1 **Cofactor symbiosis for enhanced algal growth, biofuel production, and wastewater**
2 **treatment**
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19 **Abstract**

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

41 **Key words:** Cofactor, organism interaction, symbiosis, co-culture, biofuel, wastewater
42 treatment

43 **1. Introduction**

44 Algae have gained attention for biofuel production due to their rapid growth and high
45 lipid content [1, 2]. In spite of recent advances, however, algal biofuels are still too
46 expensive to compete with conventional liquid fuels. A recent techno-economic study
47 showed that increasing algal growth rates and lipid content are the two most important
48 factors for improving the economics of algal systems [3]. Hence significant research
49 effort has focused on simultaneously increasing algal growth and lipid content [4, 5].
50 Mixotrophic algae growth, in which cells simultaneously use photosynthesis and external
51 organic carbon sources, is one approach toward achieving increased lipid productivity
52 [6]. Production of co-products [7, 8] and services such as wastewater treatment have also
53 gained attention as a means of improving both the economics [9] and environmental
54 performance of algal biofuels [10]. An algal wastewater treatment environment will
55 likely include the presence and activity of bacteria. Thus, understanding algae-bacteria
56 interaction is important for engineering processes capable of simultaneous wastewater
57 treatment and biofuel production.

58 In a previous report, we observed evidence of a symbiotic relationship in which
59 *Escherichia coli* were found to enhance the growth of *Auxenochlorella protothecoides*
60 (UTEX 2341, which was previously annotated as *Chlorella minutissima* but has since
61 been reclassified [11]) by roughly 2-6 fold under high-substrate mixotrophic conditions
62 [12]. Co-cultures also consumed organic substrates more rapidly than the two axenic
63 organisms combined. We subsequently reported that the presence of *E. coli* facilitated a

64 near doubling of neutral lipid and fatty acid content in *A. protothecoides* and enhanced
65 organic substrate uptake and nitrogen removal [13]. Moreover, the fatty acid profile
66 shifted in a manner that should improve the oxidative stability of biodiesel produced from
67 the algal lipids. These results suggest the potential of employing co-culturing as a
68 strategy to enhance water treatment performance and biofuel production in algae.

69 Given these substantial benefits, there was interest in determining the mechanisms
70 of symbiosis and whether these mechanisms extend beyond the algae-bacteria pair that
71 we studied. Such knowledge would allow for the design and operation of algal systems
72 that foster the growth of mutually beneficial organisms. It would also provide a basis for
73 understanding organism interaction in algal wastewater treatment systems, an area of
74 increasing interest [14-17]. Moreover, the engineering of synthetic co-cultures and
75 microbial communities has emerged as a new research frontier in the quest for bio-
76 derived fuels and chemicals [18].

77 At the time, we concluded that the *Auxenochlorella-E. coli* symbiotic relationship
78 was linked to substrate uptake and utilization because we did not observe similar
79 symbiosis in autotrophic cultures. We also observed low substrate utilization efficiency in
80 mixotrophic *A. protothecoides* cultures and that the presence of *E. coli* could improve this
81 efficiency under certain conditions [12]. However, the molecular mechanisms of
82 symbiosis were not elucidated. Research by others suggested three potential sources of
83 mutualistic symbiosis: exchange of primary metabolites [19, 20], exchange of cofactors
84 and hormones [15, 21, 22], and the establishment of unique physical niches within the
85 microbial ecosystem [16]. Carbon dioxide-oxygen exchange is perhaps the most-
86 discussed primary metabolite exchange in algal-bacterial systems [23]. In the ideal

87 scenario, bacteria degrade organic material in wastewater, producing carbon dioxide that
88 is then consumed by photosynthetic algae. The algae provide dissolved oxygen to
89 bacteria, further enhancing aerobic degradation of soluble organic material.

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

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

104

105 **2. Materials and Methods**

106 *2.1 Algae and bacteria cultivation*

107 Algae were pre-cultured in 1L bottles without thiamine supplementation to achieve a
108 density of roughly 10^7 cells/ml based on hemocytometer counts (~0.15 OD at 550 nm) as
109 described previously [12]. Cells were settled overnight, media was removed, and the

110 concentrated cells were used to inoculate 300 ml hybridization tubes filled to 200 ml with
111 fresh medium. N8-NH_4 medium was used for *Auxenochlorella protothecoides* (UTEX
112 2341) and *Escherichia coli* (ATCC 25922) cultures [12], and N8 medium was used for
113 *Chlorella sorokiniana* (UTEX 2805) [28]. Cultures were supplied with varying glucose
114 and glycerol (Fisher) levels as described in figure legends. Illumination (10,000 lux) was
115 provided by T5 growth lamps oriented horizontally. Stir bars (150 rpm) provided mixing,
116 and aeration was supplied (125 ml/min) without supplemental CO_2 with the exception of
117 autotrophic cultures where 2% v/v CO_2 was mixed with air. Cultures were harvested after
118 5 days of growth by centrifugation and freeze dried as previously described [12]. This
119 harvest point was selected because it generally coincided with the late log stage of
120 growth. In cases where algae were grown on residual medium from other organisms, cells
121 were initially removed by centrifugation followed by filtration through 0.2 µm filters
122 (Nalgene Rapid-Flow). Glucose and ammonium chloride, which were expected to be the
123 limiting substrates, were added to achieve the desired level, and residual medium was re-
124 inoculated with algae. Thiamine (Acros), THZ (Sigma), pantothenic acid (MP), or HMP
125 (>95%, synthesized by Arc-Pharm) were added to cultures per the experimental design.
126
127 *2.2 Neutral lipid analysis*
128 Lipids were extracted from freeze-dried algae using a modified Folch method as
129 previously described [12]. Algal neutral lipids were measured using a previously
130 described microplate assay with modifications [29]. Specifically, the Nile red solution
131 concentration was increased to 1 ug/ml (from 0.5 ug/ml) and canola oil was used as a
132 standard (rather than corn oil).

133

134 *2.3 Metabolite extraction and analysis by GC-TOF*

135 Culture samples (1 ml) were collected daily and quenched by the addition of 1 ml 70%

136 MeOH (-70 °C). Samples were centrifuged at 12,000 g for 2 minutes at 4 °C and

137 supernatant was discarded prior to freeze drying. Metabolites were extracted with 10:3:1

138 chloroform/ methanol/ water as described previously [30]. Derivatization by MSTFA and

139 GC-TOF analysis were performed as described previously [30].

140

141 *2.4 HPLC and GC-TOF analysis of pyruvate and fumarate*

142 An Aminex 87H column (Bio-rad) was used in conjunction with RID and PDA detection

143 (210 nm) to quantify glucose, pyruvate, and fumarate concentrations in media. HPLC

144 conditions have been described previously [12]. Fractions containing peaks

145 corresponding to pyruvate (RT 9.6) and fumarate (RT 13.9) were collected, freeze dried,

146 derivatized and analyzed by GC-TOF as described above.

147

148 *2.5 E. coli residual media preparation for HPLC and LCMS analysis*

149 After cell removal, residual culture media (35 ml) was freeze dried and re-suspended in 1

150 ml methanol, then centrifuged at room temperature at 6000 g to pellet salts. Supernatant

151 was recovered and either injected directly for C18 HPLC analysis/fractionation or freeze-

152 dried a second time for LCMS analyses. Re-dried material was resuspended in 100 µl

153 MeOH and centrifuged at 12000 g to pellet salts. Supernatant was recovered and injected

154 on either LC-Qtrap (AB Sciex) or LC-MSTOF (Agilent) platforms.

