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Cofactor symbiosis for enhanced algal growth, biofuel production, and wastewater treatment

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ABSTRACT

Algae have gained attention for production of fuels and chemicals, and treatment of wastewater. The high cost of algae cultivation, however, has limited industry adoption for these applications. Developing methods to increase algal growth rates and lipid content has emerged as an important strategy toward reducing production costs, and significant research effort has been exerted in this area. We have reported previously that co-culturing the green alga, *Auxenochlorella protothecoides*, with *Escherichia coli* under mixotrophic conditions led to 2–6 fold increases in algal growth, doubling of neutral lipid content, and elevated nutrient removal rates compared to axenic growth, indicative of a symbiotic relationship. In the present work, we reveal that symbiosis stems largely from *E. coli's* provision of thiamine derivatives and degradation products to *A. protothecoides*. LCMS showed that residual cell-free medium obtained from axenic *E. coli* culture contained roughly 1.15 nM thiamine pyrophosphate and 4.0–9.1 nM of the thiamine precursor and degradation product, 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP). These compounds were found to promote the growth, lipid content, and glucose uptake of *A. protothecoides*, while dramatically improving substrate utilization efficiency. Due to widespread cofactor auxotrophy among algae, the co-culture results presented here likely extend to a large number of microbial community systems. We show that algal-algal symbiosis based on cofactor exchange is also possible, opening a new frontier in algae cultivation management. These findings highlight the potential of engineered microbial communities for improved algal biofuel production and wastewater treatment.

1. Introduction

Algae have gained attention for biofuel production due to their rapid growth and high lipid content [1,2]. In spite of recent advances, however, algal biofuels are still too expensive to compete with conventional liquid fuels. A recent techno-economic study showed that increasing algal growth rates and lipid content are the two most important factors for improving the economics of algal systems [3]. Hence significant research effort has focused on simultaneously increasing algal growth and lipid content [4,5]. Mixotrophic algae growth, in which cells simultaneously use photosynthesis and external organic carbon sources, is one approach toward achieving increased lipid productivity [6]. Production of co-products [7,8] and services such as wastewater treatment have also gained attention as a means of improving both the economics [9] and environmental performance of algal biofuels [10]. An algal wastewater treatment environment will likely include the presence and activity of bacteria. Thus, understanding algae-bacteria interaction is important for engineering processes capable of simultaneous wastewater treatment and biofuel production.

In a previous report, we observed evidence of a symbiotic relationship in which *Escherichia coli* were found to enhance the growth of *Auxenochlorella protothecoides* (UTEX 2341, which was previously annotated as *Chlorella minutissima* but has since been reclassified [11]) by roughly 2–6 fold under high-substrate mixotrophic conditions [12]. Co-cultures also consumed organic substrates more rapidly than the two axenic organisms combined. We subsequently reported that the presence of *E. coli* facilitated a near doubling of neutral lipid and fatty acid content in *A. protothecoides* and enhanced organic substrate uptake and nitrogen removal [13]. Moreover, the fatty acid profile shifted in a manner that should improve the oxidative stability of biodiesel produced from the algal lipids. These results suggest the potential of employing co-culturing as a strategy to enhance water treatment performance and biofuel production in algae.

Given these substantial benefits, there was interest in determining the mechanisms of symbiosis and whether these mechanisms extend beyond the algae-bacteria pair that we studied. Such knowledge would allow for the design and operation of algal systems that foster the growth of mutually beneficial organisms. It would also provide a basis for understanding organism interaction in algal wastewater

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treatment systems, an area of increasing interest [14–17]. Moreover, the engineering of synthetic co-cultures and microbial communities has emerged as a new research frontier in the quest for bio-derived fuels and chemicals [18].

At the time, we concluded that the *Auxenochlorella-E. coli* symbiotic relationship was linked to substrate uptake and utilization because we did not observe similar symbiosis in autotrophic cultures. We also observed low substrate utilization efficiency in mixotrophic *A. protothecoides* cultures and that the presence of *E. coli* could improve this efficiency under certain conditions [12]. However, the molecular mechanisms of symbiosis were not elucidated. Research by others suggested three potential sources of mutualistic symbiosis: exchange of primary metabolites [19,20], exchange of cofactors and hormones [15, 21,22], and the establishment of unique physical niches within the microbial ecosystem [16]. Carbon dioxide-oxygen exchange is perhaps the most-discussed primary metabolite exchange in algal-bacterial systems [23]. In the ideal scenario, bacteria degrade organic material in wastewater, producing carbon dioxide that is then consumed by photosynthetic algae. The algae provide dissolved oxygen to bacteria, further enhancing aerobic degradation of soluble organic material.

Croft et al. have investigated vitamin cofactor exchange between algae and bacteria [24]. They found that provision of vitamin B_{12} by a variety of bacteria such as *Halomonas* sp. facilitated growth of algae strains that were vitamin B_{12} auxotrophs. Similar symbioses are likely to occur between bacteria and algal thiamine and biotin auxotrophs [21]. Algal-bacterial cofactor symbiosis has been recognized for decades as it pertains to the ecology of aquatic and marine habitats [25–27], however, the implications for engineering applications such as biofuel production and wastewater treatment have thus far gained little attention.

We present results here that suggest that transfer of thiamine precursors and degradation products are the most important mechanism of symbiosis between *E. coli* and *A. protothecoides*. Moreover, the results point to the importance of cofactor symbiosis for enhancing algal biofuel production and wastewater treatment. We also show that these results can extend to algal-algal symbiosis and that these findings should apply broadly due to widespread vitamin auxotrophy among algae.

