PHOTOSYNTHETIC DECOUPLING IN *CHLAMYDOMONAS REINHARDTII*

By

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A capstone project submitted for Graduation with University Honors

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ABSTRACT

Although photosynthesis sustains virtually all life on Earth and is not to be dismissed as anything short of a miracle, it is a highly inefficient process. In most photosynthetic organisms, only 1% of radiant energy is converted into biomass, and typically 3 to 4% for microalgae[2]. Naturally, one might consider alternative energy pathways that utilize technology such as photovoltaics which typically boast around 20% efficiency, with some approaching 50% in recent years[4]. Recently, a novel electro-catalytic process has been developed that is capable of fixing CO_2 and CO into multi-carbon substrates such as acetate at high efficiency rates (48% carbon to acetate)[8]. Acetate is heterotrophically viable for algal growth but is primarily produced through non sustainable petroleum or high energy intensive processes. This electro-catalytic process, however, is completely independent of photosynthesis, efficient, and can be driven with renewable energy sources such as photovoltaic energy. The effluent stream produced by this process contained cytotoxic components that were examined and treated, resulting in an acetate containing media viable for growth of *Chlamydomonas reinhardtii*, a model alga. This demonstrates the first growth of a photosynthetic organism completely independent of biological photosynthesis or photosynthetically derived substrates.

ACKNOWLEDGEMENTS

I would like to thank Dr. Robert Jinkerson, my faculty mentor and principal investigator for his guidance and support throughout this project and my undergraduate career. I would also like to thank Elizabeth Hann and Marcus Harland-Dunaway for working with me on this project and for their continued mentorship. Lastly, I want to thank Dr. Feng Jiao and Sean Overa of the University of Delaware, for collaborating with our lab on this project and providing the electrocatalytic effluent that made this coupled process possible.

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INTRODUCTION

Chlamydomonas reinhardtii is a single-celled green alga that has been determined to be able to grow in the absence of light, and therefore does not require photosynthesis, by utilizing carbon containing substrates such as acetate[6]. This is known as heterotrophic growth, where growth and propagation occur under dark conditions with metabolism of external carbon sources. This growth cannot be considered entirely decoupled from photosynthesis, however, because essentially all of these carbon substrates are derived from photosynthesis[3], including petroleum products which are the result of ancient photosynthesis.

A new, carbon fixing electrocatalytic process developed at the University of Delaware has been shown to be able to fix CO_2 and CO into acetate rich product streams[8]. This technology utilizes a copper nanosheet cathode and an IrO_2 anode to catalyze the reduction of these single carbon substrates, demonstrating a relatively high efficiency of approximately 54% conversion into acetate. Due to the high acetate content of the product stream, when incorporated into a common algal growth media, Tris-Acetate-Phosphate (TAP), a new media can be produced that can possibly harbor algal growth. This process can be powered entirely by electricity and thus, photovoltaic technology can be employed and direct comparisons regarding efficiency given a fixed solar footprint can be made.

By combining these two processes, there lies potential for developing an artificial photosynthetic system that can possibly match or exceed the efficiencies of conventional plant or algal growth, offering unforeseen advantages. Without the reliance on light, inconsistencies of sunlight due to climate variations can be remedied; this is a commonly discussed advantage of hydroponic agriculture[1]. This project has significant implications for introducing alternative

methods of agriculture that can aid in the battle against food shortage without further expanding agricultural lands.

HYPOTHESIS

I hypothesize that the high concentration of $KHCO_3$ salts in the effluent derived media will significantly hinder the growth of the algae, and with treatment utilizing dilution or salt separation, the media produced would be suitable for algal growth.

METHODS

The first experiment conducted was testing the growth of the algae on a modified version of TAP media, where the acetate was exchanged with the acetate contained within a simulated, chemically identical effluent, as the effluent produced by the University of Delaware, was not yet accessible. The purpose of this experiment was to observe if media produced utilizing the effluent can harbor algal growth in heterotrophic conditions. The amount of effluent added to supplement acetate was enough to replicate the typical acetate concentration used to grow algae (1 mL acetate / 1 L media). This also came with the potentially cytotoxic chemicals included with the effluent and was labeled as TP-Effluent media. The positive controls for this experiment were TAP-dark, and TAP-light, where the cultures were grown on TAP media in the absence and presence of light, respectively. The experimental samples were TP- Effluent dark and TP-Effluent light.