155

156 *2.6 C18 HPLC of thiamine derivatives, precursors, and degradation products*

157 A 4.6x250 mm C18 column (Zorbax SB-C18, Agilent) was used in conjunction with
158 PDA detection (254 nm) on a Prominence HPLC system (Shimadzu). Mobile phase A
159 was 100% MiliQ water and phase B was 100% HPLC-grade methanol (Fisher). Flow was
160 maintained at 1 ml/min with the following gradient: 0-4 min., 0% B, 4-10 min., ramp to
161 60% B, 10-20 min., ramp to 100% B, 20-29 min., hold 100% B, 29-30 min., ramp to 0%
162 B, 30-35 min., hold 0% B. The column was kept at 30 °C. Fractions were collected from
163 each sample over 3 replicate injections of 75 µl and pooled. Pooled fractions were freeze-
164 dried and resuspended in water and sterile filtered (0.2 µm) for addition to algae cultures.
165

166 *2.7 LC-MS/MS (QTRAP) analysis of thiamine derivatives and precursors*

167 Thiamine metabolites were quantified by LC-MS/MS using a HILIC method. An AB
168 Sciex 4000 QTrap LC/MS/MS System with Agilent Technologies 1200 Series LC
169 (degasser, binary pump, thermostated autosampler at 4 °C, column oven at 45 °C) was
170 used. The separation of thiamine, TMP, TPP, THZ and HMP was achieved on an Atlantis
171 HILIC Silica column 3 um, 2.1x100 mm, Part no 186002015 (Waters, Milford, MA)
172 using a 24-min gradient method (Solvent A, 200 mM ammonium formate/formic acid pH
173 3 in Water, Solvent B, 100% acetonitrile) starting with 70% B from 0-6 min, decreasing
174 to 30% B from 6-12 min, back to 70% B in 2 min and re-equilibrating for another 10 min
175 for a total run time of 24 min, at a flow rate of 0.3 mL/min. Analytes were detected by
176 multiple reaction monitoring (MRM) after positive mode electrospray ionization.
177 Residues were quantified against curves of authentic standards purchased from Sigma or
178 Arc Pharm, using external standard methodology.

179 *2.8 LC-MSTOF analysis of residual E. coli media*

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

191

192 *2.9 Thiamine uptake and degradation in E. coli cultures*

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

199

200 *2.10 Thiochrome assay*

201 The thiochrome assay was used for rapid detection of thiamine concentration.
202 Derivatization of thiamine to the thiochrome product was carried out based on the
203 method of Lu et al. with modifications [31]. Briefly, 120 µl of aqueous sample or
204 standard was added to a 96-well microplate followed by 40 µl of methanol to intensify
205 the signal. Background fluorescence was read on a plate reader (Spectramax M2) with
206 375 nm excitation and 435 nm emission. Samples were then derivatized by adding 75 µl
207 of freshly prepared 0.6 mM potassium ferricyanide solution dissolved in 15% (w/v)
208 aqueous NaOH. Wells were mixed by pipetting up and down and fluorescence was read
209 within 5 minutes of reagent addition. Background fluorescence was subtracted from final
210 readings. Thiamine-HCl was used as an external standard.

211

212 *2.11 Statistical analysis*

213 Paired and independent group t-tests were carried out in Excel using published equations
214 [32]. ANOVA and Tukey's HSD test were carried out in R (v. 3.1.1) using the "car" and
215 "agricolae" packages [33]. R was also used to test for homogeneity of variance using
216 Levene's test and the Brown and Forsythe test prior to carrying out ANOVA.

217

218 **3. Results**

219 *3.1 Secretion of organic acids by mixotrophic A. protothecoides*

220 We previously reported that significant acidification of media occurred under
221 mixotrophic cultivation of *A. protothecoides* on glucose and glycerol [12]. Analysis of
222 time-course media samples by HPLC revealed significant growth over time in two peaks

223 that absorbed light in the 200-210 nm range suggesting carboxylic acid secretion. These
224 peaks did not appear under autotrophic conditions. The two peaks were collected in
225 separate fractions, freeze dried, derivatized, and identified by GC-TOF as pyruvate and
226 fumarate.

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

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

240

241 *3.2 Residual E. coli media enhanced growth of mixotrophic A. protothecoides*

242 Our next line of inquiry focused on whether *E. coli* cells were required for symbiosis or if
243 effects were due to compounds released into the medium. We tested this by culturing *A.*
244 *protothecoides* on residual *E. coli* medium after cell removal. HPLC analysis was
245 employed after *E. coli* growth to ensure complete glucose removal prior to fresh

246 supplementation with 10 g/L glucose. *A. protothecoides* was then cultivated on this
247 supplemented spent medium.

248 The residual *E. coli* medium supported *A. protothecoides* growth that was nearly
249 18 fold greater than growth on fresh medium supplemented with 10 g/L glucose (Figure
250 3A). Moreover, the cultures grown on residual media also consumed all glucose after five
251 days and did not secrete any detectable pyruvate. In contrast, the control cultures
252 consumed ~1.3 g/L glucose and secreted 0.8 g/L pyruvate. This indicated that secreted
253 metabolites from *E. coli* could dramatically enhance glucose uptake and utilization by *A.*
254 *protothecoides*.

255

256 *3.3 Metabolomics analysis of mixotrophic A. protothecoides*

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

265

266 *3.4 Pyruvate dehydrogenase and thiamine deficiency*

267 Commercial pyruvate production from glucose by yeast and *E. coli* auxotrophs is
268 accomplished through aerobic fermentation in the absence of cofactors required by

269 pyruvate dehydrogenase (PDH) [34]. Enzymes involved in anaerobic pathways such as
270 pyruvate decarboxylase are likewise impaired by cofactor auxotrophy (e.g. lipoic acid).
271 The result is accumulation of intracellular pyruvate which is eventually secreted into the
272 medium. We hypothesize a similar scenario for *A. protothecoides* when grown on glucose
273 and glycerol.

274 We cultured *A. protothecoides* on glucose with exogenous addition of thiamine
275 and pantothenic acid, the precursors to two PDH cofactors. Thiamine addition resulted in
276 a 10.7 fold increase in productivity over control cultures whereas pantothenic acid had no
277 effect (Figure 3B). The combination of thiamine and pantothenic acid led to 20% greater
278 productivity than thiamine alone suggesting some synergy of the two cofactors ($p = 0.01$,
279 Tukey HSD). Cultures provided with thiamine also consumed nearly all glucose and did
280 not secrete detectable pyruvic acid. We hypothesize that *A. protothecoides* is unable to
281 synthesize sufficient thiamine, resulting in a metabolic bottleneck at pyruvate
282 dehydrogenase. The minimum level of thiamine required to achieve maximum growth on
283 8 g/L glucose was found to be ~20 nM based on fitting dose-response data to a saturation
284 model (Equation 1). Above 20 nM, no significant increase in growth was observed
285 (Figure 3C).