2. Materials and methods

2.1. Algae and bacteria cultivation

Algae were pre-cultured in 1 L bottles without thiamine supplementation to achieve a density of roughly 10^7 cells/ml based on hemocytometer counts (~0.15 OD at 550 nm) as described previously [12]. Cells were settled overnight, media was removed, and the concentrated cells were used to inoculate 300 ml hybridization tubes filled to 200 ml with fresh medium. N8-NH_4 medium was used for *A. protothecoides* (UTEX 2341) and *E. coli* (ATCC 25922) cultures [12], and N8 medium was used for *Chlorella sorokiniana* (UTEX 2805) [28]. Cultures were supplied with varying glucose and glycerol (Fisher) levels as described in figure legends. Illumination (10,000 lx) was provided by T5 growth lamps oriented horizontally. Stir bars (150 rpm) provided mixing, and aeration was supplied (125 ml/min) without supplemental CO_2 with the exception of autotrophic cultures where 2% v/v CO_2 was mixed with air. Cultures were harvested after 5 days of growth by centrifugation and freeze dried as previously described [12]. This harvest point was selected because it generally coincided with the late log stage of growth. In cases where algae were grown on residual medium from other organisms, cells were initially removed by centrifugation followed by filtration through 0.2 μm filters (Nalgene Rapid-Flow). Glucose and ammonium chloride, which were expected to be the limiting substrates, were added to achieve the desired level, and residual medium was re-inoculated with algae. Thiamine (Acros), THZ (Sigma), pantothenic acid (MP), or HMP (>95%, synthesized by Arc-Pharm) were added to cultures per the experimental design.

2.2. Neutral lipid analysis

Lipids were extracted from freeze-dried algae using a modified Folch method as previously described [12]. Algal neutral lipids were measured using a previously described microplate assay with modifications [29]. Specifically, the Nile red solution concentration was increased to 1 μg/ml (from 0.5 μg/ml) and canola oil was used as a standard (rather than corn oil).

2.3. Metabolite extraction and analysis by GC–TOF

Culture samples (1 ml) were collected daily and quenched by the addition of 1 ml 70% MeOH (−70 °C). Samples were centrifuged at 12,000 *g* for 2 min at 4 °C and supernatant was discarded prior to freeze drying. Metabolites were extracted with 10:3:1 chloroform/methanol/water as described previously [30]. Derivatization by MSTFA and GC–TOF analysis were performed as described previously [30].

2.4. HPLC and GC–TOF analysis of pyruvate and fumarate

An Aminex 87H column (Bio-Rad) was used in conjunction with RID and PDA detection (210 nm) to quantify glucose, pyruvate, and fumarate concentrations in media. HPLC conditions have been described previously [12]. Fractions containing peaks corresponding to pyruvate (RT 9.6) and fumarate (RT 13.9) were collected, freeze dried, derivatized and analyzed by GC–TOF as described above.

2.5. E. coli residual media preparation for HPLC and LCMS analysis

After cell removal, residual culture media (35 ml) was freeze dried and re-suspended in 1 ml methanol, then centrifuged at room temperature at 6000 *g* to pellet salts. Supernatant was recovered and either injected directly for C18 HPLC analysis/fractionation or freeze-dried a second time for LCMS analyses. Re-dried material was resuspended in 100 μl MeOH and centrifuged at 12,000 *g* to pellet salts. Supernatant was recovered and injected on either LC–Qtrap (AB Sciex) or LC–MSTOF (Agilent) platforms.

2.6. C18 HPLC of thiamine derivatives, precursors, and degradation products

A 4.6 × 250 mm C18 column (Zorbax SB-C18, Agilent) was used in conjunction with PDA detection (254 nm) on a Prominence HPLC system (Shimadzu). Mobile phase A was 100% MiliQ water and phase B was 100% HPLC-grade methanol (Fisher). Flow was maintained at 1 ml/min with the following gradient: 0–4 min, 0% B, 4–10 min, ramp to 60% B, 10–20 min, ramp to 100% B, 20–29 min, hold 100% B, 29–30 min, ramp to 0% B, 30–35 min, hold 0% B. The column was kept at 30 °C. Fractions were collected from each sample over 3 replicate injections of 75 μl and pooled. Pooled fractions were freeze-dried and resuspended in water and sterile filtered (0.2 μm) for addition to algae cultures.

2.7. LC–MS/MS (QTRAP) analysis of thiamine derivatives and precursors

Thiamine metabolites were quantified by LC–MS/MS using a HILIC method. An AB Sciex 4000 QTrap LC/MS/MS System with Agilent Technologies 1200 Series LC (degasser, binary pump, thermostated autosampler at 4 °C, column oven at 45 °C) was used. The separation of thiamine, TMP, TPP, THZ and HMP was achieved on an Atlantis HILIC Silica column 3 um, 2.1 × 100 mm, Part no. 186002015 (Waters) using a 24-min gradient method (Solvent A, 200 mM ammonium formate/formic acid pH 3 in Water, Solvent B, 100% acetonitrile) starting with 70% B from 0 to 6 min, decreasing to 30% B from 6 to 12 min, back to 70% B in 2 min and re-equilibrating for another 10 min for a total run time of 24 min, at a flow rate of 0.3 ml/min. Analytes were

detected by multiple reaction monitoring (MRM) after positive mode electrospray ionization. Residues were quantified against curves of authentic standards purchased from Sigma or Arc Pharm, using external standard methodology.