Table 1. Effluent simulated for the Initial Growth Experiment	
Chemical Component	Concentration (M)
Sodium Bicarbonate ($KHCO_3$)	1.0
Acetate (CH_3COOH)	0.38
Ethanol (C_2H_5OH)	0.013
Isopropanol (C_3H_8O)	0.0099
Notes: The ingredients were added to approximately 250 mL of DI H_2O, then the solution was topped off to 500 mL.	

Table 2. TP-Effluent Media for Initial Growth Experiment	
Chemical Component	Amount per 1.0 L
Effluent	175 mL
Trizma Base (TRIS)	2.42 g
TAP Salts	25.0 mL
Phosphate Solution	375 µL
Hutner's Trace Elements	1.00 mL
Notes: Ingredients were added to approximately 500 mL of MQ H_2O, then topped off to the desired volume. The pH was then adjusted using 5.0M HCl/NaOH to approximately 7.23. The Media was then sterilized using vacuum filtration.	

TAP media is typically sterilized using an autoclave, but it was discovered that the effluent contained heat or pressure sensitive chemicals that would drastically increase the pH of solution and develop white, crystalline precipitate following autoclaving. Due to this issue, after adjusting media pH utilizing 5.0M HCl and 5.0M NaOH to approximately 7.23, the media was instead vacuum filtered for both the positive controls and experimental cultures. Inside of a bio-hood, the corresponding media solutions were placed into 125 mL, pre-autoclave sterilized Erlenmeyer flasks, topped with pink caps for sterile air flow and wrapped in aluminum foil for the dark cultures. The flasks were then inoculated with 21gr- *Chlamydomonas reinhardtii* strain from an agar-plate preculture, placed under a light source and left to grow for 1 week.

Growth parameters were measured by aliquoting 1.0 mL of culture for cell count and OD_{750} analysis. For cell culture analysis, a Biorad TC20^{TM} automated cell counter was used, where 10 µL of culture was placed into both A/B sides of the slides, measured, and averaged. These parameters were measured at time 0, and at 1 week.

The second experiment aimed to determine cytotoxic chemicals in the effluent and TP-Effluent media, in order to develop potential treatment methods to improve growth. This was done by omitting chemical constituents from the simulated effluent and examining if growth improved. Because there were four chemical candidates for growth inhibition (acetic acid not included), each of the experimental effluent solutions omitted one candidate, labeled -$KHCO_3$, -Ethanol, -1-Propanol, and -Propionic Acid, respectively. Preparation of the media was identical to that of the first growth experiment.

Table 3. Effluent for Drop-Out Experiment

Chemical Component	No Drop	-KHCO3	-C_2H_5OH	-C_3H_8O	-$C_3H_6O_2$
1.0 M Sodium Bicarbonate ($KHCO_3$)	✓	-	✓	✓	✓
0.38 M Acetate (CH_3COOH)	✓	✓	✓	✓	✓
0.013 M Ethanol (C_2H_5OH)	✓	✓	-	✓	✓
0.0099 M 1-propanol (C_3H_8O)	✓	✓	✓	-	✓
0.013 M Propionic Acid ($C_3H_6O_2$)	✓	✓	✓	✓	-

Notes: Ingredients were added to approximately 250 mL of DI water to their respective flasks, then topped off to 500 mL.

Table 4. TP-Effluent Media for Drop-Out Experiment	
Chemical Component	Amount per 1.0 L
Effluent	92 mL
Trizma Base (TRIS)	2.42 g
TAP Salts	25.0 mL
Phosphate Solution	375 µL
Hutner's Trace Elements	1.00 mL
Notes: Ingredients were added to approximately 250 mL of MQ H_2O, then topped off to the desired volume. The pH was then adjusted using 5.0M HCl/NaOH to approximately 7.23. The Media was then sterilized using vacuum filtration.	