286

287 $$p = \frac{p_{max} S}{k_S + S} + p_b \qquad \text{Equation 1}$$

288

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

292

293 *3.5 Thiamine levels in E. coli medium*

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

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

314

315 *3.6 Thiamine salvage mechanisms in A. protothecoides*

316 Thiamine salvage mechanisms have been shown to exist in a variety of bacteria [37] and
317 genomic evidence exists for their presence in eukaryotic organisms [36]. However,
318 thiamine salvage mechanisms have not been thoroughly elucidated in eukaryotic algae to
319 date [26]. Thiamine is synthesized by the condensation of two precursors: 4-amino-5-
320 hydroxymethyl-2-methylpyrimidine (HMP) and 4-methyl-5-(β-hydroxyethyl)thiazole
321 (THZ) as shown in Figure 4A. These two compounds are also potential products of
322 thiamine degradation, catalyzed by thiaminases [38]. Some eukaryotic algae have been
323 shown to contain the necessary enzymes to synthesize thiamine but lack pathways to
324 produce one of the precursors [39]. To test this hypothesis, we cultured *A. protothecoides*
325 mixotrophically with HMP, THZ, or a combination of these two thiamine precursors.
326 HMP resulted in a 26 fold increase in algal growth compared to control cultures but THZ
327 had no effect (Figure 4B). Moreover, cultures provided with either thiamine or HMP had
328 more than seven-fold greater neutral lipid content than mixotrophic cultures provided
329 with only glucose (Table 1).

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

337

338 *3.7 Chromatographic fractions of residual E. coli media show growth enhancement*

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

346 To determine if multiple HMP-salvageable molecules were secreted by *E. coli*,
347 we fractionated concentrated *E. coli* medium by HPLC using a reverse-phase method. Six
348 fractions were collected from each sample over multiple injections (Figure 4C) so as to
349 concentrate each fraction. All fractions except for fraction 2 enhanced *A. protothecoides*
350 growth, with fractions 1 and 4 supporting growth on par with whole *E. coli* medium
351 (Figure 4D). Injections of individual standards revealed that TPP should elute in fraction
352 1 and HMP in fraction 4 (Figure S2A,B). Fractions three, five, and six also promoted
353 growth, suggesting that media compounds besides TPP and HMP contribute to
354 symbiosis.

355

356 *3.8 Thiamine enhances growth of autotrophic algae*

357 This work primarily focused on the benefits of thiamine symbiosis as it pertains to
358 mixotrophic algae cultures. However, thiamine supplementation was also found to
359 increase the growth of autotrophic *A. protothecoides* by 36 fold ($p < 0.001$, 2-tailed
360 independent group t-test) (Figure 5A). We also tested thiamine supplementation on

361 another green algae species, *Chlorella sorokiniana* and observed a 19% increase in
362 growth but the difference was not statistically significant (p = 0.09, 2-tailed independent
363 group t-test) (Figure 5A). We therefore concluded that *C. sorokiniana* could synthesize
364 thiamine but may receive a modest benefit from exogenous addition.

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

373

374 **4. Discussion**

375 We have shown that the previously-observed symbiosis between *A. protothecoides* and *E.*
376 *coli* appears to stem largely from exchange of TPP, HMP and other thiamine degradation
377 products. We acknowledge that other mechanisms such as carbon dioxide-oxygen
378 exchange could also play a role in symbiosis. However, an order of magnitude increase in
379 *A. protothecoides* growth could be achieved when using cell-free residual *E. coli* medium
380 suggesting that cofactor exchange is the predominant symbiotic mechanism in this case.
381 *A. protothecoides* could grow without thiamine supplementation and we previously
382 showed that its growth rates under a variety of mixotrophic conditions were comparable
383 to those reported for several other *Chlorella* species [6, 12, 40]. Nevertheless, supply of

384 the glycolytic substrates glucose and glycerol to *A. protothecoides* culture led to secretion
385 of substantial quantities of pyruvic acid. This represents a significant inefficiency in
386 substrate utilization when the goal is production of intracellular products such as lipids.
387 Pyruvate secretion could also partially explain the low substrate utilization efficiency that
388 we reported in previous studies on *A. protothecoides* [12]. Secretion of organic
389 compounds by algae is also inefficient in wastewater treatment where the goal is to
390 reduce chemical and biological oxygen demand. We have shown that *A. protothecoides's*
391 pyruvate secretion is due to a metabolic bottleneck at the PDH complex that can be
392 relieved by thiamine addition. This bottleneck may also explain why axenic *A.*
393 *protothecoides* growth exhibited saturation behavior with respect to glucose
394 concentration: providing 10 g/L glucose resulted in the same growth as 2 g/L glucose.
395 Such a bottleneck is expected to inhibit glycolysis and TCA cycle activity, consequently
396 suppressing biomass growth and substrate utilization.

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

407 presence of HMP salvage pathways in *A. protothecoides*. Such pathways have been
408 identified in bacteria [36, 41, 42] but this is the first report, to our knowledge, of potential
409 HMP salvage capability in eukaryotic algae. Further research is required to elucidate the
410 specific biochemical salvage mechanisms.

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

417 Investigation of autotrophic algae cultures showed that thiamine also conferred
418 growth benefits to *A. protothecoides.* A survey of 306 algae species by Croft et al.
419 revealed that vitamin auxotrophy is widespread among algae: 22% require thiamine, 5%
420 require biotin, and over half require exogenous cyanocobalamin [21]. Furthermore, even
421 fully autotrophic species can benefit from the presence of certain cofactors. For example,
422 *Chlamydomonas reinhardtii* has two methionine synthase genes, one of which requires
423 cyanocobalamin as a cofactor. The cofactor-requiring enzyme is more efficient and hence
424 preferentially used when cyanocobalamin is present in the environment [24]. Moreover,
425 Xie et al. showed that the cyanocobalamin-dependent methionine synthase gene was
426 more resilient to heat stress, which appeared to confer prolonged survival of
427 *Chlamydomonas reinhardtii* under high temperature conditions [43]. These results
428 suggest that cofactor symbiosis could benefit a wide range of algal species. Hence, the

429 co-culture approach that we initially developed using *A. protothecoides* and *E. coli* could
430 provide benefits to many algae-bacteria pairs through cofactor exchange.

431 Despite the benefits of thiamine toward autotrophic algal growth reported here,
432 our previous results did not show any growth benefit of co-culturing *E. coli* and *A.*
433 *protothecoides* under autotrophic conditions [12]. This outcome likely stems from low
434 thiamine levels that resulted from bacterial cell populations in autotrophic cultures that
435 were roughly three orders of magnitude lower than those in mixotrophic cultures. This
436 result suggests the need for a robust bacterial population and points to the potential
437 benefit of cultivating algae on wastewaters rather than attempting to create large-scale
438 algal monocultures. Our present results also showed that residual medium from another
439 green alga, *C. sorokiniana*, could also enhance *A. protothecoides* growth by 8.5 fold. *C.*
440 *sorokiniana* had no apparent thiamine requirement suggesting that cofactor symbiosis can
441 extend to algal-algal co-cultures. This is particularly significant if both algae species
442 produce valuable products. Moreover, algal co-cultures should confer benefits under fully
443 autotrophic conditions in contrast to the algal-bacterial co-culture studied previously.
444 This finding supports previous reports in which thiamine-producing cyanobacteria and
445 eukaryotic algae were found to exude HMP into their growth medium [42].