2.8. LC–MSTOF analysis of residual E. coli media

Samples were analyzed by HILIC with an Agilent 1290 Infinity UPLC coupled with an Agilent 6530 Accurate Mass QTOF. The HILIC method employed a Waters Acquity UPLC BEH Amide column with a 45 °C column oven temperature and a water/acetonitrile gradient. The column flow rate was 0.4 ml/min with a gradient elution of mobile phase A (water with 10 mM ammonium formate and 0.125% formic acid) and mobile phase B (acetonitrile/water with 10 mM ammonium formate and 0.125% formic acid, 95:5 v/v). The gradient elution started at 100% B, decreased to 70% B over 7.7 min, decreased to 40% B from 7.7–9.5 min, decreased to 30% B from 9.5–10.25 min, increased to 100% B from 10.25–12.75 min, and equilibrated at initial conditions until 17 min. Samples were maintained at 4 °C in a thermostated autosampler and injected at 5 μl injection volume for both positive and negative (ESI) ionization modes.

2.9. Thiamine uptake and degradation in E. coli cultures

Sterile 50 ml tubes were filled to 20 ml with N8-NH_4 medium supplemented with 10 g/l glucose. Six tubes each were supplied with either 0, 100, or 500 ng/ml thiamine-HCl and half of these were inoculated with *E. coli*, respectively. All tubes were incubated at room temperature on a shaker table (150 rpm). Samples (1 ml) were taken daily, centrifuged to pellet cells, and the supernatant was filtered (0.2 μm). The supernatant was then assayed for thiamine content using the thiochrome method.

2.10. Thiochrome assay

The thiochrome assay was used for rapid detection of thiamine concentration. Derivatization of thiamine to the thiochrome product was carried out based on the method of Lu et al. with modifications [31]. Briefly, 120 μl of aqueous sample or standard was added to a 96-well microplate followed by 40 μl of methanol to intensify the signal. Background fluorescence was read on a plate reader (Spectramax M2) with 375 nm excitation and 435 nm emission. Samples were then derivatized by adding 75 μl of freshly prepared 0.6 mM potassium ferricyanide solution dissolved in 15% (w/v) aqueous NaOH. Wells were mixed by pipetting up and down and fluorescence was read within 5 min of reagent addition. Background fluorescence was subtracted from final readings. Thiamine-HCl was used as an external standard.

2.11. Statistical analysis

Paired and independent group *t*-tests were carried out in Excel using published equations [32]. ANOVA and Tukey's HSD tests were carried out in R (v. 3.1.1) using the "car" and "agricolae" packages [33]. R was also used to test for homogeneity of variance using Levene's test and the Brown and Forsythe test prior to carrying out ANOVA.

3. Results

3.1. Secretion of organic acids by mixotrophic A. protothecoides

We previously reported that significant acidification of media occurred under mixotrophic cultivation of *A. protothecoides* on glucose and glycerol [12]. Analysis of time-course media samples by HPLC revealed significant growth over time in two peaks that absorbed light in the 200–210 nm range suggesting carboxylic acid secretion. These peaks did not appear under autotrophic conditions. The two peaks were collected in separate fractions, freeze dried, derivatized, and identified by GC–TOF as pyruvate and fumarate.

The pyruvate secretion in particular was significant, representing 20–67% of glucose uptake (w/w) under different mixotrophic conditions (Fig. 1A and B). The secretion of pyruvate initially lagged behind culture growth suggesting that cells could initially metabolize glucose efficiently into biomass but later converted glucose to pyruvate at the expense of growth (Fig. 1C). No pyruvate secretion was observed in co-cultures of *A. protothecoides* and *E. coli* (Fig. 1D) suggesting a link between pyruvate secretion and symbiosis.

We initially hypothesized that pyruvate accumulation contributes to glycolytic inhibition in *A. protothecoides* and that pyruvate consumption by *E. coli* alleviates this inhibition, thereby contributing to enhanced growth and substrate utilization. We tested the effects of exogenous pyruvate on mixotrophic *A. protothecoides* cultures but did not observe inhibition of growth or glucose consumption (Fig. S1A–C). Thus *E. coli* consumption of pyruvate is unlikely to explain the observed symbiosis.

3.2. Residual E. coli media enhanced growth of mixotrophic A. protothecoides

Our next line of inquiry focused on whether *E. coli* cells were required for symbiosis or if effects were due to compounds released into the medium. We tested this by culturing *A. protothecoides* on residual *E. coli* medium after cell removal. HPLC analysis was employed after *E. coli* growth to ensure complete glucose removal prior to fresh supplementation with 10 g/l glucose. *A. protothecoides* was then cultivated on this supplemented spent medium.

The residual *E. coli* medium supported *A. protothecoides* growth that was nearly 18 fold greater than growth on fresh medium supplemented with 10 g/l glucose (Fig. 3A). Moreover, the cultures grown on residual media also consumed all glucose after five days and did not secrete any detectable pyruvate. In contrast, the control cultures consumed ~1.3 g/l glucose and secreted 0.8 g/l pyruvate. This indicated that secreted

