Exogenous Citrate Induces Lipid Accumulation in *Chlamydomonas reinhardtii*

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Abstract

Due to the energy crisis in recent years, large-scale investments and government mandates have led to an expansion in renewable energy research. This situation provides incentive for research in microalgae as a feedstock for biofuel production. Research in our laboratory has demonstrated that nitrogen deprivation in Chamydomonas reinhardtii, a model organism, provokes the accumulation of cytosolic citrate, which is cleaved by ATP-citrate lyase (ATP-CL) into oxaloacetate and acetyl-CoA, resulting in significant accumulation of acetyl-CoA, the substrate for lipid production (Wase, et al, in submission). Therefore, we hypothesized citrate might be added to algal cultures to induce lipid accumulation. In this work, *C. reinhardtii* CC-125 strain was used to test this hypothesis. Our results demonstrate that 1) 3mM to 6mM of citrate added to TAP media enhanced cell growth in 96-well microtiter plates, while higher concentrations inhibited growth; 2) the lipid content per cell increased with citrate addition, however we also observed some growth inhibition that resulted in reduced total mass compared to control cultures; 3) the total fatty acid concentration per cell increased 1.5 fold with the supplementation of 5 mM citrate in 50mL flask cultures, relative to negative control with TAP media; 4) the lipid profile of cells treated with 5mM citrate media was significantly ($p<0.01$) different from negative and positive control with nitrogen deprivation; 5) the concentration of citrate in the media was reduced by 15% after 36h of incubation, indicating significant ($p<0.05$) uptake of extracellular citrate into cells; and 6) the amount of intracellular citrate increased significantly ($p<0.1$) to more than 100-fold relative to both negative and positive control. These data indicate citrate may be used as a chemical inducer of lipid production in algae.

Background

The microalgae are a very large and diverse group of photosynthetic organisms that have attracted global attention as a renewable energy feedstock. Various studies during 1950s and 60s indicate that nutrient stress, especially nitrogen deprivation, on microalgae induces significant lipid accumulation that might be used for the production of biofuels.

It has been shown that nitrogen deprivation in a variety of oleaginous microorganism provokes the cleavage of AMP to produce NH_4^+ as a complementary nitrogen source. The excessive decrease of intracellular AMP alters the TCA cycle, including the decline in the activity of isocitrate dehydrogenase, enzyme responsible for the transformation of isocitrate to α-ketoglutarate. The citrate, in equilibrium with accumulated isocitrate, is transported out of mitochondria and then cleaved by ATP-citrate lyase (ATP-CL) to oxaloacetate and acetyl-CoA, which is then used for fatty acid biosynthesis. *C. reinhardtii* is an important model organism for algal lipid metabolism studies since there is a vast background of information on its physiology and metabolism. Despite the fact that nitrogen deprivation induces significant lipid accumulation, it also causes serious metabolic malfunctioning, severely limiting the rate of growth, and is impractical in large-scale production due to technical and financial drawbacks. In this study, we examined effects of exogenous citrate on lipid accumulation and growth of *Chlamydomonas reinhardtii* CC-125 strain under the condition without nutrient stress and demonstrate citrate acts as an inducer of lipid storage, presumably by providing the substrate acetyl-CoA.

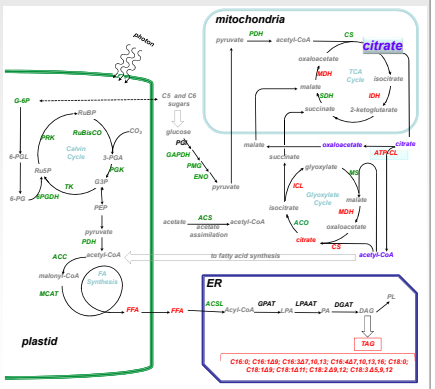


Figure 1. The lipid metabolic network in *C. reinhardtii*. As shown in this study, the increase of the concentration of extracellular citrate led to the increase of the concentration of intracellular citrate. We propose the uptake of exogenous citrate will increase the activity of ATP citrate lyase and decrease the activity of citrate synthase and malate dehydrogenase, resulting in increased cleavage of citrate, increased cytosolic malate concentration, which promote the efflux of citrate from mitochondria and thus overproduction of acetyl-CoA, which directly participates in FA synthesis.