446 Consideration must be given to cofactor synthesis capability, however, when
447 selecting organisms for synthetic algal communities. Culture conditions should also be
448 managed so as to encourage the growth of mutually beneficial organisms. Interestingly,
449 differential cofactor requirements can be used as a culture management tool, allowing for
450 the manipulation of organism abundance in mixed culture systems. Kazamia et al.
451 showed that the relative abundance of algal and bacterial populations could be

452 manipulated by controlling exogenous addition of cyanocobalamin [44]. Improved
453 understanding of algal cofactor synthesis and salvage pathways is required, however, to
454 fully realize the potential of this control strategy.

455 The present results demonstrate the value of co-culturing algae with other
456 organisms for enhanced wastewater treatment and biofuel production. Cofactors, whether
457 supplied exogenously or via other organisms, can enhance the growth and neutral lipid
458 content of algae species such as *A. protothecoides*. In the present case, we also observed
459 that thiamine and HMP could nearly eliminate pyruvic acid secretion, reducing secretion
460 of organic compounds into the medium. This complements previous work in which we
461 documented enhanced nitrogen uptake by algal-bacterial co-cultures [13], all of which
462 should benefit algal wastewater treatment. Past research on coupled biofuel production
463 and algal wastewater treatment has focused largely on the benefits of wastewater-derived
464 organic substrates toward increased growth and lipid production [45, 46]. The results
465 shown here illustrate that cofactors from bacteria found in wastewaters could be at least
466 as important toward biomass production as the organic substrate supply. This point merits
467 further study of cofactor exchange between algae and bacteria in real wastewaters.
468
469

470 **Acknowledgements**

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472 National Institute of Food and Agriculture project CA-D-BAE-2228-RR.
473

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602
603

604

605 Tables

606 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

607

608 [a] N8-NH_4 medium supplemented with 8 g/L glucose was used for all cultures. [b] Control

609 cultures had no cofactor added. [c] Glucose and ammonium chloride were added to residual

610 *C. sorokiniana* medium to restore levels to desired quantity. [d] Standard deviation is

611 shown in parenthesis based on 3 biological replicates and the following letter shows

612 significance based on Tukey's HSD test carried out a the 0.05 level. [e] Lipid productivity

613 is the product of lipid content and biomass growth rate.

614

615 Figure Captions

616

617 Figure 1: Substrate uptake and pyruvate secretion in mixotrophic cultures after 5 days of

618 growth. Substrate uptake and pyruvate secretion by axenic *A. protothecoides* cultivated

619 on (A) glucose and (B) glycerol. (C) Algal biomass (Alg) growth curve and pyruvate

620 (Pyr) secretion in axenic *A. protothecoides* culture supplemented with or without glucose.

621 No pyruvate secretion was detected in autotrophic culture. (D) Co-cultures of *A.*

622 *protothecoides* and *E. coli* exhibited high glucose consumption and minimal pyruvate

623 secretion. For bar graphs, bars with the same letter above them are not significantly
624 different at the 0.05 level based on Tukey's HSD test. Capital letters are used to denote
625 significance for substrate uptake and lowercase letters are used for pyruvate secretion.
626 Error bars are SD, n = 3 biological replicates.
627
628 Figure 2: Metabolite profile shift over time in axenic *A. protothecoides* in response to
629 mixotrophy on 10 g/L glucose (10 Glc). (A) Principle component plot based on 274
630 known and unknown metabolites excluding glucose. Relative abundances of metabolites
631 were normalized against the biomass concentration of each sample. Each point is a single
632 biological replicate. (B) Fold difference in abundance of specific metabolites of *A.*
633 *protothecoides* cultured on 10 g/L glucose for 5 days compared to autotrophic cultures.
634 The p-value reflects the significance of the difference based on an independent group t-
635 test.
636
637 Figure 3: Effects of residual media and cofactor precursors on axenic cell culture. (A) *A.*
638 *protothecoides* growth (biomass), glucose utilization (glucose), and pyruvate secretion
639 (pyruvate) after 5 days of growth on residual or fresh (control) medium supplemented
640 with 10 g/L glucose. Residual medium was prepared by culturing *E. coli* on N8-NH_4
641 medium supplemented with 2 g/L glucose for 36 hours, followed by cell removal. Error
642 bars are SD, n = 4 biological replicates. (B) *A. protothecoides* growth on N8-NH_4
643 medium supplemented with 8 g/L glucose and either 5 µM thiamine-HCl, 68 µM
644 pantothenic acid, or both. Error bars are SD, n = 3. Within each measurement type
645 (biomass growth, glucose consumption, or pyruvate secretion), bars with the same letter

646 are not significantly different at the 0.05 level based on Tukey's HSD test. (C) Dose
647 response results for *A. protothecoides* growth on varying levels of thiamine-HCl. Curve
648 fit to the saturation model was performed using Matlab's nlinfit algorithm. The half-
649 velocity constant was 4.57 nM thiamine-HCl. (D) Thiamine-HCl concentration in
650 medium monitored over time using thiochrome assay to assess thiamine degradation or
651 re-uptake. Initial doses of 0 (not shown because the level remained at 0 ng/ml throughout
652 the study), 100, and 500 ng/ml thiamine-HCl were added to N8-NH_4 medium
653 supplemented with 10 g/L glucose. Samples followed by (+) were inoculated with *E. coli*
654 and those with (-) contained no cells. Error bars are SD based on 3 biological replicates.
655
656 Figure 4: Thiamine precursor effect on *A. protothecoides* metabolism. (A) Schematic of
657 thiamine biosynthesis from HMP and THZ precursors. (B) HMP and THZ effects on *A.*
658 *protothecoides* growth and glucose metabolism. Cultures were supplied with 8 g/L
659 glucose and 100 nM of HMP and/or THZ. Within each measurement type (biomass
660 growth, glucose consumption, or pyruvate secretion), bars with the same letter are not
661 significantly different at the 0.05 level based on Tukey's HSD test. Error bars are SD
662 based on 3 biological replicates. (C) Chromatogram (254 nm absorbance) of *E. coli*
663 medium subject to fraction collection. Media from three *E. coli* biological replicates were
664 separately freeze dried, re-suspended to form a concentrate, and fractionated by HPLC
665 using a C18 column. Fractions 1-6 were collected from 2.3-4.0 min., 4.0-7.0 min., 7.0-
666 14.0 min., 14.0-21.0 min., 21.0-28.0 min., and 28.0-35.0 min., respectively. (D) Growth
667 of *A. protothecoides* on *E. coli* media fractions. Media fractions were freeze dried, re-
668 suspended, sterile filtered, and spiked into fresh glucose-free N8-NH_4 medium to supply