The inoculation protocol for this experiment was slightly different than the first, instead using liquid pre-cultures of 21gr+ *Chlamydomonas reinhardtii* grown under light for 7-days. The inoculation of the control and experimental cultures was to achieve 5.0×10^5 cells/mL cell density. The cell density of the pre-cultures was determined using the Biorad TC20^{TM} automated cell counter, diluted to the target density and inoculated into pre-autoclaved flasks similar to that of the first experiment, but instead with 50 mL of media. All of the samples were grown in triplicate, with the positive controls; TP-effluent, TAP, and the experimental flasks; $KHCO_3$, -Ethanol, -1-Propanol, and -Propionic Acid. The growth parameters used for this experiment differed slightly from the previous experiment, with the cell counting method being the same as before, but now with the inclusion of optical density measurement at 750 nm (OD_{750}) with a Molecular Devices QuickDrop^{TM} Spectrophotometer. This is a common method of quantifying algal growth. This measurement was blanked with a small aliquot of TAP media with no algae. Microscopic analysis was also performed using an Olympus BX51 Fluorescence Microscope. For each of these methods, 1.0 mL of culture was taken from each flask at time 0, and every 48 hours subsequently until 16 days total growth. On day 16, the dry biomass of the cultures by separating the cells from the media, baking overnight, and weighing the cell mass.

The third experiment was the Media Optimization Experiment, where effluent and thus acetate and cytotoxic chemical concentrations were altered to investigate the thresholds of algal growth on TP-effluent media. The percentages of acetate (relative to 1.0 mL acetate / 1 L media) used in this experiment were 25%, 50%, 75% and 100% (normal TP-Effluent media). TAP and TP were included as positive and negative controls. This experiment sought to determine how algal growth can be affected with lower concentrations of effluent, predicted to worsen growth due to less acetate but also improve growth due to lower concentrations of cytotoxic chemicals. The same effluent recipe from the drop out experiment (table 3) was used in this experiment. Inoculation and culturing protocols were very similar to that of the "Drop-Out" experiment, with the same growth parameter measurement methods, consisting of cell counts and OD_{750} every 48 hours for a 16-day period and dry biomass measurements at the very end of the experiment. The same strain of 21gr+ *Chlamydomonas reinhardtii* was used. For this experiment, the algae were grown in the dark, by wrapping the flasks with aluminum foil.

Table 5. TP-Effluent Media for Media Optimization Experiment

Chemical Component	TAP	TP	100%	75%	50%	25%
Effluent	-	-	46.0 mL/L	34.5 mL/L	23 mL/L	11.492 mL/L
Acetate (CH_3COOH)	1.0 mL/L	-	-	-	-	-
Trizma Base (TRIS)	2.42 g	2.42 g	2.42 g	2.42 g	2.42 g	2.42 g
TAP Salts	25.0 mL	25.0 mL	25.0 mL	25.0 mL	25.0 mL	25.0 mL
Phosphate Solution	375 µL	376 µL	377 µL	378 µL	379 µL	380 µL
Hutner's Trace Elements	1.00 mL	1.00 mL	1.00 mL	1.00 mL	1.00 mL	1.00 mL

Notes: Ingredients were added to approximately 250 mL of MQ H_2O, then topped off to the desired volume. The pH was then adjusted using 5.0M HCl/NaOH to approximately 7.23. The Media was then sterilized using vacuum filtration.

The fourth and final experiment was the growth experiment where the actual effluent derived from the electrocatalytic reduction process was tested. The inoculation protocol was similar to previous experiments, but cell counts for this experiment were done manually with a hemocytometer for higher fidelity. The other growth parameters measured were the OD_{750} and dry biomass. To optimize growth for this experiment, the culture flasks were placed on a shaker with a controlled temperature of 30°C. Effluents of various compositions were tested in this experiment, as the collaborating laboratory was able to produce various kinds of different compositions, some containing entirely different chemicals. The same strain of 21gr+ *Chlamydomonas reinhardtii* was grown in the dark using aluminum foil. Although the different effluents contain different acetate concentrations, the effluent was added to the media so that the final medias had the same acetate concentration (1.0 mL/L).