Study Design

- This study tested the effect of exogenous citrate supplementation to TAP media on lipid accumulation in *Chlamydomonas reinhardtii* without nutrient stress
- The **negative controls** in this study are cultures with ordinary TAP media (TAP N+), in which cells do not accumulate stored lipids and the **positive controls** are cultures without nitrogen (TAP N-), which induces lipid accumulation and storage.
- Experiments in 125 mL disposable polycarbonate flasks were performed with 3 replicates for citrate with optimum concentration of 5mM and controls.
- Experiments in 300 µL 96-well transparent plates were performed with 8 replicates for each concentration of citrate (1-10mM) and controls.
- Citrate stock solutions were buffered with Tris base to pH = 7.0

Results

1. Effects of different exogenous citrate levels on growth

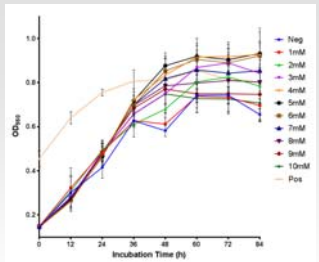


Figure 2A. The growth of cultures with different concentrations of citrate in the media. Cells in midlog phase were wash 2 × with TAP N+ and TAP N- media respectively. 100 mM citrate stock buffered with Tris base (pH = 7.0) was diluted to final concentrations of 1-10mM in each column of the 96-well plate and to a final volume of 200 µL. The plate was sealed with BreatheEasy membrane and placed on a light shelf and shaken vigorously every 12h.

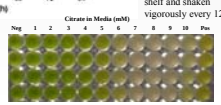


Figure 2B. Representative photograph of cultures with different concentrations of citrate in the media in a 96-well 300 µL U-bottom transparent plate. Cells treated with high concentrations (≥6mM) of citrate might be stressed by high ionic strength. This photo was taken after 48h of incubation.

2. Lipid accumulation in cells cultured with citrate

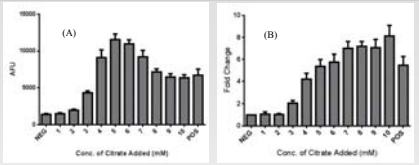
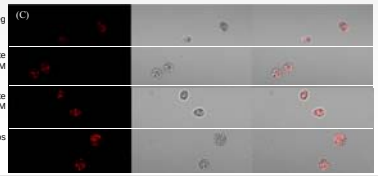


Figure 3.
(A) Lipid content of plate cultures treated with 1-10mM of citrate in media. After 48h of incubation, 2 µL of 1 mg/mL Nile Red in DMSO was added to each well. After incubated at 37 ° C for 30 min, the plate was read at excitation: 485/20 and emission: 590/35.
(B) Fold change of lipid content normalized with growth. The OD_{550} was first normalized with the average OD_{550} of the negative control. Then fluorescence, which was normalized with growth, was normalized with the average intensity of negative control. **(C) Representative confocal microscopy image of cells stained with Nile Red after 72h of incubation.**



3. Semiquantitative fatty acid analysis of 96-well plate cultures

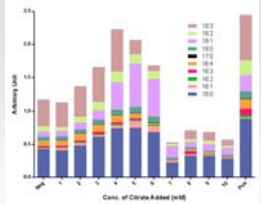


Figure 4. Relative amount FA and FA composition. Three wells of 200 µL of culture from each group was removed to 500 µL glass vials. Cells were pelleted and supernatant removed. 100 µL of tert-butyl methyl ether was added. 10 µL of 1 mg/mL nonadecanoic acid in chloroform was added as internal standard. Samples were votexed and incubated at room temperature for 5 min. 50 µL of trimethylsulfonium hydroxide (TMSH) was added and the samples incubated at room temperature for 30 min.