669 three corresponding algal biological replicates. Error bars are SD based on these three
670 biological replicates and encompass variability in *E. coli* growth, fractionation, and *A.*
671 *protothecoides* growth. Bars with the same letter are not significantly different at the 0.05
672 level based on Tukey's HSD test.
673
674 Figure 5: Effects of thiamine on autotrophic green algae. (A) Growth comparison with
675 and without exogenous thiamine-HCl (500 nM) in *A. protothecoides* and *C. sorokiniana*.
676 Error bars are SD based on 3 biological replicates. Because the two organisms were
677 cultured in separate batches, multiple comparison statistical tests were not employed. (B)
678 Growth, glucose uptake, and pyruvate secretion of *A. protothecoides* on residual medium
679 from *C. sorokiniana* supplemented with 8 g/L glucose after cell removal. Within each
680 measurement type (biomass growth, glucose consumption, or pyruvate secretion), bars
681 with the same letter are not significantly different at the 0.05 level based on Tukey's HSD
682 test. Error bars are SD based on 3 biological replicates.
683
684 Figure S1: Effect of exogenous pyruvate on *Auxenochlorella protothecoides* growth and
685 glucose metabolism. (A) Growth curves on varying glucose (glc) concentration with (+P)
686 and without 0.5 g/L exogenous pyruvate. No exogenous thiamine was provided. (B)
687 Glucose uptake (C) Pyruvate in medium.
688
689 Figure S2: Sample HPLC chromatograms using a Zorbax C18 column with water-
690 methanol gradient. (A) TPP and (B) HMP. Absorbance is 254 nm.
691

692 Figure S3: Growth of *A. protothecoides* on base-degraded thiamine. Base-degraded
693 thiamine was prepared by incubating 1M thiamine-HCl in 5M KOH at room temperature
694 for 8 days. Precipitates were removed by filtration through a 0.2 µm filter (Titan3 PTFE,
695 Thermo). Silica gel solid phase extraction columns (Fisherbrand) were rinsed and
696 equilibrated by washing with 7 ml 90:10 chloroform/ methanol. 1 ml of degraded-
697 thiamine mixture was added to the column. The column was washed with an additional 7
698 ml of 90:10 chloroform/methanol, then hydrophilic products were eluted with 1.5 ml
699 dH_2O and collected. A portion of the eluent was analyzed by LC-MSTOF. Another
700 portion of eluent was diluted 10-fold and fractionated by HPLC with a Zorbax SB-C18
701 column to further remove residual thiamine by collecting two fractions: 2.6-12.5 min (F1)
702 and 12.5-27.0 min (F2) over the course of 3x 75 µl injections (Figure S3A). The resulting
703 fractions were freeze-dried, re-suspended in dH_2O, and sterile filtered prior to addition to
704 algae cultures. The two fractions were tested for thiamine using the thiochrome assay and
705 trace residual thiamine was detected in both fractions. Upon addition of degraded
706 samples to algae cultures, the resulting thiamine concentrations were 0.08 and 0.38 nM
707 for fractions 1 and 2, respectively. Control cultures were prepared with 0.37 nM thiamine
708 to compensate for incomplete degradation. The degraded thiamine fractions supported
709 more than two fold faster growth, roughly 50% more glucose consumption, and near
710 elimination of pyruvate secretion compared to the control cultures (Figure S3B). LC-
711 MSTOF analysis of base-degraded thiamine revealed the presence of HMP and THZ.
712

Acknowledgements

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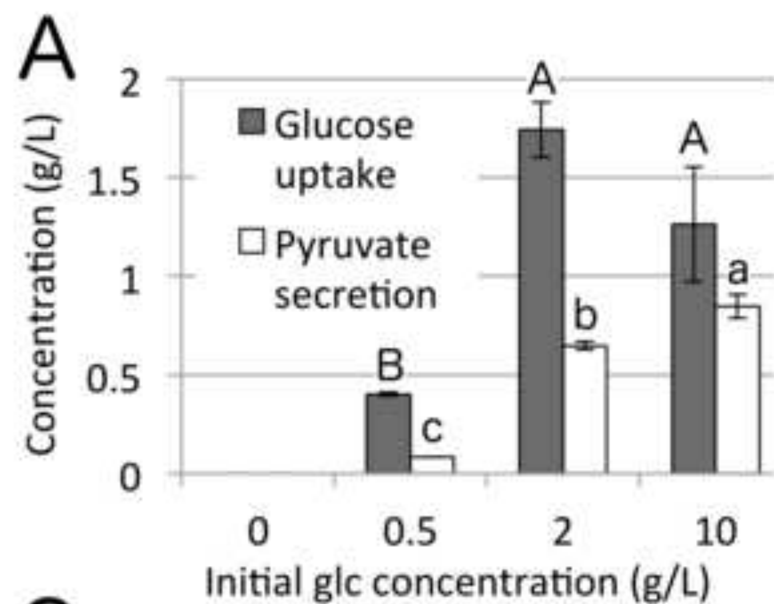
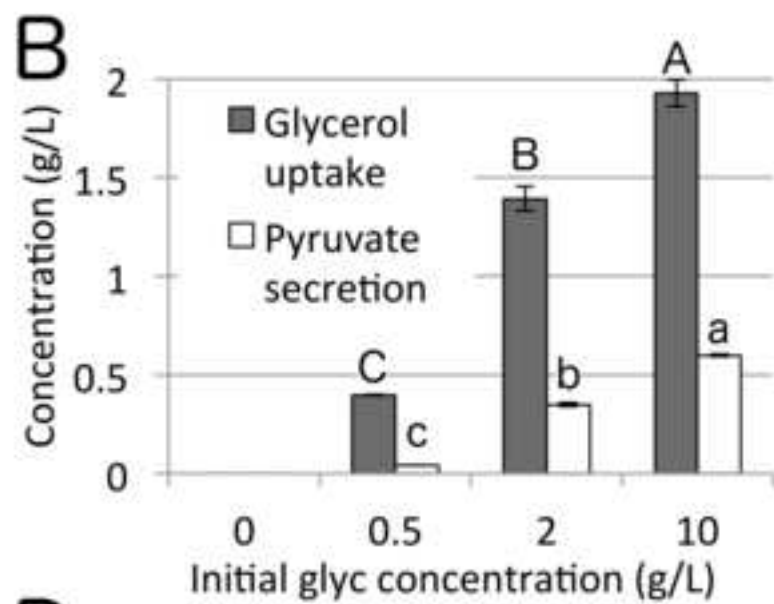
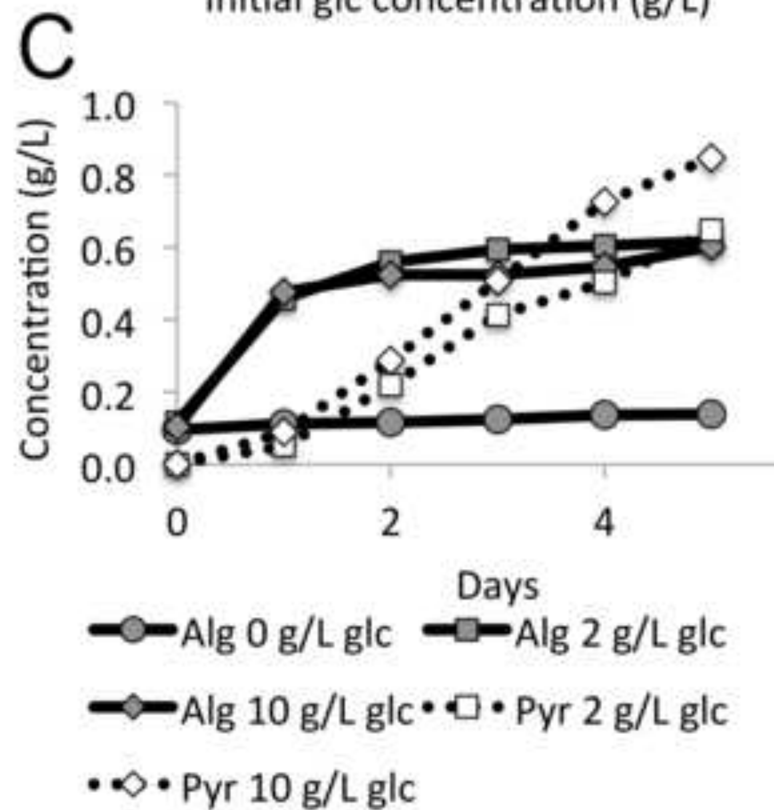
Figure 1