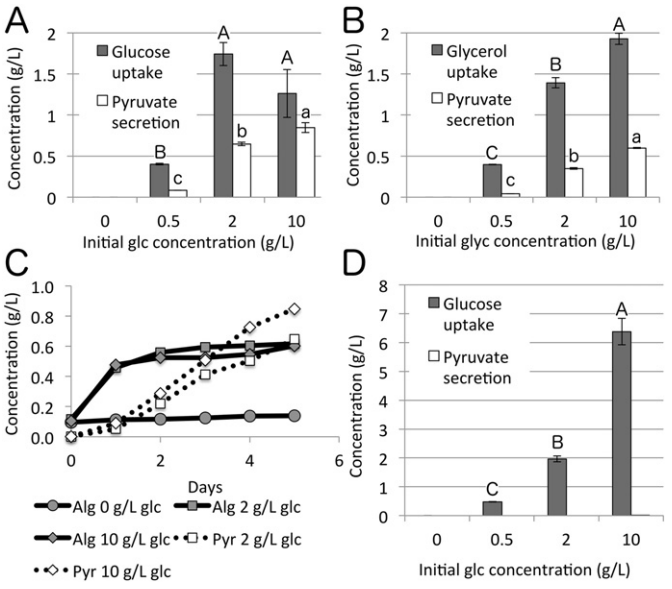


Fig. 1. Substrate uptake and pyruvate secretion in mixotrophic cultures after 5 days of growth. Substrate uptake and pyruvate secretion by axenic *A. protothecoides* cultivated on (A) glucose and (B) glycerol. (C) Algal biomass (Alg) growth curve and pyruvate (Pyr) secretion in axenic *A. protothecoides* culture supplemented with or without glucose. No pyruvate secretion was detected in autotrophic culture. (D) Co-cultures of *A. protothecoides* and *E. coli* exhibited high glucose consumption and minimal pyruvate secretion. For bar graphs, bars with the same letter above them are not significantly different at the 0.05 level based on Tukey's HSD test. Capital letters are used to denote significance for substrate uptake and lowercase letters are used for pyruvate secretion. Error bars are SD, n = 3 biological replicates.

metabolites from *E. coli* could dramatically enhance glucose uptake and utilization by *A. protothecoides*.

3.3. Metabolomics analysis of mixotrophic A. protothecoides

Untargeted metabolomics analysis was performed on axenic *A. protothecoides* to better understand intracellular metabolite accumulation under mixotrophic (glucose) conditions. Cells were grown on freshly prepared media with or without glucose and samples were collected daily, quenched, extracted, and analyzed by GC–TOF to obtain time-course changes in the central metabolome. Glucose supplementation resulted in a large shift in the metabolome including a 634 fold increase in the intracellular pyruvate concentration compared to autotrophic cells (Fig. 2). While it was apparent that a significant quantity of pyruvate exited the cell, the exact mechanism of transport was not elucidated.

3.4. Pyruvate dehydrogenase and thiamine deficiency

Commercial pyruvate production from glucose by yeast and *E. coli* auxotrophs is accomplished through aerobic fermentation in the absence of cofactors required by pyruvate dehydrogenase (PDH) [34]. Enzymes involved in anaerobic pathways such as pyruvate decarboxylase are likewise impaired by cofactor auxotrophy (e.g. lipoic acid). The result is accumulation of intracellular pyruvate which is eventually secreted into the medium. We hypothesize a similar scenario for *A. protothecoides* when grown on glucose and glycerol.

We cultured *A. protothecoides* on glucose with exogenous addition of thiamine and pantothenic acid, the precursors to two PDH cofactors. Thiamine addition resulted in a 10.7 fold increase in productivity over control cultures whereas pantothenic acid had no effect (Fig. 3B). The combination of thiamine and pantothenic acid led to 20% greater productivity than thiamine alone suggesting some synergy of the two cofactors ($p = 0.01$, Tukey HSD). Cultures provided with thiamine also consumed nearly all glucose and did not secrete detectable pyruvic acid. We hypothesize that *A. protothecoides* is unable to synthesize sufficient thiamine, resulting in a metabolic bottleneck at pyruvate dehydrogenase. The minimum level of thiamine required to achieve maximum growth on 8 g/l glucose was found to be ~20 nM based on fitting dose-response data to a saturation model (Eq. (1)). Above 20 nM, no significant increase in growth was observed (Fig. 3C).

$$p = \frac{p_{max} S}{k_S + S} + p_b \quad (1)$$

where p is expected culture productivity (mg/l/d), p_{max} is maximum productivity, k_S is the half-velocity constant, S is the concentration of thiamine-HCl, and p_b is the baseline productivity with no exogenous thiamine.

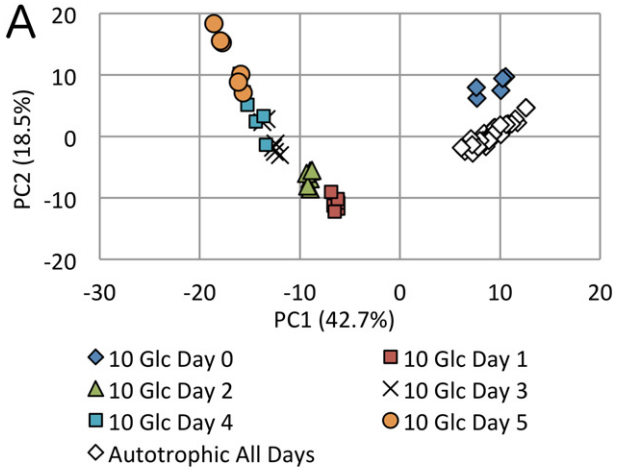
3.5. Thiamine levels in E. coli medium

E. coli are known to synthesize thiamine [35], and using targeted LC–MS/MS (QTRAP) analysis, we could detect thiamine, thiamine monophosphate (TMP), and thiamine pyrophosphate (TPP) in *E. coli* cell extracts. However, when we tested the residual medium from axenic *E. coli* culture after cell removal, we detected very little thiamine or its phosphate derivatives. The medium contained only 0.06 nM thiamine (SD = 0.002 nM), no detectable TMP, and 1.15 nM TPP (SD = 0.12 nM) based on three biological replicates. The values reported here are not corrected for losses during sample processing and are therefore likely to be conservative. Nevertheless, these concentrations are far below the levels required to achieve the growth effects observed in *A. protothecoides*.

