Algal Research 33 (2018) 197–208

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Impact of thiamine metabolites and spent medium from *Chlorella sorokiniana* on metabolism in the green algae *Auxenochlorella protothecioides*

Brendan T. Higgins[a,*], Qichen Wang[a], Sandon Du[b], Marie Hennebelle[c,d], Ameer Y. Taha[c], Oliver Fiehn[e], Jean S. VanderGheynst[b]

[a] Biosystems Engineering, Auburn University, Auburn, AL 36849, United States
[b] Biological and Agricultural Engineering, University of California, Davis, CA 95616, United States
[c] Food Science, University of California, Davis, CA 95616, United States
[d] Present address: Laboratory of Food Chemistry, Wageningen University and Research, Wageningen, The Netherlands
[e] University of California Davis, Genome Center, Davis, CA 95616, United States

ARTICLE INFO

Keywords:
Microalgae
Thiamine
Metabolism
Symbiosis
Substrate utilization

ABSTRACT

Auxenochlorella protothecoides is a known thiamine auxotroph but our past work has shown that it can synthesize thiamine if provided with the precursor molecule 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP). Partial thiamine auxotrophy is common in microalgae with important ramifications for global phytoplankton productivity as well as engineering applications of algae. While thiamine deficiency can greatly depress algae growth and lipid content, the detailed metabolic impacts of thiamine deficiency are not well understood. We used metabolomics to study the response to thiamine-limited and replete conditions in mixotrophic *A. protothecoides*. We also investigated the impacts of exogenous HMP addition and the use of spent medium from another green algae, *C. sorokiniana*, as a source of thiamine metabolites. This is the first study, to our knowledge, that addresses metabolic impacts of thiamine deficiency and alleviation in green microalgae. Thiamine deficient cultures exhibited accumulation of pyruvate and α-ketoglutarate, indicating bottlenecks at the pyruvate dehydrogenase (PDH) and oxoglutarate dehydrogenase (OGDH) complexes. Both PDH and OGDH require thiamine pyrophosphate (TPP) as a cofactor. Transketolase also requires TPP but we only observed build-up of ribose-5-phosphate when glucose was supplied as a substrate. As expected, thiamine and HMP addition could alleviate these metabolic bottlenecks while greatly increasing algal growth, neutral lipid and starch content. Spent medium from *C. sorokiniana* only appeared to partially alleviate thiamine deficiency and resulted in build-up of isocitrate and glycolate, metabolites that appeared relatively unaffected by the presence or absence of thiamine. Interestingly, longer culture time of *C. sorokiniana* when preparing the spent medium led to much higher availability of thiamine metabolites. Thus, under the right conditions, it may be possible to co-culture mutually beneficial algae species and/or recycle spent cultivation medium to overcome auxotrophy in algae.

1. Introduction

The green algae, *Auxenochlorella protothecoides*, holds great potential for lipid production and wastewater treatment, even in the presence of wastewater microbes [1,2]. Under optimized heterotrophic conditions, it can accumulate 55% of biomass as lipids [3]. This strain also grows very rapidly under mixotrophic conditions in which both photosynthesis and an organic carbon source provide energy [4,5]. *A. protothecoides* is understood to be a thiamine auxotroph, but our past work has shown that it can synthesize thiamine if provided with the precursor molecule 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) [6]. Specifically, *A. protothecoides* can synthesize the thiazole precursor of thiamine but lacks the HMP synthase gene. A recent study has shown that loss of HMP synthase is common among a range of marine algae species [7].

Green algae use thiamine to synthesize thiamine pyrophosphate (TPP). TPP is a known cofactor in carbohydrate and amino acid metabolism [8]. It is required by pyruvate dehydrogenase (PDH), which converts pyruvate into acetyl-CoA. The latter can enter the tricarboxylic acid (TCA) cycle or can be used for fatty acid synthesis. TPP is also a cofactor for oxoglutarate dehydrogenase (OGDH), which converts α-ketoglutarate into succinyl-CoA in the TCA cycle and for transketolase (TK), which catalyzes reversible reactions in the pentose phosphate pathway as well as the Calvin-Benson cycle for CO_2 fixation. In addition

* Corresponding author at: Department of Biosystems Engineering, 203 Corley Building, Auburn, AL 36849, United States.
E-mail address: bth0023@auburn.edu (B.T. Higgins).

https://doi.org/10.1016/j.algal.2018.05.019
Received 25 March 2018; Received in revised form 20 May 2018; Accepted 20 May 2018

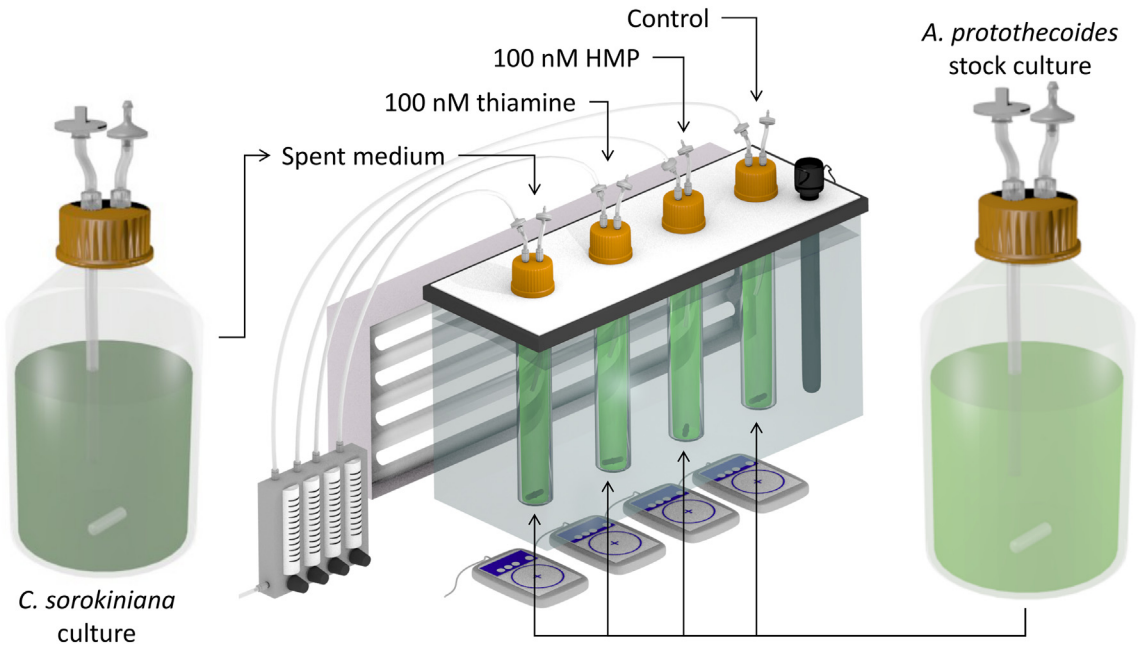

Fig. 1. Scheme of experimental set-up showing 1 L bottles used for algae stock cultures and 200 mL airlift reactors for culture experiments. The experimental scheme shown was carried out in triplicate. HMP is 4-amino-5-hydroxymethyl-2-methylpyrimidine.

to providing ribose-5-phosphate for nucleic acid synthesis, the pentose phosphate pathway also produces NADPH, the principle supplier of electrons for lipid biosynthesis. Finally, TPP is a known cofactor for enzyme complexes that catalyze reactions of keto acids generated during catabolism of the branched amino acids, leucine, isoleucine, and valine. Much of our understanding of thiamine nutrition stems from extensive research in humans and animals [9,10]. In a recent review, Helliwell raised the critical question of how transferable this knowledge is to the microbial realm, particularly that of phytoplankton [11]. This is a highly relevant question given that half of algae are likely B-vitamin auxotrophs with roughly 22% exhibiting thiamine auxotrophy [12]. In nature, these auxotrophs are understood to scavenge B-vitamin metabolites from bacteria [12–14] whose populations can vary with environmental conditions. The consequences of B-vitamin deficiency can lead to vast changes in phytoplankton community abundance and consequent marine primary production [15]. Likewise, B-vitamin deficiency can lead to significant impact on algal growth for engineering applications including biofuels, bioproducts, and wastewater treatment [6].

In past research, we showed that supplementation of *A. protothecoides* cultures with exogenous thiamine and HMP resulted in rapid growth compared to un-supplemented control cultures [6]. Under mixotrophic conditions, thiamine supplementation increased neutral lipid content significantly and eliminated pyruvate secretion by *A. protothecoides*, a common side-effect of thiamine deficiency in this organism. Similar effects were observed when co-culturing *Escherichia coli* with *A. protothecoides* [5,16]. In these conditions, *E. coli* provided thiamine metabolites to *A. protothecoides*, particularly the thiamine precursor, HMP. Thus co-culturing algae with bacteria is a promising engineering strategy to overcome B-vitamin deficiency.

The use of mixed cultures to promote algae growth through mutualistic interaction is an emerging research frontier in algal biotechnology [17]. Emulating natural symbioses, engineers can design processes that utilize mutually beneficial organisms with biotechnological applications. As an example of this, we have cultured *A. protothecoides* on spent medium from another industrially-relevant green algae, *Chlorella sorokiniana* [6]. *C. sorokiniana* is an autotrophic strain and has the ability to synthesize thiamine de novo, similar to *E. coli*. Co-culturing multiple green algae species, as a means of overcoming thiamine deficiency, is highly attractive for engineering applications of algae, especially when both strains can be used for biofuel and bioproduct synthesis. Our results showed that *C. sorokiniana* spent medium supported faster growth than control cultures but that the growth rate appeared to fall short of thiamine-replete cultures [6]. This raised questions about whether *C. sorokiniana* could fully alleviate thiamine deficiency in *A. protothecoides*.

Thiamine deficiency has been shown to decrease growth rates, nutrient uptake, and lower cellular lipid and starch contents. However, detailed impacts of exogenous thiamine and cellular secretions on *A. protothecoides* metabolism have not been investigated. Moreover, extensive database searches revealed no studies that specifically address metabolic impacts of thiamine deficiency and alleviation in microalgae. The objective of the work presented here was to determine how thiamine deficiency and alleviation in *A. protothecoides* impacts its metabolome and conversion of organic substrates into biomass components. We used a combination of culture studies and metabolomics to reveal the impact of exogenous thiamine and HMP on mixotrophic metabolism in *A. protothecoides*. We also tested the metabolic impacts of culturing *A. protothecoides* on spent *C. sorokiniana* medium as a potential source of algae-derived thiamine metabolites. This is the first study, to our knowledge, to examine the effects of exudates from one green algae species on the metabolome of another.