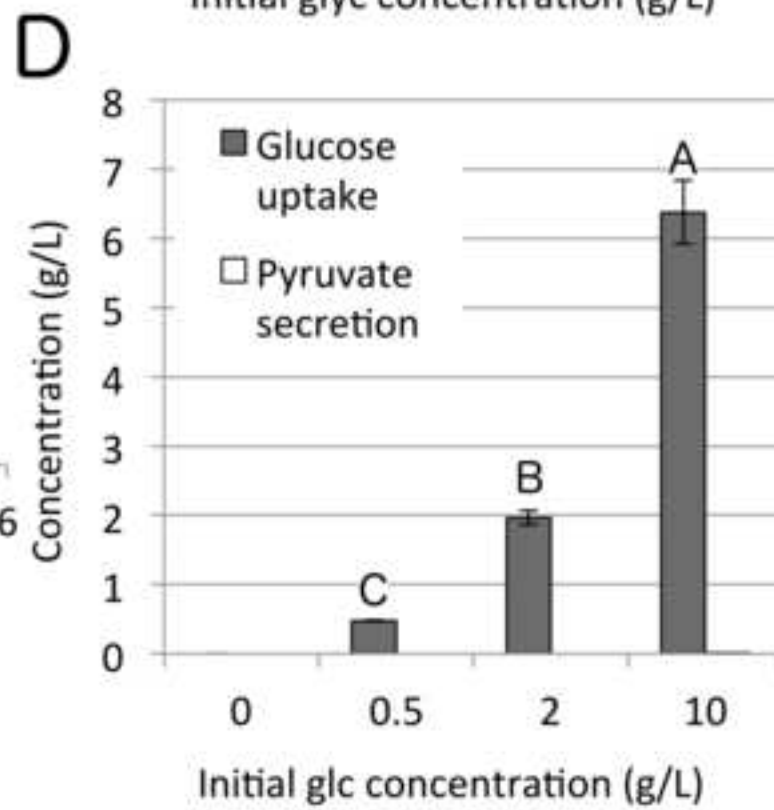
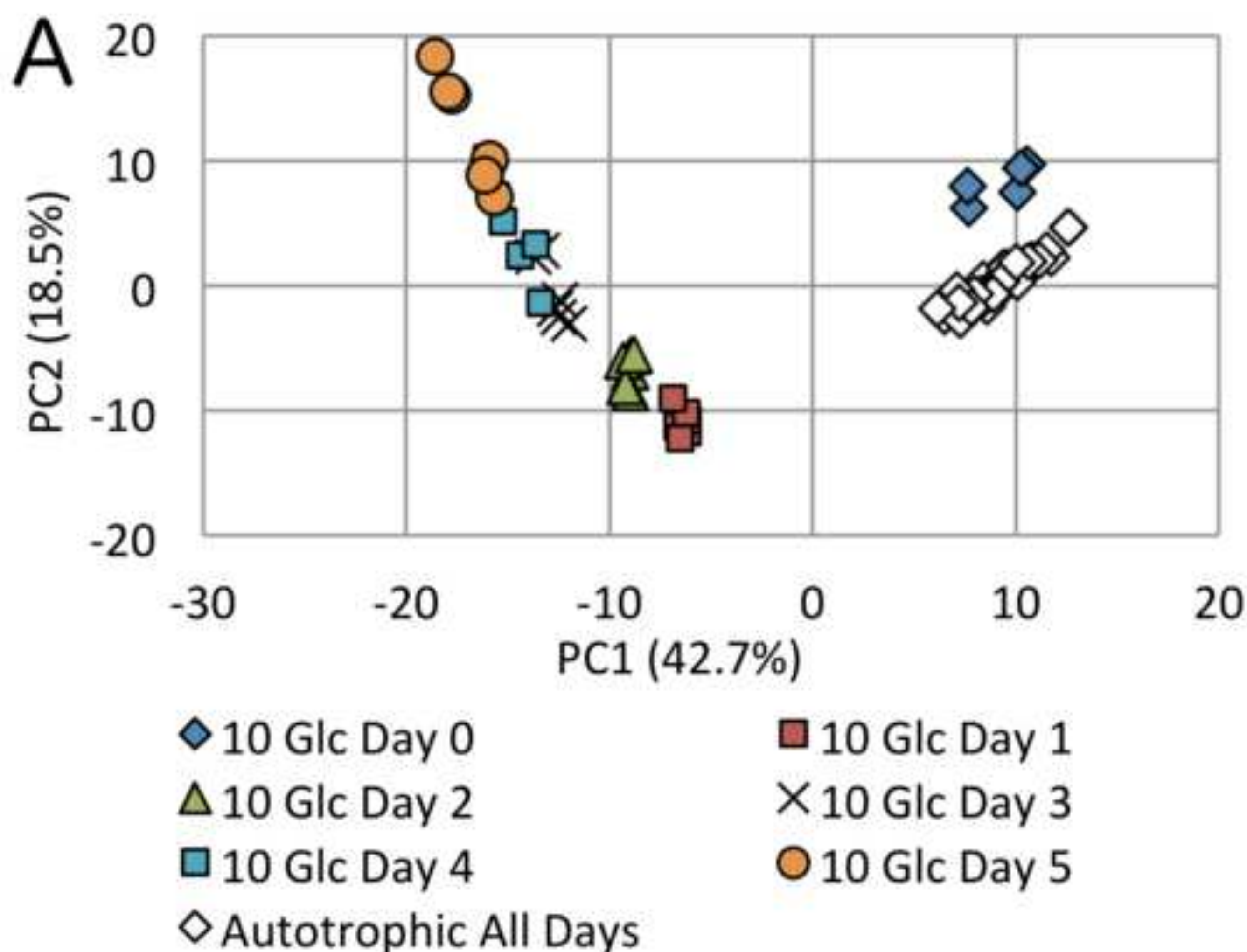