Thiamine and its phosphate derivatives are known to degrade easily [36] and molecules released into the medium upon cell lysis could be reacquired by other *E. coli* cells. To test the latter effect, we added exogenous thiamine at 100 ng/ml and 500 ng/ml to *E. coli* cultures and observed depletion of medium thiamine concentration that exceeded cell-free controls (Fig. 3D). We also observed small but statistically significant declines in thiamine concentration ($p = 0.011$ and $p = 0.018$, 2 tailed paired *t*-tests) in cell free controls initially provided with 100 and 500 ng/ml thiamine-HCl, respectively. These results could explain the low concentration of thiamine and its phosphate derivatives in the residual medium. However, we hypothesized that degraded thiamine products were present in the residual *E. coli* medium and that these products could provide benefits to *A. protothecoides*.

3.6. Thiamine salvage mechanisms in A. protothecoides

Thiamine salvage mechanisms have been shown to exist in a variety of bacteria [37] and genomic evidence exists for their presence in eukaryotic organisms [36]. However, thiamine salvage mechanisms have not been thoroughly elucidated in eukaryotic algae to date [26]. Thiamine is synthesized by the condensation of two precursors: 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) and 4-methyl-5-(β-hydroxyethyl)thiazole (THZ) as shown in Fig. 4A. These two compounds are also potential products of thiamine degradation, catalyzed by thiaminases [38]. Some eukaryotic algae have been shown to contain the necessary enzymes to synthesize thiamine but lack pathways to produce one of the precursors [39]. To test this hypothesis, we cultured



B

Metabolite	Fold Difference	p-value
Pyruvate	634.3	< 0.001
Homoserine	18.2	< 0.001
Lysine	16.5	< 0.001
Fumarate	15.7	< 0.001
Ornithine	11.7	< 0.001
Glc-6P	11.1	< 0.001
Malate	9.3	< 0.001
Stearic acid	-2.8	< 0.001
Phosphate	-6.6	0.0011

Fig. 2. Metabolite profile shift over time in axenic *A. protothecoides* in response to mixotrophy on 10 g/l glucose (10 Glc). (A) Principle component plot based on 274 known and unknown metabolites excluding glucose. Relative abundances of metabolites were normalized against the biomass concentration of each sample. Each point is a single biological replicate. (B) Fold difference in abundance of specific metabolites of *A. protothecoides* cultured on 10 g/l glucose for 5 days compared to autotrophic cultures. The p-value reflects the significance of the difference based on an independent group *t*-test.

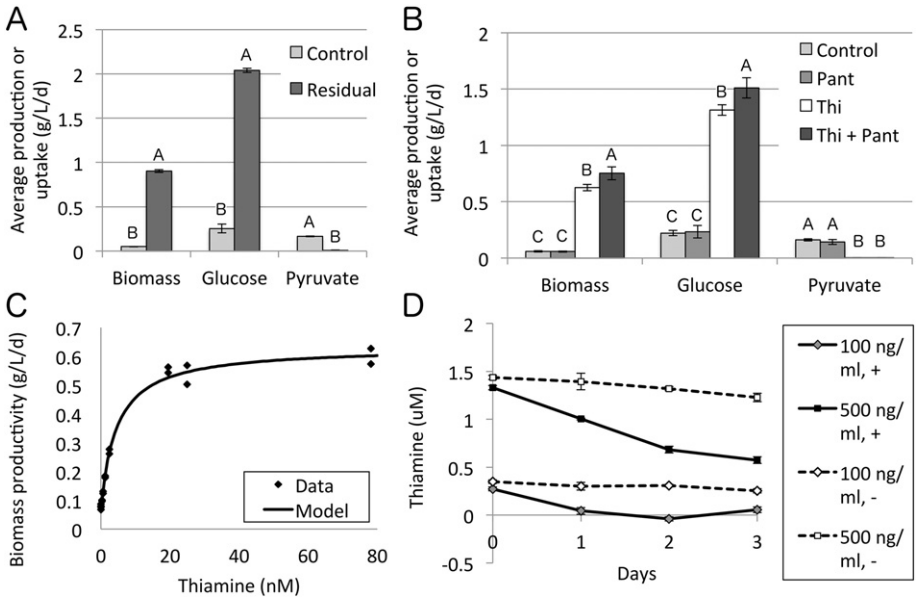


Fig. 3. Effects of residual media and cofactor precursors on axenic cell culture. (A) *A. protothecoides* growth (biomass), glucose utilization (glucose), and pyruvate secretion (pyruvate) after 5 days of growth on residual or fresh (control) medium supplemented with 10 g/l glucose. Residual medium was prepared by culturing *E. coli* on N8-NH_4 medium supplemented with 2 g/l glucose for 36 h, followed by cell removal. Error bars are SD, n = 4 biological replicates. (B) *A. protothecoides* growth on N8-NH_4 medium supplemented with 8 g/l glucose and either 5 μM thiamine-HCl, 68 μM pantothenic acid, or both. Error bars are SD, n = 3. Within each measurement type (biomass growth, glucose consumption, or pyruvate secretion), bars with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test. (C) Dose response results for *A. protothecoides* growth on varying levels of thiamine-HCl. Curve fit to the saturation model was performed using MATLAB's nlinfit algorithm. The half-velocity constant was 4.57 nM thiamine-HCl. (D) Thiamine-HCl concentration in medium monitored over time using thiochrome assay to assess thiamine degradation or re-uptake. Initial doses of 0 (not shown because the level remained at 0 ng/ml throughout the study), 100, and 500 ng/ml thiamine-HCl were added to N8-NH_4 medium supplemented with 10 g/l glucose. Samples followed by (+) were inoculated with *E. coli* and those with (−) contained no cells. Error bars are SD based on 3 biological replicates.