2. Methods

2.1. Culture experiments

A. protothecoides (UTEX 2341 [18]) was cultivated in lab-scale bioreactors to understand the impacts of exogenous thiamine, HMP, or spent *C. sorokiniana* medium on algae growth, composition, and metabolism (Fig. 1). All reactors contained N8-NH_4 medium supplemented with either 8 g/L glucose or 8 g/L acetate as described in previous work [5]. Control reactors were filled with fresh medium only. For treatment reactors, fresh N8-NH_4 medium was supplemented with either 100 nM thiamine or 100 nM HMP, levels that were previously determined to fully alleviate thiamine deficiency in *A. protothecoides* [6]. To prepare spent medium, *C. sorokiniana* was cultured in bottles filled with N8-NH_4 medium without any organic carbon source. Culture time was five days

and cell settling time was three days. The resulting supernatant was collected, augmented with 8 g/L organic substrate, supplemented with ammonium chloride, and its pH readjusted to 7.2. The level of ammonium chloride supplementation was determined based on calculated consumption by *C. sorokiniana*. Briefly, biomass concentration was estimated by optical density and used to estimate ammonium chloride removal based on 8% N content of biomass, a typical value for *C. sorokiniana* under non-stress conditions [19]. Spent medium was re-supplemented with ammonium chloride to restore it to its initial level. The medium was then sterile filtered using a 0.22 μm filtration apparatus and used immediately. Three replicate bioreactors were used for controls and each of the three experimental treatments. The batch experiment was carried out first with acetate and then glucose as the organic substrate. A variety of biomass composition assays (detailed below) and time course metabolomics analysis were carried out on samples collected from these culture experiments.

A follow-up experiment was carried out to understand the impact of *C. sorokiniana* culture time when preparing spent medium. For this experiment, *C. sorokiniana* were cultured on N8 medium either for 5 days (as above) or 15 days. N8 medium [20] uses nitrate rather than ammonium as the nitrogen source. This was chosen because *C. sorokiniana* can utilize nitrate but *A. protothecoides* cannot [1], thus, eliminating the need to calculate how much ammonium to add to spent medium. Spent N8 medium was supplemented with ammonium chloride equivalent to the amount in N8-NH_4 medium. These different spent media were then tested with *A. protothecoides* in quadruplicate and compared to four control cultures using fresh N8-NH_4 medium. All of these cultures were supplied with 8 g/L glucose.

2.2. Algae cultivation methods

Cultivation methods were similar to those employed previously for *A. protothecoides* and *C. sorokiniana* [1,5]. Briefly, cultures were started from ATCC #5 agar plates, followed by stock culture production in 1 L bottles for ~7 days under autotrophic conditions. Once culture optical density reached ~0.2 at 550 nm (0.11 g/L) the stock culture was allowed to settle for 24–48 h. The supernatant was removed and the concentrated algae was used to inoculate the experimental reactors.

Culture experiments were carried out over 5 days in airlift bioreactors filled with 200 mL medium. These tubular reactors were suspended in a water bath (28 °C), mixed by stir bar (~150 rpm), illuminated horizontally with fluorescent lamps (135 μmol/m^2/s operating on a 16:8 light/dark cycle), and bubbled with 125 ml/min air. Two samples were taken daily from each reactor and reactors were rotated to a new incubator position each day. The first sample was used to measure optical density and pH. This sample was then centrifuged to pellet cells and filtered through a 0.2 μm filter for HPLC analysis of organic substrate uptake and product secretion into the medium. The second sample was used for metabolomics analysis. This second sample was mixed 1:1 with cold methanol (−80 °C), and centrifuged to pellet cells for 2 min at 12,000*g*. The supernatant was removed and the fixed cell pellet was frozen at −80 °C prior to freeze drying and metabolite extraction as detailed below.

At the end of the 5 day culture period, 160 mL of the culture was harvested from the hybrid tube reactors by centrifugation at 6000 *g*. The cell pellet was washed three times with dH_2O to remove medium salts. The resulting pellet was then freeze dried and used for subsequent biomass composition analyses.

2.3. Lipid analysis

Lipid extraction was carried out on 20 mg of freeze dried biomass using a modified Folch method as discussed previously [5]. The resulting lipid extract was then used to carry out a neutral lipid assay [21] with slight modifications: specifically 1 μg/mL Nile Red solution was used rather than 0.5 μg/mL as discussed in the publication. Canola oil was used as a standard. This extract was also used to perform total FAME analysis on the lipids.

Two milliliters of lipid extract containing 133.5 nmol of 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (17:0 PC) as an internal standard, were dried under a stream of nitrogen gas and reconstituted in 400 μL of toluene. Lipids were transesterified by adding 3 mL pure methanol and 600 μL 8% HCl in methanol and by heating the samples at 90 °C for 1 h. Once cooled down, 1 mL of hexane and 1 mL of dH_2O were added. Samples were vortexed and allowed to sit for 10 min to allow the separation of the hexane and aqueous layers. Eight hundred microliters of the hexane upper layer was transferred to a new tube containing 400 μL of dH_2O, vortexed and centrifuged 1 min at 15,871 *g* at room temperature. The hexane upper-layer was transferred to a new tube, dried under nitrogen and reconstituted in 150 μL hexanes for gas chromatography – flame ionization detection (GC-FID) analysis.

Fatty acid methyl esters (FAMEs) were analyzed on a Varian 3800 gas-chromatography system equipped with a DB-23 fused silica capillary column (Length: 30 m; ID: 0.25 mm; film: 0.25 μm; Agilent Technologies, Santa Clara, CA, USA), as previously described [22]. The injector was set at 250 °C and the detector at 300 °C. The oven program was as followed: held at 50 °C for 2 min, increased to 180 °C at 10 °C/min, held at 180 °C for 5 min, increased to 240 °C at 5 °C/min and held at 240 °C fir 5 min. Helium was the carrier gas and was maintained at a 1.3 mL/min flow rate. Identification of the individual FAME was made by comparison with a custom mix of FAME standards.

2.4. Starch and cell wall analysis

After lipid extraction, the lipid-free cell pellet was washed three times each with acetone and water as described previously [5]. This pellet was then suspended in water and gelatinized at 80 °C for 30 min. An enzyme cocktail containing α-amylase (15 U/ml) and α-amiloglucosidase (6 U/ml) was prepared in 100 mM acetate buffer with 0.04% sodium azide. This was added to each cell pellet sample as described previously [5]. These enzymes break down starch and glycogen, liberating glucose in the process. The latter was assayed using the dinitrosalicylic acid assay with glucose as a standard [23]. The amount of glucose released was multiplied by 0.9 to determine the glycogen content of the cell pellet. The remaining pellet, which contains the cell wall, was then washed twice with distilled water, freeze dried, and its mass recorded.

2.5. Total nitrogen and protein analysis

The HACH Total Nitrogen assay was used to measure nitrogen content of the biomass as described previously [16]. Briefly, 2 mg of freeze dried algae was suspended in 1.5 mL of dH_2O. This was then diluted by a factor of 4 to create a dilute algae suspension which was assayed per the manufacturer's instructions. The resulting nitrogen content of the algal biomass was calculated and multiplied by 6.25 to obtain a crude protein content [19].

2.6. Medium composition analysis by high pressure liquid chromatography (HPLC)

HPLC was used to monitor uptake of glucose and acetate by algae. It was also used to monitor secretion of products, particularly pyruvic acid. An HPLC system (Prominence, Shimadzu, Japan) was used to carry out the analysis as described previously [1]. An Aminex 87H column (Bio-Rad, USA) was used for the analysis as described previously [1].

2.7. Sequencing 16S rRNA gene of contaminating bacteria

One of the cultures grown in spent *C. sorokiniana* medium showed visible signs of contamination and had much faster growth than its

Table 1
Biomass composition of *A. protothecoides* under varying medium conditions.

	Acetate as organic substrate				Glucose as organic substrate			
	Control[b]	Thiamine[c]	HMP[d]	Spent[e]	Control	Thiamine	HMP	Spent
Total biomass productivity[a] (mg/L/d)	4 (1) c[f]	573 (20) a	546 (31) a	103 (5) b	36 (< 1) c	575 (125) a	494 (107) a	149 b
Neutral lipid (%)[g]	1.3 (0.1) b	2.6 (0.4) a	2.8 (0.2) a	3.2 (0.1) a	0.6 (< 0.1) b	17.2 (5.8) a	18.2 (4.9) a	1.0 b
Total FA[h] (%)	6.2 (1.5) a	10.8 (3.4) a	16.3 (8.9) a	12.7 (1.8) a	6.2 (0.7) b	18.6 (1.1) a	13.1 (3.4) a	6.9 b
Starch (%)	0.8 (0.1) b	5.7 (0.2) a	6.7 (0.5) a	0.3 (0.01) b	3.2 (0.2) b	6.1 (1.9) ab	9.0 (1.9) a	8.9 a
Cell wall (%)	47 (2) a	34 (3) b	38 (2) b	33 (2) b	36 (1) a	29 (2) b	31 (5) ab	36 ab
Crude protein (%)	40 (1) ab	33 (6) b	32 (3) b	43 (2) a	40 (3) a	23 (5) c	24 (5) bc	37 ab
Other biomass (%)	11 (2) b	25 (6) a	21 (4) ab	21 (3) ab	20 (2) a	25 (13) a	18 (6) a	17 a
Neutral lipid productivity (mg/L/d)	0.1 (0.01) c	14.8 (2.4) a	15.5 (1.1) a	3.3 (0.1) b	0.2 (0.01) c	94.2 (8.5) a	93 (46) a	1.4 b
Total FA productivity (mg/L/d)	0.2 (0.05) c	62 (18) a	91 (53) a	13.1 (2.1) b	2.2 (0.2) c	106 (20) a	66 (30) a	10.1 b
Starch productivity (mg/L/d)	0.03 (0.01) c	32.6 (2.4) a	36.8 (4.6) a	0.3 (0.02) b	1.2 (0.1) c	33.9 (7.5) a	43.3 (2.3) a	12.6 b
Cell wall productivity (mg/L/d)	1.9 (0.2) c	196 (20) a	206 (13) a	34.4 (1.3) b	13.0 (0.3) c	164 (31) a	150.2 (9.1) a	53.3 b
Crude protein productivity (mg/L/d)	1.6 (0.2) c	189 (41) a	173.1 (7.1) a	43.9 (2.9) b	14.2 (1.0) c	125.7 (4.1) a	113.2 (7.0) a	55.7 b
Other biomass productivity (mg/L/d)	0.5 (0.2) c	141 (33) a	115 (25) a	21.2 (3.4) b	7.2 (0.8) b	160 (100) a	90 (50) a	25.5 ab

[a] Average total biomass productivity measured over 120 h.
[b] Control medium is N8-NH4 medium with 8 g/L of organic substrate.
[c] Thiamine dose of 100 nM added to control medium.
[d] HMP (4-amino-5-hydroxymethyl-2-methylpyrimidine) dose of 100 nM added to control medium.
[e] Spent medium from *C. sorokiniana* after 5 days of culture.
[f] Values are expressed as mean (standard deviation, $n = 3$); for each component, values with the same letter within a substrate group (acetate or glucose) are not significantly different at the 0.05 level. Three biological replicates were used except for glucose spent medium where one culture was lost to contamination.
[g] Mass basis of dry weight.
[h] Fatty acid.

replicate cultures. Plating of this culture revealed white colonies. Several of these colonies were scraped from the plate and suspended separately in 1 mL of dH_2O. A portion of this suspension (0.2 ml) was used for DNA extraction using the Fast DNA spin kit per the manufacturer's instructions (MP Biomedicals, USA). The bacterial extraction buffer and lysing matrix A tubes were used.