The samples were analyzed on GCMS with full-scan mode. The peak areas of each species were normalized with the peak areas of the internal standard and shown on the graph. All values were based on 3 replicates of 200 µL (single well) cultures. The total FA of cells treated by 5 mM citrate increased by 1.5 folds. Note the decrease of total FA of cells treated with citrate ≥6mM was resulted from inhibition of growth.

4. Quantitative fatty acid analysis of flask cultures

Total FA (n=3) nmol / 10^6 cells			
Citrate	37.2	30.1	34.9
Pos	29.6	31.5	36.9
Neg	23.4	25	24.6

Table 1. Total FA of controls and 5 mM citrate treated cells in flasks at 72h normalized with cell count. Cells supplemented with 5 mM of extracellular citrate increased lipid accumulation by 1.5 folds.

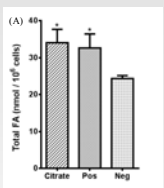
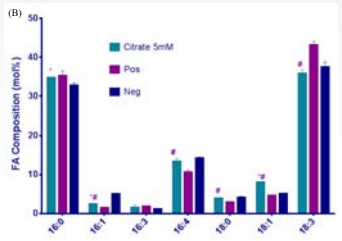


Figure 5. 1 mL of culture was centrifuged after 72h of incubation. Media was removed and cells were washed 2 × with water. The pellet was dried in SpeedVac and 250 µL B&D extraction mixture was added with glass beads. After vigorously vortexed for 2 min, the mixture was incubated at room temperature for at least 2 hours and then centrifuged at 15000 rpm for 5 min. Supernatant was removed to a vial and cells were washed with another 250 µL of B&D extraction mixture. 10 µg heptadecanoic acid was supplemented as internal standard. Extraction mixtures were pooled and dried under a stream of N_2 and redissolved in 50 µL of tert-butyl methyl ether. 50 µL of trimethylsulfonium hydroxide (TMSH) was added and incubated at room temperature for 30 min. The samples were analyzed on GCMS using full-scan mode.
(A) Total FA of controls and 5 mM citrate treated cells in flasks at 72h normalized with cell count. * indicates $p<0.05$ versus negative control.
(B) Fatty acid composition of controls and 5 mM citrate flasks treated cells. In citrate treated cells, slightly more 16:0 were accumulated relative to negative control and less 18:3, less 16:1 and more 18:1 were accumulated relative to both controls. * indicates $p<0.05$ versus negative control. # indicates $p<0.05$ versus positive control.



5. Quantitative citrate analysis of flask cultures

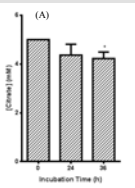


Figure 5(A) Citrate level change in the 5 mM citrate media in 36h of incubation. * indicates $p<0.05$ versus that at 0h.

Time(h)	[citrate] (mmol/mL media)		
	Citrate 5mM	Pos	Neg
0.5	5.00	ND	ND
24	4.36	ND	ND
36	4.22	ND	ND

Table 2. Change of concentrations of citrate in the media in the first 36h of incubation. The concentration of citrate in the media decreased by 15% relative to that at 0h when 5 mM of citrate was supplemented, indicating the uptake of citrate. ND: not detected.

Time (h)	Citrate (nmol/10^6 cells) (n=3)					
	Citrate 5mM			Neg		
0.5	0.191	0.047	0.040	0.0123	0.0046	0.0037
24	0.130	0.067	0.057	0.0039	0.0019	0.0027
36	0.995	0.769	0.265	0.0016	0.0012	0.0012
48	0.191	0.051	0.003	0.0121	0.0027	NA

NA: sample not available

Table 3 Change of concentrations of citrate in the cells in the first 48h of incubation. Although the data has a large variability, the citrate accumulation in treated cells increased in the first 36h and was significantly higher at 36h and decreased at 48h.