A. protothecoides mixotrophically with HMP, THZ, or a combination of these two thiamine precursors. HMP resulted in a 26 fold increase in algal growth compared to control cultures but THZ had no effect (Fig. 4B). Moreover, cultures provided with either thiamine or HMP had more than seven-fold greater neutral lipid content than mixotrophic cultures provided with only glucose (Table 1).

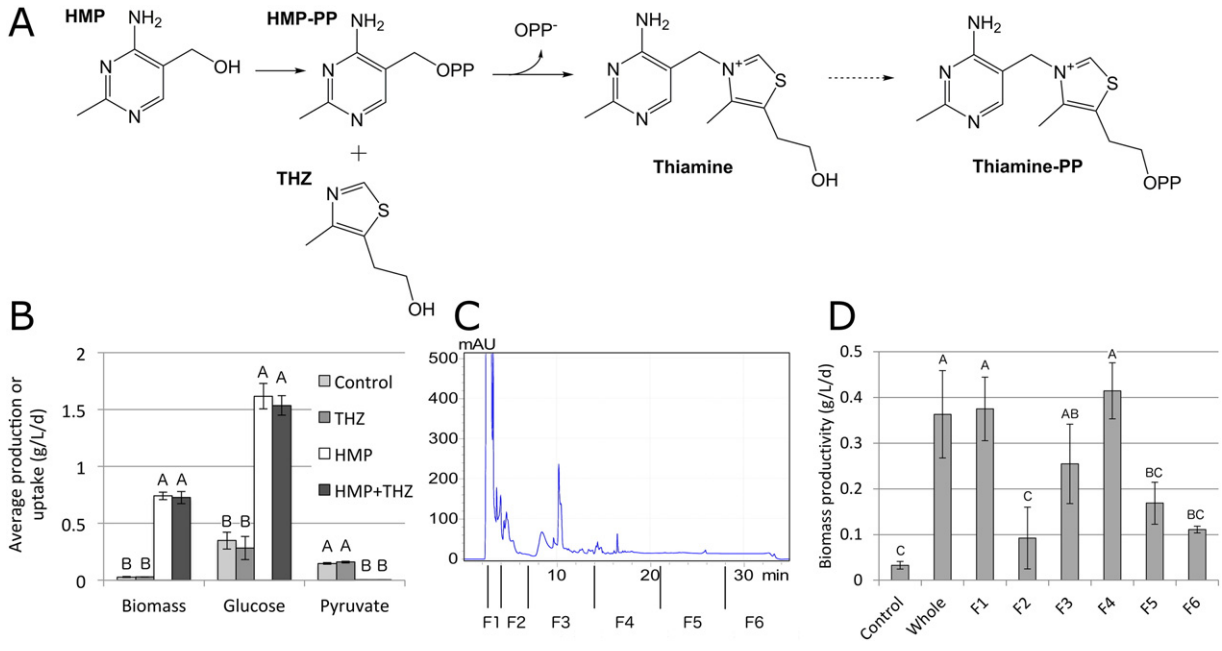


Fig. 4. Thiamine precursor effect on *A. protothecoides* metabolism. (A) Schematic of thiamine biosynthesis from HMP and THZ precursors. (B) HMP and THZ effects on *A. protothecoides* growth and glucose metabolism. Cultures were supplied with 8 g/l glucose and 100 nM of HMP and/or THZ. Within each measurement type (biomass growth, glucose consumption, or pyruvate secretion), bars with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test. Error bars are SD based on 3 biological replicates. (C) Chromatogram (254 nm absorbance) of *E. coli* medium subject to fraction collection. Media from three *E. coli* biological replicates were separately freeze dried, re-suspended to form a concentrate, and fractionated by HPLC using a C18 column. Fractions 1–6 were collected from 2.3–4.0 min., 4.0–7.0 min., 7.0–14.0 min., 14.0–21.0 min., 21.0–28.0 min., and 28.0–35.0 min., respectively. (D) Growth of *A. protothecoides* on *E. coli* media fractions. Media fractions were freeze dried, re-suspended, sterile filtered, and spiked into fresh glucose-free N8-NH_4 medium to supply three corresponding algal biological replicates. Error bars are SD based on these three biological replicates and encompass variability in *E. coli* growth, fractionation, and *A. protothecoides* growth. Bars with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

Table 1
Neutral lipid analysis of *A. protothecoides*.

Growth medium treatment[a]	Lipid content (%)[d]	Lipid productivity (mg/l/d)[d,e]
Control[b]	1.8 (0.1) C	1.0 (0.1) B
Thiamine (5 μM)	13.0 (0.8) B	81.5 (4.8) A
HMP (100 nM)	12.7 (2.4) B	93.4 (13.1) A
C. sorokiniana residual[c]	30.9 (1.4) A	84.2 (8.7) A

[a] N8-NH_4 medium supplemented with 8 g/l glucose was used for all cultures.
[b] Control cultures had no cofactor added.
[c] Glucose and ammonium chloride were added to residual *C. sorokiniana* medium to restore levels to desired quantity.
[d] Standard deviation is shown in parentheses based on 3 biological replicates and the following letter shows significance based on Tukey's HSD test carried out at the 0.05 level.
[e] Lipid productivity is the product of lipid content and biomass growth rate.