The extracted DNA was diluted ten-fold for polymerase chain reaction (PCR) amplification of the 16S rRNA gene using the 27F and 1492R universal primers. Reaction volume was 25 µl, primer concentrations were 100 nM, and template volume added was 2 µl. The thermocycler ran for 30 cycles: 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. The amplified DNA was cleaned using ExoSap-IT reagent (Thermo, USA) according to the manufacturer's instructions. Sanger sequencing was performed using both forward and reverse primers at the UC Davis DNA Sequencing Facility. Partial sequences were aligned and trimmed using the Geneious software package (Biomatters Inc., USA) to obtain a ~1400 bp sequence. All colonies analyzed revealed matching sequence to each other. The sequence was entered into BLAST to determine the closest matching organism.

2.8. Metabolomics extraction and analysis

Metabolomics extraction was carried out on freeze dried algae pellets per a previously established method [24]. Briefly, pellets were frozen in liquid nitrogen and pulverized in a ball mill. Metabolites were then extracted using a mixture of 10:3:1 chloroform/methanol/water. Extracts were dried in a centrivap and frozen until analysis. Samples were then derivatized by N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and run on a gas chromatograph with tandem quadrupole and time of flight mass spectrometer (GCMS-TOF) for metabolite profiling and BinBase data processing [24].

2.9. Data analysis and statistics

Statistical analyses, including ANOVA and Tukey's HSD test, were carried out in R (R Project, USA) [25] using the “agricolae” and “car” packages. The Brown and Fosythe test, along with data visualization, were used to assess homogeneity of variance. In certain cases, data were transformed to ensure compliance with the assumption of homogenous variance [26]. These analyses were used throughout the study to understand impacts of treatments on growth and biomass composition.

Principle component (PCA) analysis was used to analyze metabolomics datasets given the large number of metabolites (~130 known and ~200 unknown metabolites quantified per sample). Each experimental batch generated 72 samples (12 cultures over 5 days), however, some analyses failed and were not included in the final data. Prior to statistical analyses, metabolite abundance values were normalized by the sample biomass concentration, providing a relative abundance per unit of biomass. These normalized data were log-scaled prior to PCA analysis in R. In addition, data from the 72 h time point underwent further analysis. This time point was chosen because it was roughly the midpoint of exponential growth. For each metabolite of interest, abundance was normalized against the average of abundance for that metabolite across all twelve culture samples. This provides a glimpse of how up- or down-regulated a metabolite was relative to the experimental average. These values were then integrated into metabolic maps of central metabolism for visualization and comparison.

Non-linear regression was used to fit growth data to a saturation model for substrate limited growth (Eq. 1). This was carried out in MATLAB (MathWorks, USA) using the “nlinfit” function.

$$P = \frac{P_{max} S}{k_S + S} \quad (1)$$

where P is the biomass growth rate (mg/L/d), P_{max} is the maximum specific growth rate (mg/L/d), S is the concentration of the limited substrate (thiamine, nM), and k_S is the half velocity constant (nM).

3. Results

3.1. Algae growth and composition

Addition of exogenous thiamine and its precursor, HMP, led to 13 to 140 fold increases in *A. protothecoides* growth over control cultures (Table 1). The increase was more pronounced when acetate was the organic substrate rather than glucose. Spent medium from *C. sorokiniana* that was subsequently refortified with ammonium and organic substrate also led to 4–25 fold higher growth rates compared to control cultures. However, growth on spent medium was significantly less than

Table 2
Total fatty acid methyl ester concentration (mg/g of biomass) under varying medium conditions.

Fatty acid	Name	Acetate as organic substrate				Glucose as organic substrate			
		Control[a]	Thiamine[b]	HMP[c]	Spent[d]	Control	Thiamine	HMP	Spent
C12:0	Lauric acid	0.59 (0.24) a[e]	–	–	0.08 (0.14) b	–	–	–	–
C13:0	Tridecylic acid	0.55 (0.22) a	0.59 (0.32) a	1.1 (0.8) a	0.09 (0.10) a	0.12 (0.20) a	0.32 (0.12) a	–	–
C14:0	Myristic acid	1.5 (0.4) a	1.6 (0.48) a	2.1 (1.1) a	1.5 (0.5) a	1.0 (0.1) b	2.6 (0.3) a	1.9 (0.4) ab	1.4 b
C14:1	Myristoleic acid	0.72 (0.26) a	0.58 (0.26) a	0.77 (0.41) a	0.21 (0.22) a	0.32 (0.02) a	–	–	0.42 a
C15:0	Pentadecylic acid	0.23 (0.23) a	0.19 (0.17) a	0.06 (0.1) a	–	0.08 (0.13) a	0.25 (0.01) a	0.22 (0.19) a	–
C15:1	10-pentadecenoate	–	0.26 (0.30) a	0.38 (0.43) a	0.25 (0.22) a	–	0.31 (0.08) a	0.11 (0.19) a	1.1 a
C16:0	Palmitic acid	15.4 (1.1) c	18.9 (0.8) bc	19.7 (1.4) ab	23.2 (1.5) a	15.8 (0.6) b	35.1 (2.0) a	30.0 (3.7) a	16.3 b
C16:1	Palmitoleic acid	2.2 (0.7) a	1.4 (0.7) a	1.9 (1.7) a	1.5 (1.0) a	0.95 (1.1) a	1.0 (0.1) a	0.90 (0.24) a	1.6 a
C18:0	Stearic acid	0.88 (0.33) a	0.85 (0.52) a	1.3 (0.9) a	0.55 (0.12) a	0.61 (0.16) a	0.19 (0.17) b	–	–
C18:1 n-9	Oleic acid	4.7 (1.4) a	26.2 (11.3) a	42.5 (25.8) a	41.8 (7.1) a	5.3 (0.9) b	101 (10.4) a	69.3 (22.1) a	12.6 b
C18:2 n-6	Linoleic acid	15.4 (4.9) a	40.8 (16.7) a	60.0 (37.6) a	37.5 (6.2) a	17.7 (2.5) b	35.8 (1.7) a	22.1 (6.5) b	18.6 b
C18:3 n-3	Linolenic acid	19.4 (6.2) a	16.9 (6.6) a	33.5 (21.0) a	20.8 (3.7) a	19.9 (3.0) a	7.8 (0.6) bc	5.2 (1.6) c	16.6 ab
C20:2 n-6	11,14-Eicosadienoic acid	–	–	–	–	–	0.56 (0.43) a	0.70 (0.44) a	–
C20:3	Homo-gamma-linolenic acid	–	–	–	–	–	0.74 (0.70) a	1.2 (1.0) a	–
C20:5 n-3	Eicosapentaenoic acid	–	–	–	–	–	0.18 (0.15) a	–	–
Total FA		61.6 (15.1) a	108 (33.6) a	163 (89.5) a	127 (18) a	61.8 (6.7) b	186 (11) a	131 (34) a	68.7 b

[a] Control medium is N8-NH_4 medium with 8 g/L of organic substrate.
[b] Thiamine dose of 100 nM added to control medium.
[c] HMP (4-amino-5-hydroxymethyl-2-methylpyrimidine) dose of 100 nM added to control medium.
[d] Spent medium from *C. sorokiniana* after 5 days of culture.
[e] Units of mg/g biomass; values are mean (standard deviation, $n = 3$); for each fatty acid, values with the same letter within a substrate group (acetate or glucose) are not significantly different at the 0.05 level.

thiamine-supplemented cultures in all cases ($p < 0.001$).

Similar to growth rates, provision of thiamine metabolites roughly doubled and, in some cases, tripled neutral lipid and starch content compared to controls. However, crude protein content and cell wall content tended to be lower than in control cultures. Cultivation on spent medium led to biomass compositions that were unique from both control cultures and cultures supplemented with thiamine metabolites. In acetate cultures, spent medium led to lipid and cell wall contents similar to thiamine-supplemented cultures. However, protein and starch content were closer to levels observed in control cultures. In glucose cultures, spent medium resulted in lipid, protein, and cell wall contents similar to control cultures. However, starch content was similar to that in thiamine and HMP-supplemented cultures, a contrast to what was observed in acetate cultures.

3.2. Fatty acid profiles

Because of their value for biofuel, food, and feed applications, we analyzed the fatty acid content and composition (Table 2). When cultured on acetate, there were nominal differences among culture treatments but most were not statistically different due to relatively wide standard deviations. However, palmitate was significantly higher in the presence of HMP and spent medium compared to control cultures. Addition of thiamine and HMP led to a 5-fold and 9-fold increase in oleic acid content. Spent *C. sorokiniana* medium also led to a 9-fold increase in *A. protothecoides* oleic acid content. Linoleic acid content also increased, although to a lesser extent, under these treatment conditions.

When cultured on glucose, the addition of thiamine and HMP doubled and tripled total fatty acid content, respectively. Specifically, palmitic, oleic, and, to a lesser extent, linoleic acid content all increased under thiamine and HMP treatments. Oleic acid content in particular increased by 18-fold and 13-fold under thiamine and HMP treatment, respectively. Interestingly, the α-linolenic acid content significantly decreased under those same treatments. Culture on spent medium yielded little change in the fatty acid profile compared to control cultures.