Figure 5. 1 mL of culture was centrifuged at selected time points. The supernatant was carefully removed with a needle and filtered through a 0.22 µm syringe driven filter. The cell pellets were washed 2 × with water, flash frozen with liquid nitrogen and stored at -80° C. The cell pellet was lysed in 600 µ of cold MCW (MeOH:$CHCl_3$:H_2O=10:3:1) with the presence of glass beads on TissueLyser for 2 min and 1 µg of sorbitol was added as internal standard. The tubes were then vigorously shaken in ice for more than 30 min and centrifuged at 4 ° C. 500 µL of supernatant was removed and dried in SpeedVac. 100 µL of the filtered supernatant (media) was also dried for the determination of citrate level in the media. 5 µL of 40 mg/mL methoxyamine hydrochloride was added and incubated at 30° C, 400 rpm for 90 min. 45 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was added and incubated at 37° C, 400 rpm for 30min. Samples were analyzed on GCMS with selected ions 273 and 147.

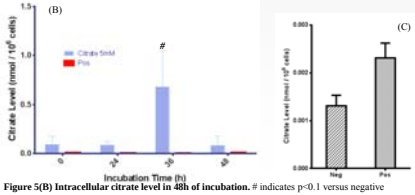


Figure 5(B) Intracellular citrate level in 48h of incubation. # indicates $p<0.1$ versus negative control. **Figure 5(C) Intracellular citrate level at 36h of negative and positive control. $p<0.05$**

6. Growth and Nile Red assay of flask cultures

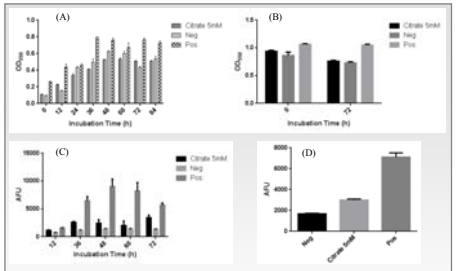


Figure 5. Cells in midlog phase were wash 2 × with TAP N+ and TAP N- media respectively. 100 mM citrate stock buffered with Tris base (pH = 7.0) was diluted to final concentrations of 5mM in the media. The 50 mL culture was started in polycarbonate disposable flask with 0.22 µm filter cap on. The flasks were shaken at 120 rpm and 25 ° C. 600 µL of culture was transfer to 3 wells in a 96-well plate . 2 µL of 1 mg/mL Nile Red in DMSO was added to each well. After incubated at 37 ° C for 30 min, the plate was read at excitation: 485/20 and emission: 590/35. **(A) The growth of cells with a low starting cell density (B) The growth of cells with a high starting cell density . (C) The lipid accumulation in cells with a low starting cell density (D) The lipid accumulation in cells with a high starting cell density after 72h of incubation.**

Conclusions

- 3mM to 6mM of citrate in TAP media enhanced cell growth in 96-well microtiter plates, while higher concentrations inhibited growth;
- Lipid content per cell increased as higher concentration of citrate was supplemented in the media, though lipid content per unit volume of culture decreased due to limited growth;
- Total fatty acid per cell increased by up to 1.5 folds with the supplementation of 5 mM citrate in 50mL flask cultures, relative to negative control;
- The lipid profile of cells treated with 5mM citrate media was significantly ($p<0.01$) different from negative control (TAP media) and positive control (nitrogen deprivation);
- The concentration of citrate in the media reduced by 15% after 36h of incubation, indicating significant ($p<0.05$) uptake of extracellular citrate into cells.
- The amount of intracellular citrate increased significantly ($p<0.1$) by 5 folds relative to the time of start and by more than 100 folds relative to both negative and positive control.

Future Directions

- Continue to assess the uptake of extracellular citrate in CC-125 cells at more time points with more consistent quantification
- Determine the total FA normalized with dry cell weight and triglycerides compositions
- Perform enzyme assays on ATP citrate lyase, citrate synthase, malate dehydrogenase, isocitrate dehydrogenase and isocitrate lyase.
- Analyze the utilization of extracellular citrate using radioactive isotope labeled citrate
- Test of extracellular citrate as the only carbon source (without acetate) on the lipid accumulation in CC-125 cells and its uptake
- Test the effect of extracellular citrate on the lipid accumulation in other algae

References

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