Analysis of residual *E. coli* medium by LC–MS/MS (QTRAP) revealed HMP concentrations of 4.0, 5.4, and 9.1 nM over three biological replicates. Untargeted metabolite analysis in *E. coli* medium by LC–qTOF also showed the presence of HMP. While this level of HMP may not be sufficient to achieve the growth levels observed in *A. protothecoides*, the presence of HMP suggests that thiamine and its phosphate derivatives are likely degraded in culture. A variety of other thiamine degradation products could also be associated with the observed symbiosis.

3.7. Chromatographic fractions of residual E. coli media show growth enhancement

Jenkins et al. reported a novel thiamine salvage pathway in bacteria in which base-degraded thiamine products could be salvaged to re-form HMP [36]. We tested *A. protothecoides* growth on medium that was supplemented with base-degraded thiamine and found that these degraded products substantially increased algal growth (Fig. S3). However, further analysis of these degraded products by LCMS revealed that HMP was highly abundant, complicating efforts to determine if alternative growth-promoting degradation products exist.

To determine if multiple HMP-salvageable molecules were secreted by *E. coli*, we fractionated concentrated *E. coli* medium by HPLC using a reverse-phase method. Six fractions were collected from each sample over multiple injections (Fig. 4C) so as to concentrate each fraction. All fractions except for fraction 2 enhanced *A. protothecoides* growth, with fractions 1 and 4 supporting growth on par with whole *E. coli* medium (Fig. 4D). Injections of individual standards revealed that TPP should elute in fraction 1 and HMP in fraction 4 (Fig. S2A, B). Fractions three, five, and six also promoted growth, suggesting that media compounds besides TPP and HMP contribute to symbiosis.

3.8. Thiamine enhances growth of autotrophic algae

This work primarily focused on the benefits of thiamine symbiosis as it pertains to mixotrophic algae cultures. However, thiamine supplementation was also found to increase the growth of autotrophic *A. protothecoides* by 36 fold ($p < 0.001$, 2-tailed independent group *t*-test) (Fig. 5A). We also tested thiamine supplementation on another green algae species, *C. sorokiniana* and observed a 19% increase in growth but the difference was not statistically significant ($p = 0.09$, 2-tailed independent group *t*-test) (Fig. 5A). We therefore concluded that *C. sorokiniana* could synthesize thiamine but may receive a modest benefit from exogenous addition.

Given that *A. protothecoides* benefits from co-culture with *E. coli* based on cofactor exchange, we wished to know if similar benefits could be achieved using *C. sorokiniana* as a source of thiamine derivatives. To test this, we cultivated mixotrophic *A. protothecoides* on residual *C. sorokiniana* medium. We found that this residual medium enhanced *A. protothecoides* growth 8.5 fold compared to freshly prepared medium and that pyruvate secretion was nearly eliminated (Fig. 5B). We also observed that cultures grown on residual medium contained roughly 17-fold greater neutral lipid content compared to control cultures (Table 1).

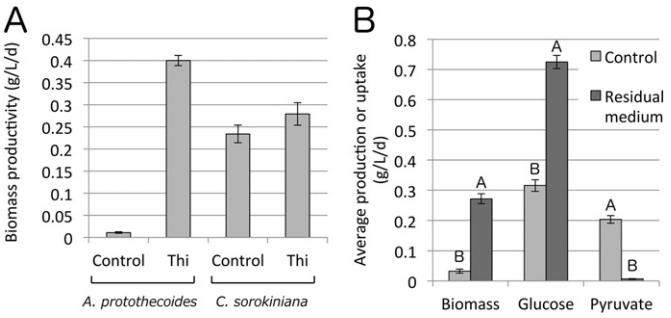


Fig. 5. Effects of thiamine on autotrophic green algae. (A) Growth comparison with and without exogenous thiamine-HCl (500 nM) in *A. protothecoides* and *C. sorokiniana*. Error bars are SD based on 3 biological replicates. Because the two organisms were cultured in separate batches, multiple comparison statistical tests were not employed. (B) Growth, glucose uptake, and pyruvate secretion of *A. protothecoides* on residual medium from *C. sorokiniana* supplemented with 8 g/l glucose after cell removal. Within each measurement type (biomass growth, glucose consumption, or pyruvate secretion), bars with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test. Error bars are SD based on 3 biological replicates.

4. Discussion

We have shown that the previously-observed symbiosis between *A. protothecoides* and *E. coli* appears to stem largely from exchange of TPP, HMP and other thiamine degradation products. We acknowledge that other mechanisms such as carbon dioxide-oxygen exchange could also play a role in symbiosis. However, an order of magnitude increase in *A. protothecoides* growth could be achieved when using cell-free residual *E. coli* medium suggesting that cofactor exchange is the predominant symbiotic mechanism in this case. *A. protothecoides* could grow without thiamine supplementation and we previously showed that its growth rates under a variety of mixotrophic conditions were comparable to those reported for several other *Chlorella* species [6,12,40]. Nevertheless, supply of the glycolytic substrates glucose and glycerol to *A. protothecoides* culture led to secretion of substantial quantities of pyruvic acid. This represents a significant inefficiency in substrate utilization when the goal is production of intracellular products such as lipids. Pyruvate secretion could also partially explain the low substrate utilization efficiency that we reported in previous studies on *A. protothecoides* [12]. Secretion of organic compounds by algae is also inefficient in wastewater treatment where the goal is to reduce chemical and biological oxygen demand. We have shown that *A. protothecoides's* pyruvate secretion is due to a metabolic bottleneck at the PDH complex that can be relieved by thiamine addition. This bottleneck may also explain why axenic *A. protothecoides* growth exhibited saturation behavior with respect to glucose concentration: providing 10 g/l glucose resulted in the same growth as 2 g/l glucose. Such a bottleneck is expected to inhibit glycolysis and TCA cycle activity, consequently suppressing biomass growth and substrate utilization.