3.3. Substrate consumption and product secretion

In order to understand substrate utilization, substrate uptake from the medium was measured over time (Fig. 2a–b). Provision of exogenous thiamine and HMP yielded significantly faster acetate and glucose uptake compared to control cultures which had minimal substrate consumption ($p < 0.01$). As with growth rates, spent medium fell somewhere between the control and thiamine-provided cultures.

Our past research has shown that thiamine-deficient *A. protothecoides* cultures secrete pyruvate when supplied with organic substrates [6]. We, therefore, measured this secretion for each culture to better understand loss of this key metabolite from the cell. The results show that cultures supplied with thiamine or HMP had lower ($p < 0.05$ for acetate, $p < 0.001$ for glucose) pyruvate secretion than control and spent medium cultures (Fig. 2c-d). Overall, pyruvate secretion in acetate cultures was about an order of magnitude lower than pyruvate secretion in glucose cultures.

Interestingly, the spent medium cultures had higher pyruvate secretion than control cultures regardless of whether acetate or glucose was used as an organic substrate. This may have been due in part to the fact that algae grew much faster, and consumed more substrate, when cultured on spent medium compared to controls. Therefore, we normalized the pyruvate secretion by the reactor algae concentration (Fig. 2e–f). This showed that the pyruvate secretion per unit algae was actually significantly lower in spent medium compared to control cultures ($p < 0.001$).

3.4. Metabolite profiling

Given the role of thiamine and HMP in the synthesis of TPP, we chose to investigate their impact on the *A. protothecoides* metabolome. Study of the metabolome can reveal potential metabolic bottlenecks and shed light on underlying causes of the macro-compositional differences observed.

Metabolomics data were uploaded to Metabolomics Workbench. PCA plots were generated based on time-course metabolomics analysis (Fig. 3). These plots show the first two principle components based on analysis of 338 metabolites for acetate cultures and 317 metabolites for glucose cultures. The plots show clear separation of treatments from the

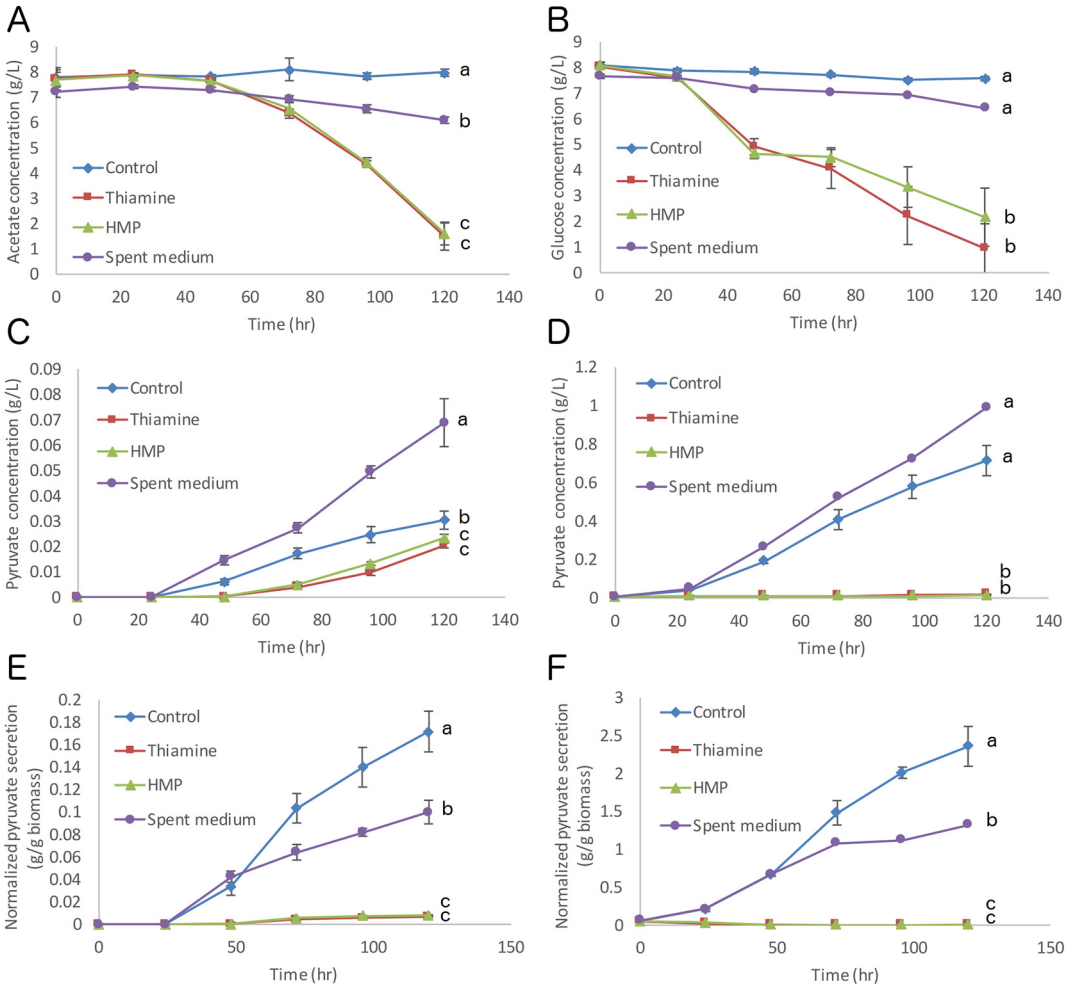

Fig. 2. Time-course substrate utilization and pyruvate secretion. Panels on the left correspond to cultures supplied with acetate and panels on the right correspond to cultures supplied with glucose. A) Acetate consumption in cultures supplied with 8 g/L acetate. B) Glucose consumption in cultures supplied with 8 g/L glucose. C) Pyruvate concentration in medium for cultures supplied with acetate. D) Pyruvate concentration in medium for cultures supplied with glucose. E) Pyruvate concentration in acetate cultures normalized by culture density on a mass basis. F) Pyruvate concentration in glucose cultures normalized by culture density on a mass basis. Error bars are standard deviation based on $n = 3$ biological replicates. For the final time points, values with the same letter are not significantly different at the 0.05 level. HMP is 4-amino-5-hydroxymethyl-2-methylpyrimidine.

control with movement of the scores in an arc-like fashion over time. Scores are only shown for 0, 72, and 120 h to maintain figure clarity. These time points roughly correspond to culture acclimation, exponential growth, and late-log growth, respectively.

The control cultures moved only slightly over time from the initial metabolic state. The thiamine and HMP cultures both moved in a clear arc over the time course. The spent medium cultures moved initially with the thiamine and HMP cultures but then stopped near the top of the arc. The exception to this observation was one spent medium culture (identified by arrows in Fig. 3b) that became contaminated with bacteria. This culture continued to track alongside the thiamine-provided cultures, had a growth rate and composition similar to thiamine-provided cultures, and showed no evidence of pyruvate secretion (Table S1). We isolated and partially sequenced the 16S rRNA gene of this bacteria and ambiguously identified it as either *Bacillus megaterium* or *Bacillus aryabhattai* based on a blast search (100% sequence match to both species). The sequencing result has been uploaded to GenBank (MH108943). Other than the PCA plots, data from this culture were discarded from other analyses to prevent bias of results.

3.5. Impact of thiamine metabolites and spent medium on the central metabolome

The PCA plots show a high-level view of how thiamine metabolites and spent medium impact algal metabolism but do not reveal molecular-level information. We undertook more detailed analysis of metabolite abundance during the exponential growth phase (72 h time point) given the likely industrial relevance of this culture state. Figs. 3 and 4 show a map of central metabolism including enzymes that utilize TPP as a cofactor: pyruvate dehydrogenase, oxoglutarate dehydrogenase, and transketolase. In addition to the pentose-phosphate pathway shown, transketolase catalyzes the same reaction in the Calvin-Benson cycle. TPP is also employed by 2-oxoisovalerate dehydrogenase E1 in branched amino acid catabolism. The metabolomics analysis did not capture the substrates or products of this enzyme complex and it was not included in the analysis.

3.5.1. Acetate cultures

Fig. 4 shows the relative abundance of each metabolite in each experimental treatment for algae grown on acetate. Substrates for the three TPP-dependent enzymes generally accumulated in control and spent medium cultures. There was clear accumulation of intracellular pyruvate in control cultures, mirroring high levels of pyruvate secretion

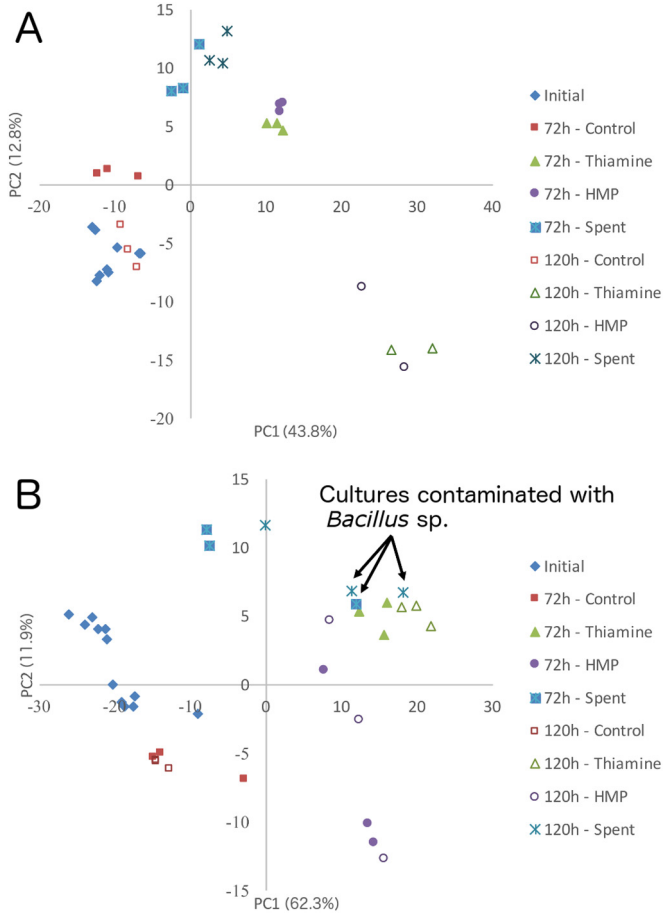

Fig. 3. PCA plots for central metabolome of *A. protothecoides* grown on medium containing A) acetate and B) glucose. Points are shown for time zero (initial), after 72 h (72 h) and after 120 h (120 h) of culture time. The PCA plot for acetate cultures was based on 138 known and 200 unknown metabolites. The PCA plot for glucose cultures was based on 127 known and 190 unknown metabolites. HMP is 4-amino-5-hydroxymethyl-2-methylpyrimidine.

into the culture medium. Cultures grown on spent medium also had significantly higher intracellular pyruvate than cultures supplied with thiamine or HMP. Likewise, there was accumulation of α-ketoglutarate in both the control and spent medium cultures. Fig. S1 shows time course data for intracellular pyruvate and α-ketoglutarate concentrations. Ribose-5P, a substrate of transketolase, also had marginally higher abundance in the spent medium and control cultures compared to thiamine-provided cultures.