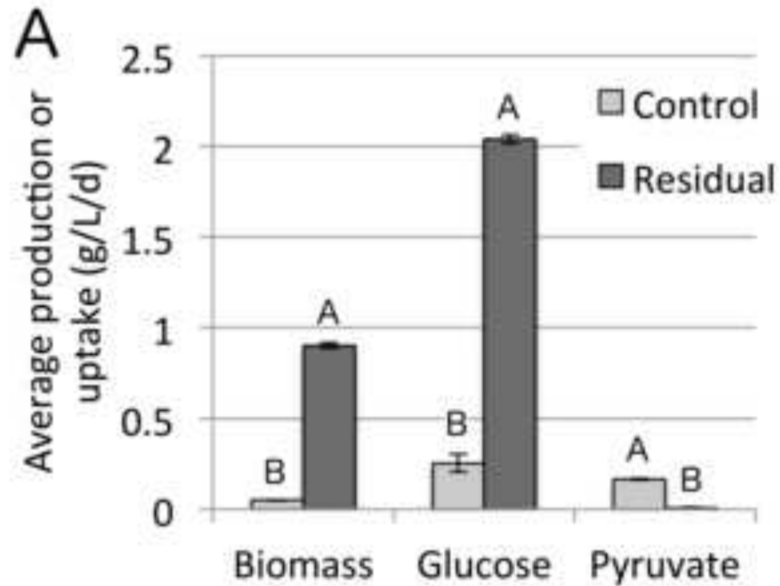
Figure 2

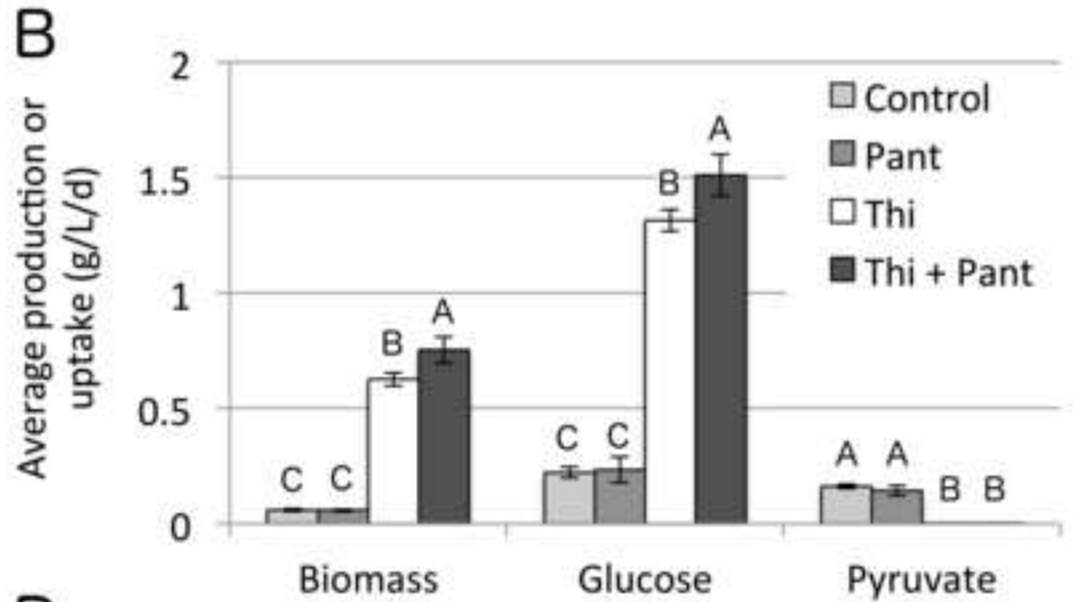


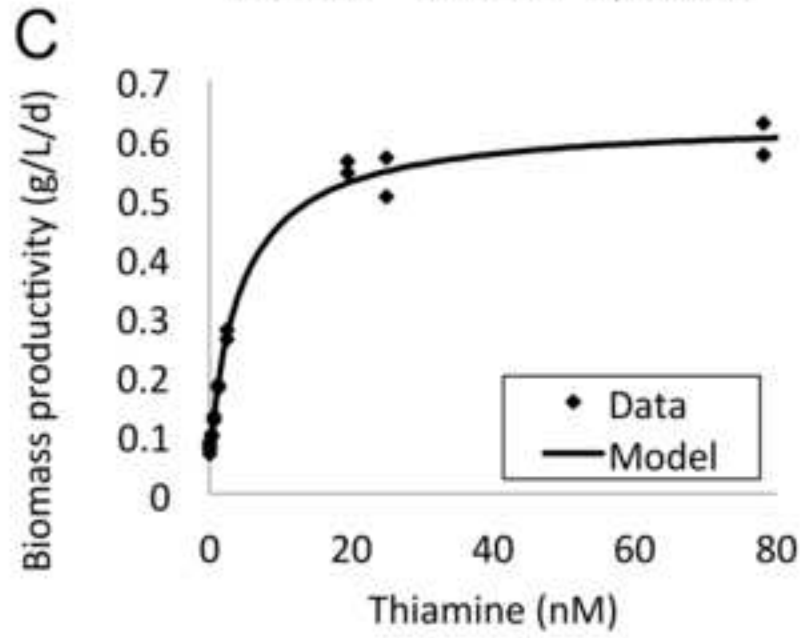
B

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