Further study revealed that the thiamine precursor HMP provided similar metabolic benefits to exogenous thiamine but the addition of THZ did not, suggesting that *A. protothecoides* has retained genes to synthesize THZ. Hence it is likely that *A. protothecoides* once was able to synthesize thiamine de-novo but salvage mechanisms for HMP have allowed the organism to persist without a de novo HMP pathway. This also implies that *A. protothecoides* relies on thiamine and degraded thiamine products obtained from other organisms in its environment. We hypothesize that *E. coli* release thiamine, TMP, and TPP into the medium upon cell lysis and that these molecules degrade in the medium to form HMP and other products. We showed that multiple chromatographically-separated fractions support enhanced algae growth suggesting the presence of HMP salvage pathways in *A. protothecoides*. Such pathways

have been identified in bacteria [36,41,42] but this is the first report, to our knowledge, of potential HMP salvage capability in eukaryotic algae. Further research is required to elucidate the specific biochemical salvage mechanisms.

Thiamine auxotrophs must obtain the requisite precursors from other organisms in nature, however, thiamine is unstable under many conditions including water-soil mixtures [36]. Hence, salvage mechanisms should confer an evolutionary advantage to thiamine auxotrophs. For example, Karunakaran et al. showed that the soil bacteria *Rhizobium leguminosarum* could develop pin colonies on thiamine-free agarose plates and attributed this minimal growth to thiamine salvage [41].

Investigation of autotrophic algae cultures showed that thiamine also conferred growth benefits to *A. protothecoides*. A survey of 306 algae species by Croft et al. revealed that vitamin auxotrophy is widespread among algae: 22% require thiamine, 5% require biotin, and over half require exogenous cyanocobalamin [21]. Furthermore, even fully autotrophic species can benefit from the presence of certain cofactors. For example, *Chlamydomonas reinhardtii* has two methionine synthase genes, one of which requires cyanocobalamin as a cofactor. The cofactor-requiring enzyme is more efficient and hence preferentially used when cyanocobalamin is present in the environment [24]. Moreover, Xie et al. showed that the cyanocobalamin-dependent methionine synthase gene was more resilient to heat stress, which appeared to confer prolonged survival of *C. reinhardtii* under high temperature conditions [43]. These results suggest that cofactor symbiosis could benefit a wide range of algal species. Hence, the co-culture approach that we initially developed using *A. protothecoides* and *E. coli* could provide benefits to many algae-bacteria pairs through cofactor exchange.

Despite the benefits of thiamine toward autotrophic algal growth reported here, our previous results did not show any growth benefit of co-culturing *E. coli* and *A. protothecoides* under autotrophic conditions [12]. This outcome likely stems from low thiamine levels that resulted from bacterial cell populations in autotrophic cultures that were roughly three orders of magnitude lower than those in mixotrophic cultures. This result suggests the need for a robust bacterial population and points to the potential benefit of cultivating algae on wastewaters rather than attempting to create large-scale algal monocultures. Our present results also showed that residual medium from another green alga, *C. sorokiniana*, could also enhance *A. protothecoides* growth by 8.5 fold. *C. sorokiniana* appears to be capable of synthesizing thiamine, suggesting that cofactor symbiosis can extend to algal-algal co-cultures. This is particularly significant if both algae species produce valuable products. Moreover, algal co-cultures should confer benefits under fully autotrophic conditions in contrast to the algal-bacterial co-culture studied previously. This finding supports previous reports in which thiamine-producing cyanobacteria and eukaryotic algae were found to exude HMP into their growth medium [42].

Consideration must be given to cofactor synthesis capability, however, when selecting organisms for synthetic algal communities. Culture conditions should also be managed so as to encourage the growth of mutually beneficial organisms. Interestingly, differential cofactor requirements can be used as a culture management tool, allowing for the manipulation of organism abundance in mixed culture systems. Kazamia et al. showed that the relative abundance of algal and bacterial populations could be manipulated by controlling exogenous addition of cyanocobalamin [44]. Improved understanding of algal cofactor synthesis and salvage pathways is required, however, to fully realize the potential of this control strategy.

The present results demonstrate the value of co-culturing algae with other organisms for enhanced wastewater treatment and biofuel production. Cofactors, whether supplied exogenously or via other organisms, can enhance the growth and neutral lipid content of algae species such as *A. protothecoides*. In the present case, we also observed that thiamine and HMP could nearly eliminate pyruvic acid secretion, reducing secretion of organic compounds into the medium. This complements previous work in which we documented enhanced nitrogen uptake by algal-bacterial co-cultures [13], all of which should benefit algal wastewater treatment. Past research on coupled biofuel production and algal wastewater treatment has focused largely on the benefits of wastewater-derived organic substrates toward increased growth and lipid production [45,46]. The results shown here illustrate that cofactors from bacteria found in wastewaters could be at least as important toward biomass production as the organic substrate supply. This point merits further study of cofactor exchange between algae and bacteria in real wastewaters.

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