L)	Cell yield (mg/mg)
200	0.04	34.9 a	24.7 a	3.3 b	404 b	7.0 b	0.29 a
400	0.04	25.0 b	19.6 ab	5.7 a	439 b	6.8 b	0.32 a
200	2.0	27.0 ab	24.0 a	3.2 b	739 a	9.4 a	0.39 a
400	2.0	12.6 c	17.1 b	6.1 a	566 ab	7.5 b	0.38 a

Co-cultures were grown on 10 g/L glucose and nitrogen was supplied as ammonium chloride. Total gas flow rate was 125 ml/min for all reactors; only ambient air was provided to cultures with 0.04% CO_2. Values are the means of 3 biological replicates except for the condition of 200 mg/L nitrogen with 0.04% CO_2 which had 2 biological replicates. Glucose consumption was measured over a five day period and the cell yield is the ratio of biomass growth to glucose consumption on a mass basis. Values followed by the same letter within a column are not significantly different at the 0.05 level. Cell yield differences were not significantly different ($p = 0.138$ for one-way ANOVA)

Table V: Effect of nitrogen and CO_2 levels on axenic autotrophic *C. minutissima*

			Composition as % of total fatty acid (mass basis)						
Culture condition	Neutral lipid content (%)	FA content (%)	C16:0	C16:1	C18:0	C18:1,9	C18:1,11	C18:2	C18:3
200 mg/L N, air	6.9 b	7.9 b	13.7 a	0.0 a	1.4 a	0.2 c	0.0 a	11.3 b	73.4 a
200 mg/L N, 2% CO_2	5.9 b	9.0 b	11.9 a	0.0 a	0.7 a	7.1 b	0.0 a	42.8 a	37.4 b
0 mg/L N, 2% CO_2	17.8 a	16.6 a	12.7 a	0.0 a	1.8 a	27.6 a	0.0 a	42.6 a	15.3 c

A portion of cultures grown on 200 mg/L N were subsequently transferred to nitrogen free media to induce nitrogen stress responses. Values are the mean of 4 biological replicates and values followed by the same letter within a column are not significantly different. Attempts to culture *C. minutissima* on air without nitrogen were also made but resulted in minimal algae growth such that a nitrogen stress response could not be achieved.

Table VI: Fatty acid compositions of neutral and polar lipid fractions obtained from autotrophic and mixotrophic cultures

		Composition as % of total fatty acid (mass basis)						
Culture condition	Lipid class	C16:0	C16:1	C18:0	C18:1,9	C18:1,11	C18:2	C18:3
Axenic, autotrophic, 0.04% CO_2, 200 mg/L N	Polar	15.8	0.0	9.7	0.0	0.0	7.3	67.2
	TAG	*Not quantifiable*						
Axenic, 10 g/L glucose, 0.04% CO_2, 200 mg/L N	Polar	18.8	0.0	4.0	0.0	0.0	27.6	49.6
	TAG	36.7	0.0	30.6	9.5	0.0	23.2	0.0
Co-culture, 10 g/L glucose, 0.04% CO_2, 200 mg/L N	Polar	24.2	0.0	12.6	9.1	0.0	20.2	33.9
	TAG	14.0	0.0	1.3	62.2	0.0	18.1	4.3
Axenic, autotrophic, 2% CO_2, 200 mg/L N	Polar	9.3	0.0	4.6	4.8	0.0	46.8	34.5
	TAG	*Not quantifiable*						
Axenic, autotrophic, 2% CO_2, 0 mg/L N	Polar	15.2	0.0	6.5	8.5	0.0	41.6	28.1
	TAG	10.5	0.0	0.0	47.2	0.0	34.9	7.4

Polar and TAG bands were scraped from TLC plates and biological duplicates were pooled for transesterification. Vertical boundaries for the scraped bands are denoted in Figure 1 by brackets.

Table S1: Effects of nitrogen and CO_2 supplementation on fatty acid composition in mixotrophic co-cultures

	Composition as % of total fatty acid (mass basis)						
Culture condition	C16:0	C16:1	C18:0	C18:1,9	C18:1,11	C18:2	C18:3
200 mg/L N, Air	15.4 b	1.2 b	2.2 a	49.7 a	0.0 a	23.3 c	8.2 c
400 mg/L N, Air	17.2 ab	0.9 b	1.9 a	43.8 b	0.0 a	24.8 bc	11.5 ab
200 mg/L N, 2% CO2	17.5 ab	2.5 a	2.1 a	39.3 b	0.0 a	28.1 ab	10.5 bc
400 mg/L N, 2% CO2	18.5 a	3.2 a	1.7 a	33.2 c	0.0 a	30.6 a	12.8 a

Fatty acid composition data as percent of total fatty acids. This data accompanies that presented in Table IV. Values are the mean of 3 biological replicates except for the condition of 200 mg/L nitrogen with air which had 2 biological replicates. Values followed by the same letter within a column are not significantly different at the 0.05 level.

Figure Captions:

Figure 1: TLC plates showing polar and neutral lipid bands obtained from extracts of *C. minutissima* and *E. coli* harvested after 5 days cultivation. All algal extracts and corn oil standards were dissolved in chloroform. A) Lane 1: corn oil, Lanes 2-3: axenic autotrophic *C. minutissima*, Lanes 4-5: axenic mixotrophic (10 g/L glucose) *C. minutissima*, Lanes 6-7: mixotrophic co-culture (10 g/L glucose). All cultures were grown using air at 0.5 vvm. B) Lane 1: corn oil, Lane 2: axenic autotrophic *C. minutissima* grown using air, Lane 3: *E. coli* grown on 10 g/L glucose with air, Lanes 4-5: axenic autotrophic *C. minutissima* grown on air with 2% CO_2 with 200 mg/ml nitrogen as ammonium, Lanes 6-7 axenic autotrophic *C. minutissima* grown with 2% CO_2 in nitrogen free medium.

Figure 2: Concentration of ammonium ions over time in media during co-culture growth on glucose. Concentrations were measured by cation chromatography. Cultures were supplied with air.

Figure 3: Correlation between *E. coli* content (mg dry weight *E. coli* per mg total dry biomass) as quantified by qPCR and vaccenic acid.