Looking at closely-related metabolites to those mentioned above, we found that isocitrate levels partially mirrored those of α-ketoglutarate. However, other TCA cycle intermediates were not heavily impacted by experimental treatments. Given upregulation of α-ketoglutarate, it was interesting that glutamate levels were lower in control cultures compared to other conditions. Likewise, proline, aspartate, and alanine were all depressed in control cultures whereas glycine concentration was elevated. The concentration of free fatty acids, palmitate and stearate were significantly higher in control cultures compared to cultures supplied with thiamine and HMP. Note that these are free fatty acids, in contrast to the fatty acids associated with acyl lipids discussed previously. Time course data shows that biomass normalized abundance of free fatty acids was initially high in all cultures but declined over time in cultures supplied with thiamine and HMP (Fig. S2). Spent medium fell somewhere between the controls and thiamine treatments.

Spent medium appeared to lower intracellular pyruvate concentrations compared to controls. It also led to amino acid abundance comparable to cultures provided with thiamine or HMP in most instances. However, abundance of α-ketoglutarate was similar to control cultures. Spent medium also appeared to induce metabolic changes that appeared to be disconnected from the presence or absence of thiamine. For example, isocitrate and glycolic acid concentrations were elevated in spent medium but were relatively unaffected by thiamine (Fig. S3). Glycolate concentrations were initially low in spent medium cultures, peaked around 72 h and then declined. Likewise, the glycolytic substrates glucose-6-phosphate and 3-phosphglycerate were also upregulated under spent medium in comparison to control and thiamine-provided cultures.

3.5.2. Glucose cultures

Compared to acetate cultures, the impact of adding thiamine and HMP to glucose cultures was even more dramatic (Fig. 5). Exogenous thiamine greatly depressed cellular concentrations of pyruvate, ribose-5P, and α-ketoglutarate. Interestingly, spent medium, while not as effective as thiamine, also significantly reduced intracellular pyruvate concentrations compared to control cultures. Spent medium also yielded nominal reductions in ribose-5P and α-ketoglutarate compared to controls but the differences were not statistically significant. With the exception of proline, addition of thiamine, HMP, or spent medium led to lower free amino acid concentrations compared to controls. Like acetate cultures, free fatty acid concentrations were elevated in control cultures. In this case, normalized fatty acid abundance declined over time in control cultures but not as much as the other treatments (Fig. S2).

In contrast to acetate cultures, there appears to be accumulation of malate and fumarate in control cultures supplied with glucose. This suggests a build-up of TCA cycle intermediates in the latter half of the cycle. This build-up is alleviated by thiamine addition. Likewise, thiamine appears to lower intermediate concentrations in glycolysis but the results are not statistically significant. Interestingly, HMP cultures did not track identically with thiamine cultures, showing greater intracellular pyruvate, α-ketoglutarate, and ribose-5P abundance.

3.6. Impact of culture age in spent medium production

The culture characteristics of *A. protothecoides* grown on spent *C. sorokiniana* medium seemed to exhibit characteristics that lie somewhere between the control cultures and the cultures provided with thiamine. This was apparent with regard to the growth rate, organic substrate consumption, cell-normalized pyruvate secretion, and metabolomics analysis. This suggests that there are some useable thiamine metabolites present in this spent medium, however, the level is likely limiting. In a past study, we found that *C. sorokiniana* spent medium could nearly eliminate pyruvate secretion from *A. protothecoides* that was cultured on glucose. One possible explanation for the apparent discrepancy with the present results is the length of time used for *C. sorokiniana* culture when preparing spent medium. We, therefore, carried out a bioassay to better understand the impact of culture time on spent-medium properties. This bioassay depends on the fact that thiamine-deficient *A. protothecoides* secrete pyruvate into the medium when supplied with excess glucose.

Fig. 6A shows the algae growth response of *A. protothecoides* when supplied with varying doses of exogenous thiamine. Average growth rates are reported over a 5-day period. The dose response-curve follows saturation behavior and a saturation model was fit in MATLAB. P_{max} was 822 mg/L/d, and K_s was 4.57 nM. Pyruvate secretion was also measured over 5 days and normalized by the algae cell mass. Pyruvate secretion had an inverse relationship with exogenous thiamine concentration (Fig. 6C). This assay was then applied to *A. protothecoides* cultured on spent *C. sorokiniana* media.

Spent media were prepared by culturing *C. sorokiniana* for either 5 or 15 days. The resulting spent media were supplemented with glucose and used for *A. protothecoides* cultivation. The productivity of *A.*

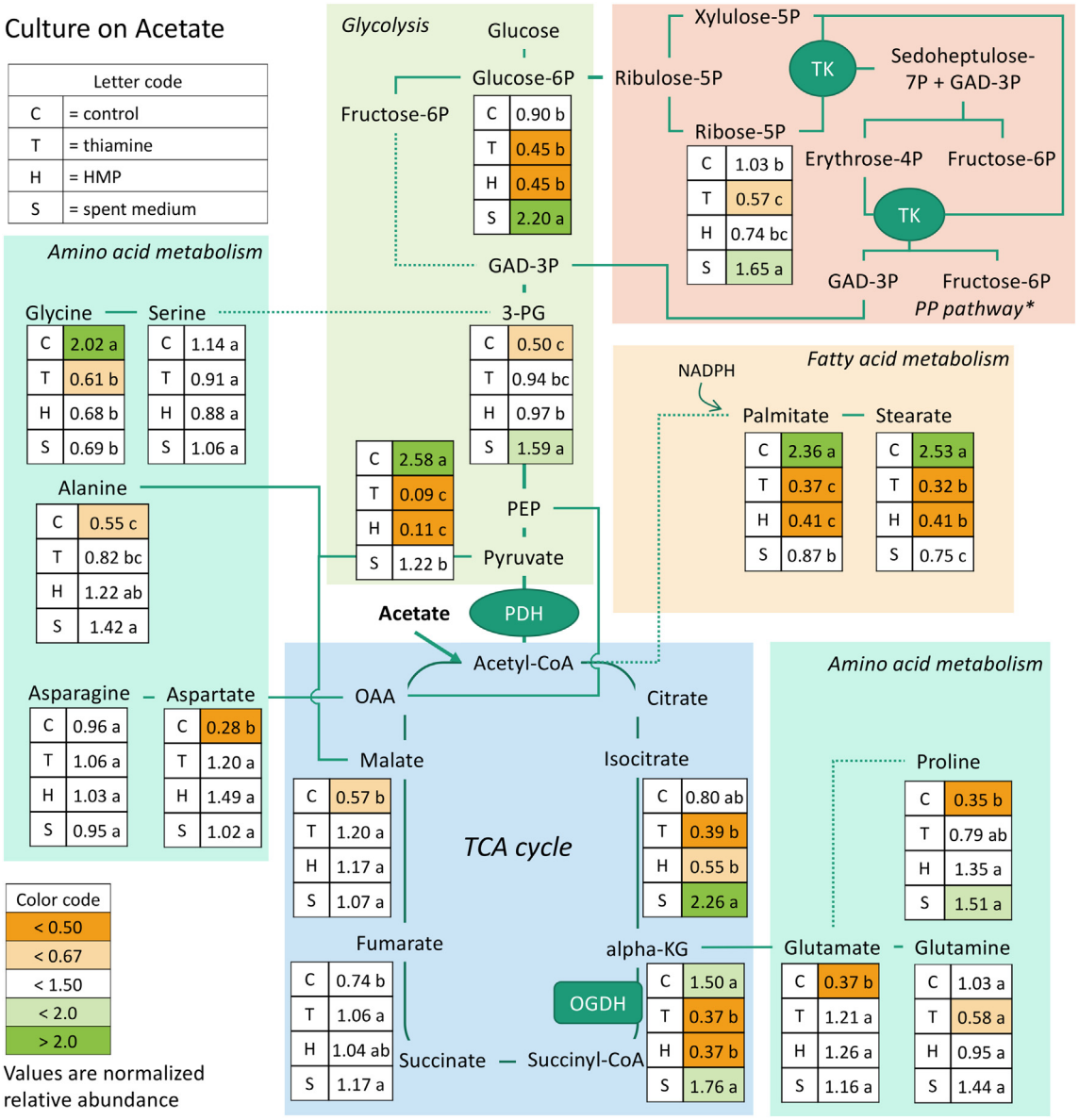

Fig. 4. Map of metabolite abundance for *A. protothecoides* after 72 h cultivation on acetate. Dashed lines indicate multiple enzymatic steps. Experimental treatments included dosing with 100 nM of thiamine (T), 100 nM HMP (H), or cultivation on spent medium from 5-day *C. sorokiniana* culture (S). Control cultures did not receive any supplementation with thiamine derivatives. Tables of relative metabolite abundance are shown below the metabolite name for each experimental treatment (except for pyruvate which is shown to the left). Within a table, numbers followed by the same letter are not significantly different at the 0.05 level (n = 3 biological replicates). *Pentose phosphate pathway with similar reactions catalyzed by TK in the Calvin-Benson cycle. Acronyms: GAD-3P: glyceraldehyde-3-phosphate, 3-PG: 3-phosphoglycerate, PEP: phosphoenolpyruvate, NADPH: Nicotinamide adenine dinucleotide phosphate, PDH: pyruvate dehydrogenase, OAA: oxaloacetate, CoA: coenzyme A, KG: ketoglutarate, 4P: 4-phosphate, 5P: 5-phosphate, 6P: 6-phosphate.

protothecoides increased nearly 6-fold when it was cultured in 15-day *C. sorokiniana*'s spent medium compared to either 5-day spent medium or freshly prepared medium (Fig. 6B). Glucose consumption (4.7 g/L) was higher in 15-day spent medium than 5-day spent medium (2.3 g/L) and control cultures (1.3 g/L). Pyruvate secretion (0.13 g/L) was lower in 15-day spent medium than in 5-day spent medium (0.9 g/L) and control cultures (0.8 g/L). Biomass normalized pyruvate secretion followed this same trend (Fig. 6D).