Figure 3







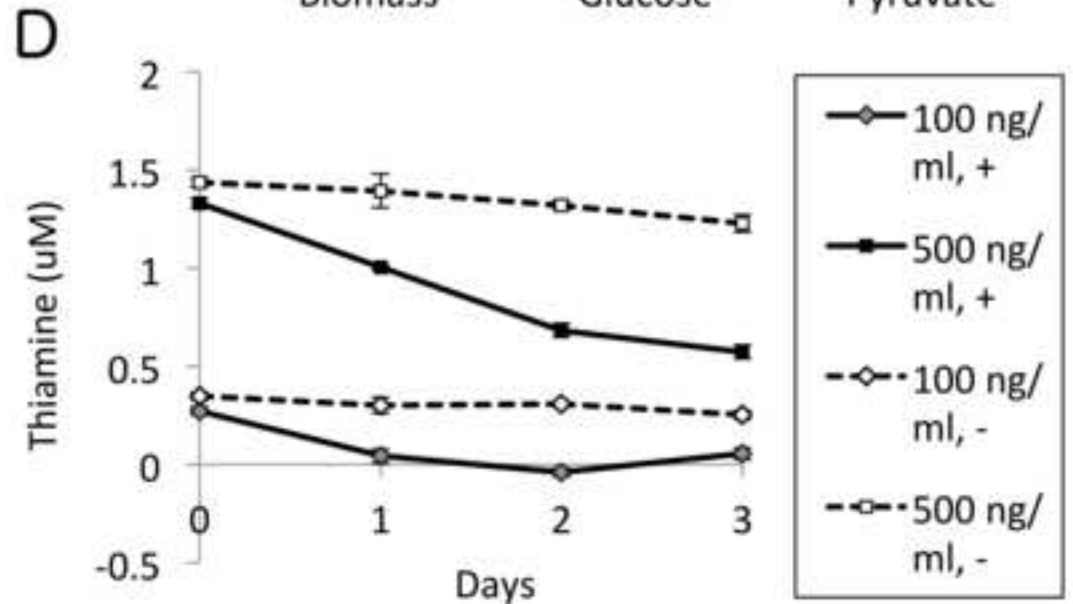


Figure 4

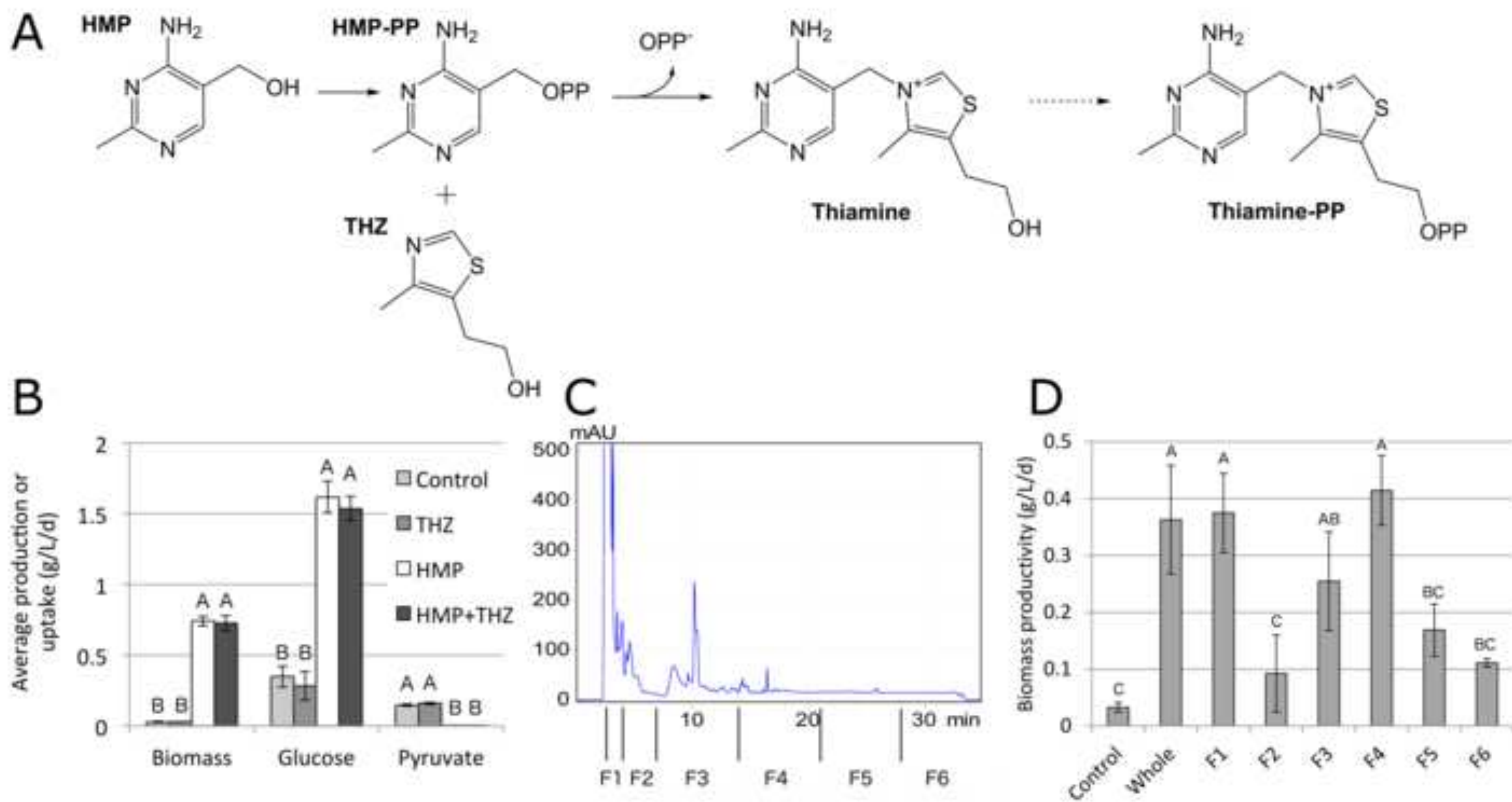
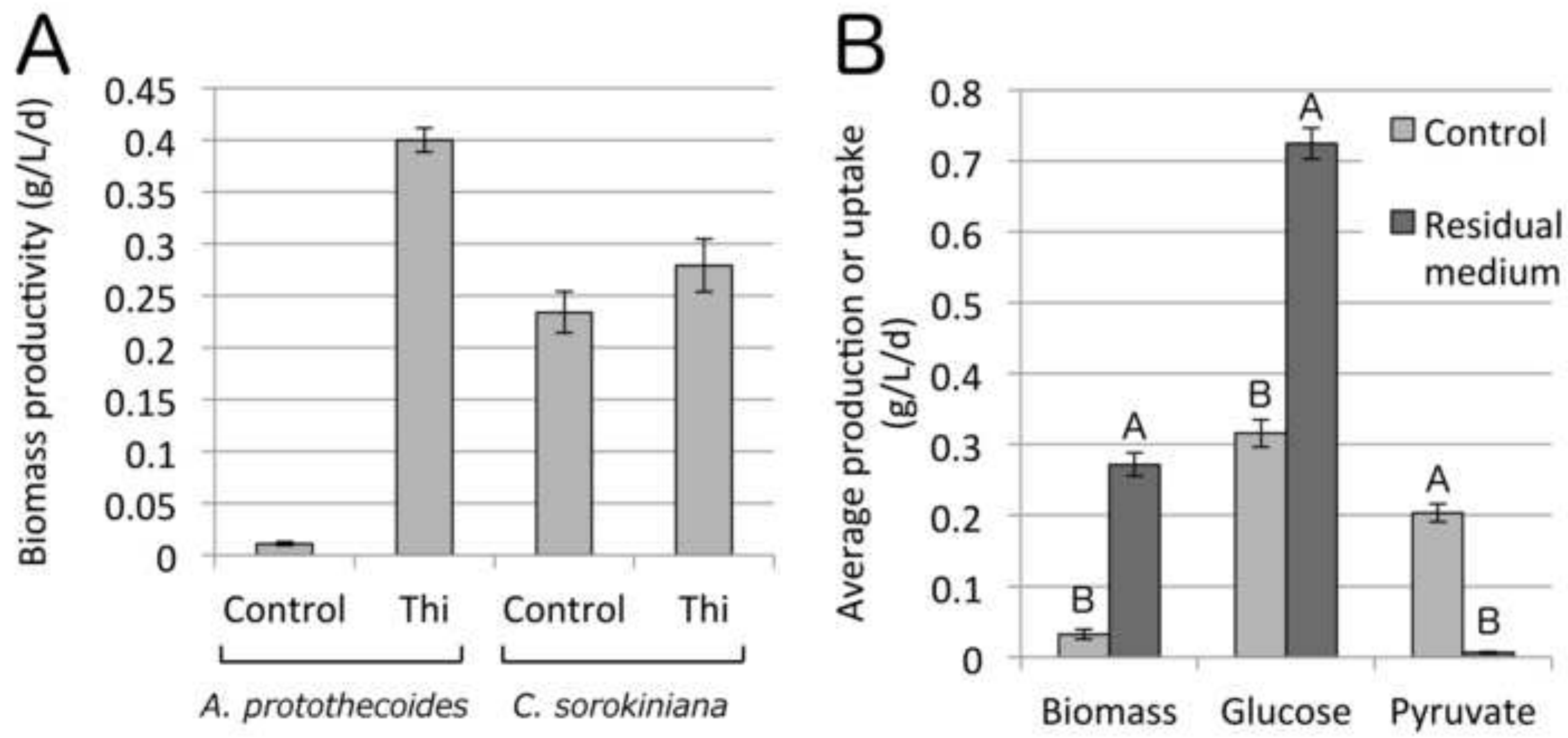


Figure 5



Graphical Abstract