4. Discussion

4.1. Thiamine metabolites impact macro-composition of A. protothecoides

The results show clear impacts of thiamine, HMP, and spent *C. sorokiniana* medium on *A. protothecoides* metabolism. This was apparent both through macro-compositional analysis and at the level of individual central metabolites. Thiamine deficiency and its alleviation through exogenous additions leads to compositional outcomes consistent with our past findings on co-cultures of *A. protothecoides* and *E. coli* [5,27]. In those studies, we found that addition of *E. coli* to *A. protothecoides* culture supplied with glucose and acetate led to large increases in neutral lipid content, starch content, and very large increases in oleic and linoleic acid content. We later discovered that *E. coli* was likely contributing significant amounts of HMP to *A. protothecoides* [6]. It is apparent from the present results that this HMP provision is likely responsible for those significant changes in algae composition.

HMP and thiamine are precursors to thiamine monophosphate (TMP) synthesis [28]. TMP is then phosphorylated to TPP, an active cofactor for key enzymes in central metabolism. Our results show that

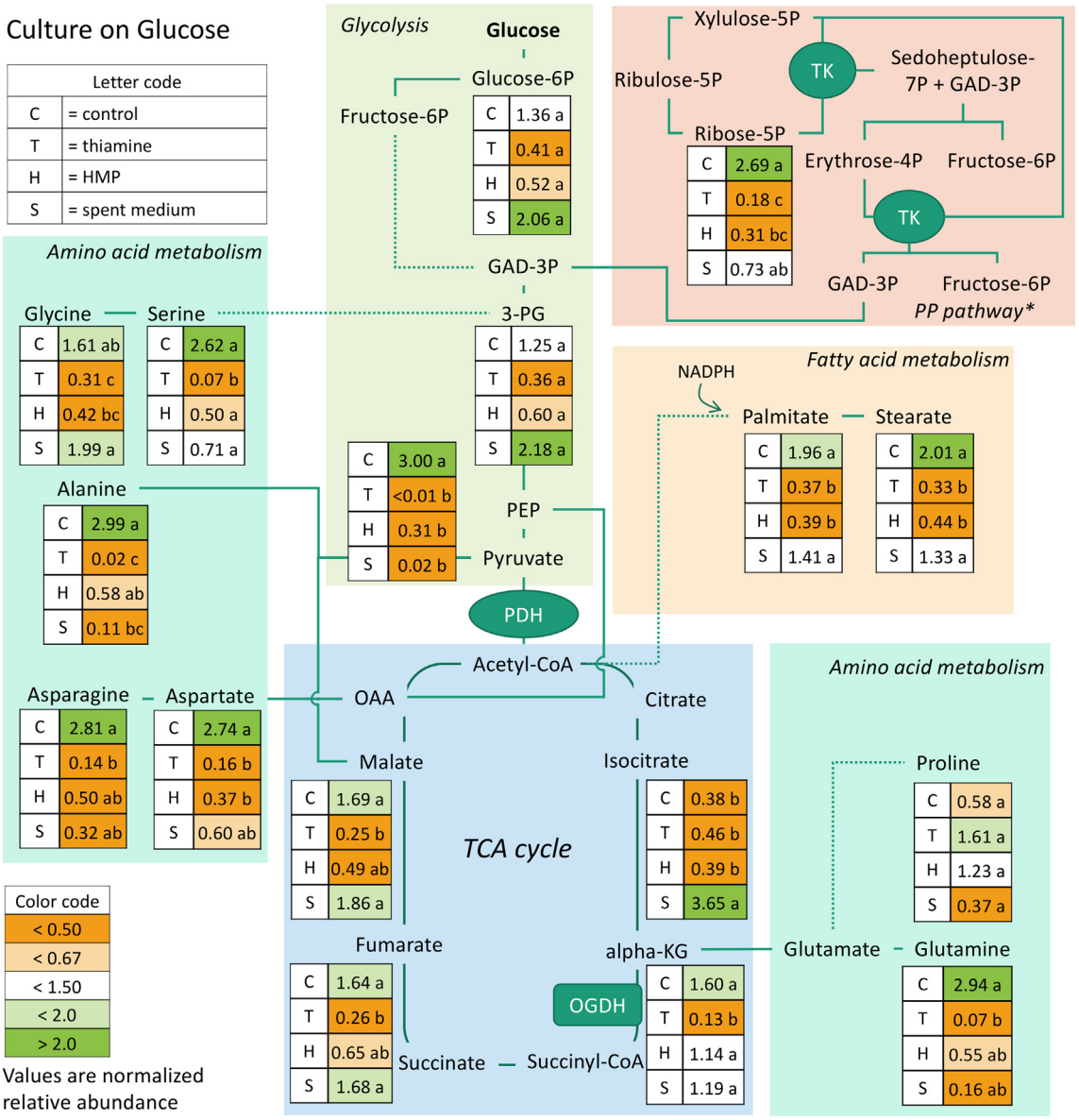

Fig. 5. Map of metabolite abundance for *A. protothecoides* after 72 h cultivation on glucose. Dashed lines indicate multiple enzymatic steps. Experimental treatments included dosing with 100 nM of thiamine (T), 100 nM HMP (H), or cultivation on spent medium from 5-day *C. sorokiniana* culture (S). Control cultures did not receive any supplementation with thiamine derivatives. Tables of relative metabolite abundance are shown below the metabolite name for each experimental treatment (except for pyruvate which is shown to the left). Within a table, numbers followed by the same letter are not significantly different at the 0.05 level (n = 3 biological replicates except for spent medium where $n = 2$). *Pentose phosphate pathway with similar reactions catalyzed by TK in the Calvin-Benson cycle. Acronyms: GAD-3P: glyceraldehyde-3-phosphate, 3-PG: 3-phosphoglycerate, PEP: phosphoenolpyruvate, NADPH: Nicotinamide adenine dinucleotide phosphate, PDH: pyruvate dehydrogenase, OAA: oxaloacetate, CoA: coenzyme A, KG: ketoglutarate, 4P: 4-phosphate, 5P: 5-phosphate, 6P: 6-phosphate.

thiamine deficiency led to significant metabolic bottlenecks around PDH, OGDH, and TK. The bottlenecks were more pronounced under exogenous glucose addition than they were under acetate addition. This was expected because glucose is more directly linked to both PDH and TK via glycolysis and the pentose phosphate pathway. In contrast, carbon from acetate must first undergo gluconeogenesis before encountering these two enzymes.

4.2. Impacts of thiamine and HMP on algal central metabolism in acetate cultures

Acetate enters central metabolism through conversion into acetyl-CoA. From there, its carbon can pass into the TCA cycle, generating reduced electron carriers for oxidative phosphorylation and intermediates for amino acid synthesis. One of the TCA cycle intermediates, oxaloacetate (OAA), can also undergo decarboxylation to produce phosphenolpyruvate, thereby facilitating gluconeogenesis. Interestingly, we observed some pyruvate accumulation in the thiamine-deficient control cultures but not the thiamine or HMP-supplemented cultures. This suggests a bottleneck in PDH conversion of pyruvate to acetyl-CoA. This could be driven by significant gluconeogenic activity followed by glycolysis. It could also be due to photosynthetic generation of glucose, which then passes through glycolysis.

Build-up of α-ketoglutarate in thiamine-deficient control cultures also suggests that the TCA cycle is operating primarily in the clockwise direction with a metabolic bottleneck at OGDH. However, other TCA metabolites are relatively unaffected. This is likely because the TCA cycle enzymes are reversible. Carbon can enter the TCA cycle from the reverse direction via action of malic enzyme, which reversibly catalyzes production of malate from pyruvate at the expense of NADH [29].

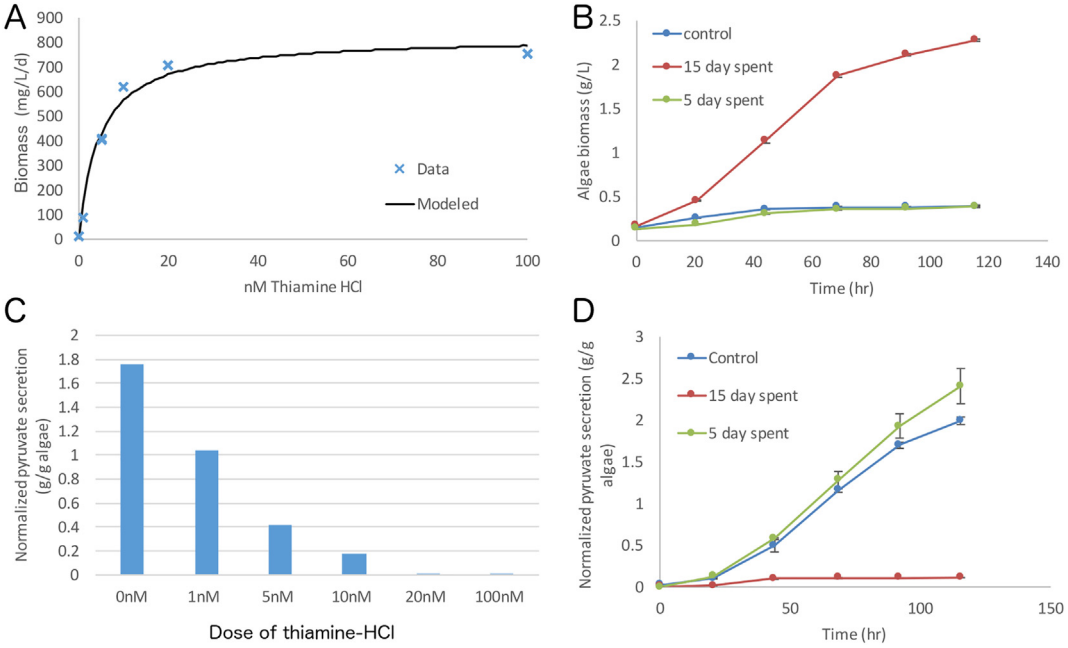

Fig. 6. Thiamine metabolites in spent medium impact *A. protothecoides* growth and pyruvate secretion. All cultures were supplied with 8 g/L glucose. A) Saturation curve for growth response of *A. protothecoides* as a function of thiamine dose. The curve was fit to the Monod equation with thiamine as the limiting substrate. B) *A. protothecoides* growth on 5-day vs. 15-day spent medium from *C. sorokiniana*. C) Biomass-normalized pyruvate secretion by *A. protothecoides* in response to thiamine dose. D) Pyruvate secretion by *A. protothecoides* when cultured on 5-day vs. 15-day spent medium.

However, this is one approach to bypassing the bottleneck at OGDH.

It is understood that algae possess isocitrate lyase and malate synthase, enzymes necessary to carry out the glyoxylate cycle [30]. The glyoxylate cycle plays an essential role in acetate metabolism and also allows for bypass of OGDH. Plancke et al. created a mutant of the green algae *Chlamydomonas* that lacked an isocitrate lyase gene [31]. They found that this mutant strain lost its ability to grow on acetate. Investigation of the *A. protothecoides* transcriptome by Gao et al. [32] revealed the presence of partial mRNA sequences that mapped to annotated genes for isocitrate lyase and malate synthase. Unfortunately, our metablomics analysis was unable to determine abundance of glyoxylate, the key metabolic intermediate in this pathway. Therefore, the impact of thiamine deficiency and alleviation cannot be assessed for the glyoxylate cycle.

In addition to PDH and OGDH, thiamine deficiency should also create a bottleneck at transketolase (TK). TK is involved in both the pentose phosphate pathway and the Calvin-Benson Cycle, although the direction of the reaction is typically opposite in these two processes [33]. Because metabolites are extracted from all cellular compartments, it is nominally difficult to make conclusions about the specific directionality and reaction rates in each of these TK-dependent pathways. Build-up of TK dependent metabolites on one side of the enzyme under thiamine-limited conditions, however, can reveal information about the net direction of metabolic flows. According to our results for acetate cultures, the impact of thiamine and HMP addition on metabolites related to TK was not particularly clear. This is partially due to only one measurable metabolite (ribose-5P) that is a reactant or product of TK. Thiamine deficiency showed only mild accumulation of this metabolite compared to cultures provided with exogenous thiamine.

It is interesting to note that the free fatty acids, palmitate and stearate accumulated significantly under thiamine deficient conditions. It is possible that acetyl-CoA was pushed into this pathway due to the downstream bottleneck at OGDH. These two saturated free fatty acids are precursors to unsaturated fatty acids and are the building blocks of neutral lipids, e.g. triacylglycerol, and polar lipids. Despite the higher concentration of free fatty acids, we observed lower neutral lipid and total fatty acid levels in these same thiamine-deficient cultures. Note that the total FAME measurements account for all acyl lipids, in addition to the free fatty acids; the latter of which account for a small fraction of total fatty acids in the cell. The reason for this apparent discrepancy is not clear from the results obtained in this study.

4.3. Impacts of thiamine and HMP on algal central metabolism in glucose cultures

Compared to cultures supplied with acetate, thiamine and HMP provision had a more pronounced impact on central metabolism in cultures supplied with glucose. Glucose enters the central metabolism via the glycolytic pathway. From there, its carbon can proceed to the pentose phosphate pathway, amino acid synthesis, and the TCA cycle. There was evidence of some glycolytic metabolite accumulation in thiamine-deficient control cultures. Moreover, this accumulation carried over to amino acids, namely glycine, serine, and alanine that are derived from glycolytic intermediates. Intracellular pyruvate accumulation was very dramatic in thiamine-deficient cultures, which is consistent with our past findings for cultures of *A. protothecoides* supplied with glucose [6]. Because glucose enters metabolism via glycolysis, unlike acetate, it is expected that a bottleneck at PDH would lead to significant pyruvate accumulation. Our results showed that pyruvate secretion under glucose conditions was roughly an order of magnitude greater than that under acetate conditions. It is apparent that exogenous thiamine and HMP could largely overcome the bottleneck at PDH as was expected. Only minimal secretion of pyruvate was detected in the medium. These findings in *A. protothecoides* are consistent with findings from mutant *E. coli* that have been transformed into thiamine auxotrophs [34].

Accumulation of malate and fumarate occurred under thiamine deficiency, but was alleviated by addition of thiamine or HMP. In contrast, isocitrate was relatively unaffected by thiamine addition. These results suggest that the TCA cycle may have been partially operating in the counterclockwise direction with pyruvate being converted into malate via malic enzyme. This conclusion is supported by metabolic flux analysis carried out by Wu et al. on heterotrophic *A. protothecoides* supplied with glucose [35]. This is a reasonable response

given the need to supply carbon to the TCA cycle without a fully-functional PDH or OGDH complex. Moreover, malic enzyme in animals has been shown to undergo allosteric activation in both the reverse and forward direction in the presence of malate and fumarate [36]. Both of these metabolites were found to have high intracellular concentrations under thiamine deficiency. Interconversion between malate and fumarate is reversible, resulting in accumulation of both metabolites in the cell. In past work, we have observed fumarate secretion when *A. protothecoides* was cultured on glucose [6]. This is consistent with the high intracellular fumarate levels observed under thiamine deficiency. Xiong et al. carried out metabolic flux analysis on *A. protothecoides* grown on ^{13}C-glucose in the presence of exogenous thiamine [37]. They concluded that there was no malic enzyme activity based on the labeling pattern. Rather, they found that there was significant phosphoenolpyruvate (PEP) carboxylase activity with net conversion of PEP to OAA under nitrogen-replete conditions. Like malic enzyme, PEP carboxylase can allow for the bypass of PDH, but there is a loss in opportunity to produce ATP from PEP [29]. Xiong et al. did not investigate the impact of thiamine deficiency on partitioning between malic enzyme and PEP carboxylase, making it difficult to apply their result to the present study. Typically PEP carboxylase undergoes allosteric inhibition when asparate levels are high [29], as they were under thiamine deficiency. Therefore, this anapleurotic reaction may have been less important than malic enzyme with regard to shuttling pyruvate into the TCA cycle under thiamine deficiency. Metabolic flux analysis is required to conclusively address this hypothesis.

Interestingly, α-ketoglutarate levels were higher in control cultures than in thiamine supplied cultures suggesting there was also a bottleneck at OGDH in the clockwise direction of the TCA cycle. As with acetate-supplied cultures, the impact of thiamine deficiency and alleviation on the glyoxylate cycle was not clear but flux analysis by others has shown this pathway to be inactive in *A. protothecoides* supplied with glucose [35,37].

In contrast to acetate cultures, thiamine deficiency led to an apparent bottleneck at TK. Ribose-5-phosphate accumulated in thiamine-free controls but not when exogenous HMP and thiamine were added. This also suggests that the pentose phosphate pathway was dominant compared to the Calvin-Benson cycle for carbon fixation. In the pentose phosphate pathway, Ribose-5-phosphate is a reactant, whereas in the Calvin-Benson cycle it is a product of the reaction catalyzed by TK. This result is not surprising; the cell preferentially uses exogenous glucose over photosynthesis to power its metabolic needs. This is consistent with past findings about mixotrophic green algae [4]. As the pentose phosphate pathway is a primary path for NADPH generation under conditions of low photosynthesis [35], a bottleneck in this pathway could explain low lipid content under thiamine-deficient conditions. NADPH provides the reducing power needed for fatty acid synthesis.

4.4. Impact of spent algae medium on A. protothecoides growth and metabolism

The results suggest that spent medium from *C. sorokiniana* may partially alleviate thiamine deficiency. However, there are likely other factors at play. Spent medium obtained after 5 days of *C. sorokiniana* culture did increase the growth rate and organic substrate uptake. It also reduced, but did not eliminate, the biomass-normalized pyruvate secretion when compared to the thiamine-free control cultures. However, none of these effects were as strong as those in cultures with exogenous HMP and thiamine addition. This suggests that spent *C. sorokiniana* medium obtained from 5-day-old cultures provided limited alleviation of thiamine deficiency. A dose response study showed that mixotrophic *A. protothecoides* could achieve half its maximum growth rate at ~4.5 nM thiamine concentration and deficiency could be completely eliminated at ~20 nM. This was very similar to our past finding with autotrophic growth of *A. protothecoides* [6].

Examination of the central metabolome revealed some interesting findings that suggest partial thiamine alleviation by spent medium. Interestingly, spent medium lowered intracellular pyruvate concentrations (consistent with normalized pyruvate secretion data). However, spent medium seemed to have little impact on metabolic bottlenecks in the TCA cycle, particularly at OGDH. The impacts of spent medium on TK were clear for glucose cultures; the amount of ribose-5P fell somewhere between the control (high) and the thiamine-provided cultures (low). With acetate as a substrate, spent medium led to higher ribose-5P levels, opposite of what is expected from thiamine alleviation. It is difficult to gauge the TK bottleneck from a single substrate, particularly because TK serves in both the pentose phosphate pathway and Calvin Benson cycle. Overall, 5-day spent medium appears to offer limited thiamine metabolites. This finding is consistent with a hypothesis posited by Bashan et al. in which *C. sorokiniana* provides thiamine to *Azospirillum* bacteria in co-culture [38].

Furthermore, *C. sorokiniana*'s spent medium composition appears to change as the culture ages. In past research, we observed that spent *C. sorokiniana* medium could almost completely alleviate pyruvate secretion [6]. In the present study, 5-day spent medium yielded only partially alleviated pyruvate secretion. This discrepancy can likely be explained by the age of the culture used to generate spent medium. Spent medium from a 15-day culture appears to provide enough thiamine metabolites to nearly alleviate deficiency in *A. protothecoides*. This was observed by much faster growth rates, glucose uptake, and lower pyruvate secretion compared to use of 5-day spent medium. It is unlikely that the algae secrete thiamine as a product, rather we hypothesize that degraded thiamine products are released by cells that have died and lysed. In humans and animals it is understood that thiamine is tightly bound within cells, resulting in low free concentrations of thiamine metabolites [10]. We, therefore, expect uncompromised algae cells to also tightly bind and internally recycle thiamine.

Spent medium appears to result in other impacts that cannot be explained by thiamine metabolites alone. These include high concentrations of isocitrate and glycolate, which appeared to be unaffected by the presence of thiamine. Glycolate in particular is understood to be a product of photosynthesis, but the literature on this topic is generally old [39–41]. This raises the question of whether secretions in spent medium alter photosynthetic activity in mixotrophic *A. protothecoides*. *C. sorokiniana* likely secretes a wide range of metabolites into the spent medium, many of which could impact *A. protothecoides* metabolism in unexpected ways. The composition of *C. sorokiniana*'s "phycosphere" of secreted metabolites is an area of ongoing investigation.

That spent medium from *C. sorokiniana* could generally increase growth of *A. protothecoides* relative to control cultures, suggests possible benefits of co-culturing these organisms. Additionally, these results also demonstrate the possibility of recycling the medium of one algae species for growth of another. The latter concept is attractive because it reduces waste of nutrients and water, improving the life cycle environmental performance of algae cultivation [42–44].

5. Conclusion

This study showed that exogenous thiamine and its precursor, HMP, led to significant changes in *A. protothecoides* composition and metabolism compared to thiamine-deficient control cultures. Specifically, thiamine and HMP relieved metabolic bottlenecks around the PDH and OGDH complexes, as well as transketolase. This resulted in greater substrate uptake and limited secretion of pyruvate into the culture medium. While these results are consistent with how TPP is utilized by mammals and bacteria, the net outcome of these metabolic changes was higher lipid and starch content in *A. protothecoides*. Cultivation of *A. protothecoides* on spent *C. sorokiniana* medium also led to partial alleviation of thiamine deficiency, the extent of which depended on the age of the culture used to prepare the spent medium. *C. sorokiniana* spent medium also led to metabolic changes that were unique from thiamine or HMP addition alone, indicating the presence of other mechanisms.

The study of algal-algal symbiosis has received minimal attention to date but our results suggest there could be benefit in co-culturing algae species and recycling spent algae cultivation medium under the right process conditions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2018.05.019.

Competing interest statement

The authors declare no competing interests.

Acknowledgements

This work was supported by the USDA National Institute of Food and Agriculture Hatch Project ALA0HIGGINS, the Auburn University Office of the Vice President for Research, and NSF grants CBET-1438211 and MCB-1139644.

Author contributions

B.T.H and J.S.V. designed the experiments. B.T.H., Q.W., and S.D. conducted culture experiments and assays. O.F. oversaw metabolomics analysis. B.T.H. analyzed processed metabolomics datasets and LC datasets. M.H. and A.T. carried out FAME analysis. B.T.H. wrote the manuscript. J.S.V., Q.W., M.H., and A.T. also contributed to the manuscript. All authors have read and approve of the final manuscript.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

References

[1] B. Higgins, et al., Algal-bacterial synergy in treatment of winery wastewater, Nature Clean Water, 2017 (In Press).
[2] E. Sforza, et al., Integration of *Chlorella protothecoides* production in wastewater treatment plant: from lab measurements to process design, Algal Res. 6 (2014) 223–233.
[3] X. Miao, Q. Wu, Biodiesel production from hetertrophic microalgal oil, Bioresour. Technol. 97 (2006) 841–846.
[4] Y. Wang, et al., Mixotrophic continuous flow cultivation of *Chlorella protothecoides* for lipids, Bioresour. Technol. 144 (Supplement C) (2013) 608–614.
[5] B. Higgins, J. VanderGheynst, Effects of *Escherichia coli* on mixotrophic growth of *Chlorella minutissima* and production of biofuel precursors, PLoS One 9 (5) (2014) e96807.
[6] B.T. Higgins, et al., Cofactor symbiosis for enhanced algal growth, biofuel production, and wastewater treatment, Algal Res. 17 (2016) 308–315.
[7] M.A. Gutowska, et al., Globally important haptophyte algae use exogenous pyrimidine compounds more efficiently than thiamin, MBio 8 (5) (2017).
[8] D. Lonsdale, A review of the biochemistry, metabolism and clinical benefits of thiamin(e) and its derivatives, Evid. Based Complement. Alternat. Med. 3 (1) (2006) 49–59.
[9] S. Manzetti, J. Zhang, D. van der Spoel, Thiamin function, metabolism, uptake, and transport, Biochemistry 53 (5) (2014) 821–835.
[10] C.E. Kraft, E.R. Angert, Competition for vitamin B1 (thiamin) structures numerous ecological interactions, Q. Rev. Biol. 92 (2) (2017) 151–168.
[11] K.E. Helliwell, The roles of B vitamins in phytoplankton nutrition: new perspectives and prospects, New Phytol. 216 (1) (2017) 62–68.
[12] M.T. Croft, M.J. Warren, A.G. Smith, Algae need their vitamins, Eukaryot. Cell 5 (8) (2006) 1175–1183.
[13] M.T. Croft, et al., Algae acquire vitamin B12 through a symbiotic relationship with bacteria, Nature 438 (7064) (2005) 90–93.
[14] E. Kazamia, et al., Mutualistic interactions between vitamin B12 -dependent algae and heterotrophic bacteria exhibit regulation, Environ. Microbiol. 14 (6) (2012) 1466–1476.
[15] R.W. Paerl, et al., Vitamin B1 ecophysiology of marine picoeukaryotic algae: strain-specific differences and a new role for bacteria in vitamin cycling, Limnol. Oceanogr. 60 (1) (2015) 215–228.
[16] B. Higgins, Co-culturing green algae with bacteria for enhanced growth and production of biofuel precursors, in Biological and Agricultural Engineering, University of California, Davis, CA, 2014.
[17] F.J. Choix, C.G. Lopez-Cisneros, H.O. Mendez-Acosta, *Azospirillum brasilense* increases CO_2 fixation on microalgae *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Chlamydomonas reinhardtii* cultured on high CO_2 concentrations, Microb. Ecol. (2018), http://dx.doi.org/10.1007/s00248-017-1139-z.
[18] B.T. Higgins, et al., Informatics for improved algal taxonomic classification and research: a case study of UTEX 2341, Algal Res. 12 (2015) 545–549.
[19] R.W. Hunt, et al., Effect of biochemical stimulants on biomass productivity and metabolite content of the microalga, *Chlorella sorokiniana*, Appl. Biochem. Biotechnol. 162 (8) (2010) 2400–2414.
[20] O.U. Tanadul, et al., The impact of elevated CO_2 concentration on the quality of algal starch as a potential biofuel feedstock, Biotechnol. Bioeng. 111 (7) (2014) 1323–1331.
[21] B. Higgins, et al., Microplate assay for quantitation of neutral lipids in extracts from microalgae, Anal. Biochem. 465 (2014) 81–89.
[22] C.E. Richardson, et al., Lipidomic analysis of oxidized fatty acids in plant and algae oils, J. Agric. Food Chem. 65 (9) (2017) 1941–1951.
[23] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (3) (1959) 426–428.
[24] D. Lee, O. Fiehn, High quality metabolomic data for *Chlamydomonas reinhardtii*, Plant Methods 4 (1) (2008) 7.
[25] Team, R.C, R: A language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2013.
[26] G.E.P. Box, D.R. Cox, An Analysis of Transformations, J. R. Stat. Soc. Ser. B 26 (2) (1964) 211–252.
[27] B. Higgins, J.M. Labavitch, J.S. VanderGheynst, Co-culturing *Chlorella minutissima* with *Escherichia coli* can increase neutral lipid production and improve biodiesel quality, Biotechnol. Bioeng. 112 (9) (2015) 1801–1809.
[28] T.P. Begley, et al., Thiamin biosynthesis in prokaryotes, Arch. Microbiol. 171 (5) (1999) 293–300.
[29] R.H. Garrett, C.M. Grisham, Biochemistry, 5th Edition, Cengage Learning, Mason, Ohio, 2012, pp. 578–730.
[30] A. Chalima, et al., Utilization of volatile fatty acids from microalgae for the production of high added value compounds, Fermentation 3 (4) (2017).
[31] C. Plancke, et al., Lack of isocitrate lyase in *Chlamydomonas* leads to changes in carbon metabolism and in the response to oxidative stress under mixotrophic growth, Plant J. 77 (3) (2014) 404–417.
[32] C. Gao, et al., Oil accumulation mechanisms of the oleaginous microalga *Chlorella protothecoides* revealed through its genome, transcriptomes, and proteomes, BMC Genomics 15 (2014) 582.
[33] M. Pasquini, et al., Structural basis for the magnesium-dependent activation of transketolase from *Chlamydomonas reinhardtii*, Biochim. Biophys. Acta 1861 (8) (2017) 2132–2145.
[34] T.B. Causey, et al., Engineering *Escherichia coli* for efficient conversion of glucose to pyruvate, Proc. Natl. Acad. Sci. U. S. A. 101 (8) (2004) 2235–2240.
[35] C. Wu, et al., Genome-based metabolic mapping and ^{13}C flux analysis reveal systematic properties of an oleaginous microalga *Chlorella protothecoides*, Plant Physiol. 167 (2) (2015) 586.
[36] W.E. Karsten, et al., *Ascaris suum* NAD-malic enzyme is activated by l-malate and fumarate binding to separate allosteric sites, Biochemistry 42 (32) (2003) 9712–9721.
[37] W. Xiong, et al., 13C-tracer and gas chromatography-mass spectrometry analyses reveal metabolic flux distribution in the oleaginous microalga *Chlorella protothecoides*, Plant Physiol. 154 (2010) 1001–1011.
[38] O.A. Palacios, et al., Tryptophan, thiamine and indole-3-acetic acid exchange between *Chlorella sorokiniana* and the plant growth-promoting bacterium *Azospirillum brasilense*, FEMS Microbiol. Ecol. 92 (6) (2016) fiw077.
[39] G.E. Fogg, C. Nalewajo, W.D. Watt, Extracellular products of phytoplankton photosynthesis, Proc. R. Soc. London, Ser. B 162 (989) (1965) 517.
[40] N.E. Tolbert, L.P. Zill, Excretion of glycolic acid by algae during photosynthesis, J. Biol. Chem. 222 (2) (1956) 895–906.
[41] J.A. Bassham, M. Kirk, The effect of oxygen on the reduction of CO_2 to glycolic acid and other products during photosynthesis by *Chlorella*, Biochem. Biophys. Res. Commun. 9 (5) (1962) 376–380.
[42] M.A. Borowitzka, N.R. Moheimani, Sustainable biofuels from algae, Mitig. Adapt. Strateg. Glob. Chang. 18 (1) (2013) 13–25.
[43] A.F. Clarens, et al., Environmental life cycle comparison of algae to other bioenergy feedstocks, Environ. Sci. Technol. 44 (2010) 1813–1819.
[44] F. Hadj-Romdhane, et al., Development and validation of a minimal growth medium for recycling *Chlorella vulgaris* culture, Bioresour. Technol. 123 (2012) 366–374.