Table 6. Effluent for True Growth Experiment

Chemical Component	$KHCO_3$ #1	$KHCO_3$ #2	KOH
Sodium Bicarbonate ($KHCO_3$)	0.33 M	0.25 M	-
Acetate (CH_3COOH)	0.157 M	0.162 M	0.691 M
Ethanol (C_2H_5OH)	0.018 M	0.007 M	-
n-propanol (C_3H_8O)	0.006 M	0.003 M	-
Propionic Acid ($C_3H_6O_2$)	0.004 M	-	0.042 M
Potassium Hydroxide (KOH)	-	-	1.0 M
Notes: All chemical constituents were in DI H_2O solution			

RESULTS

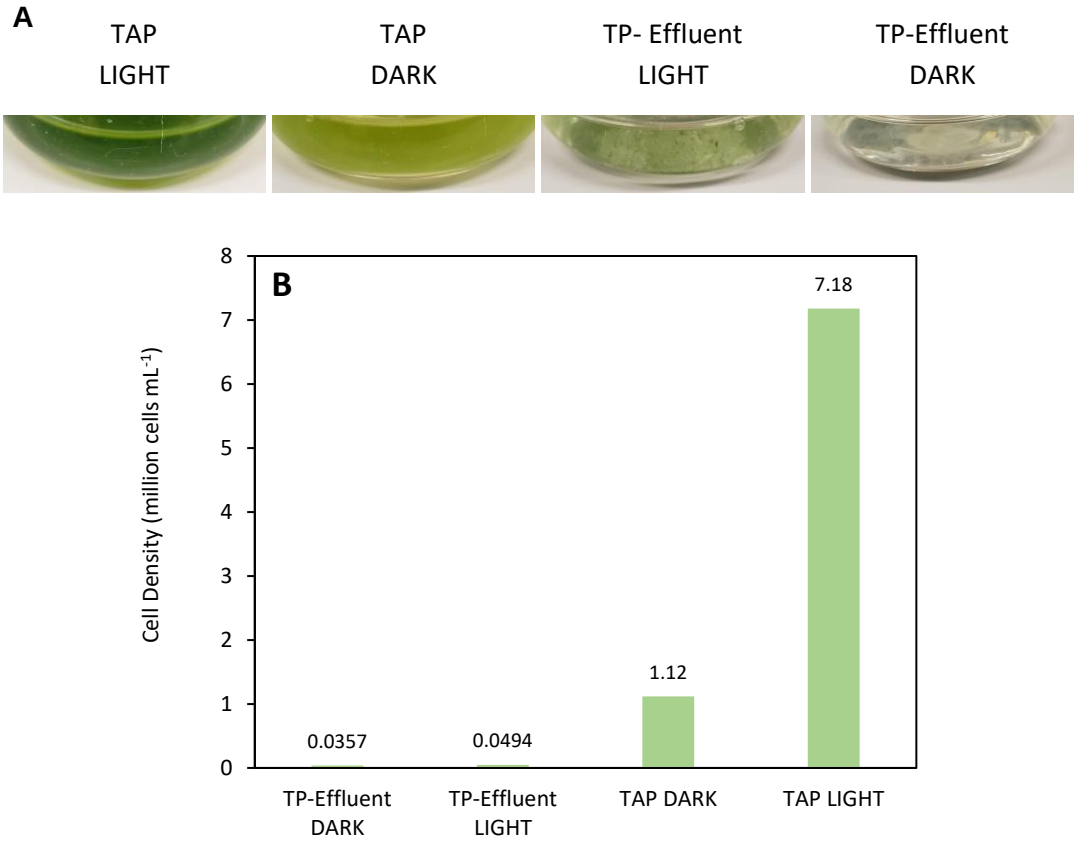
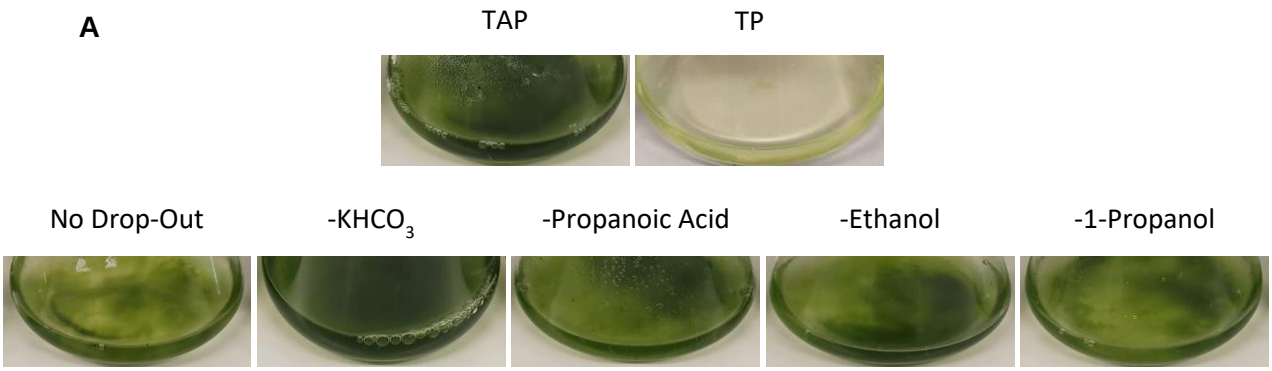
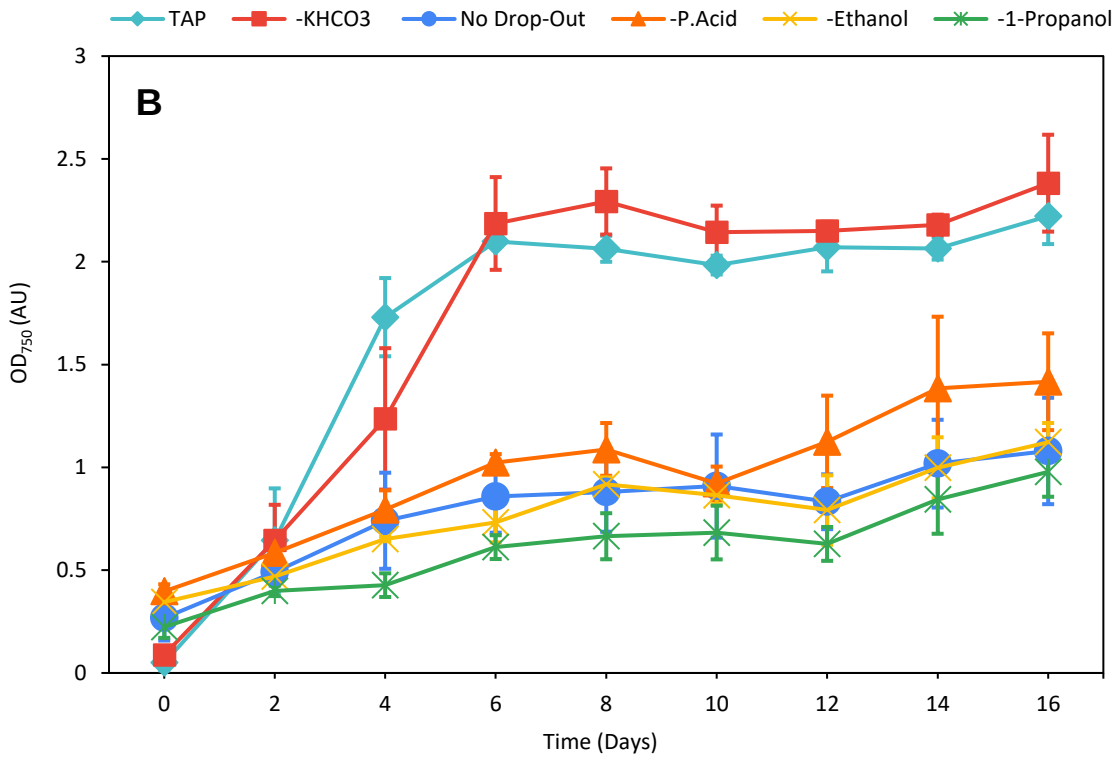
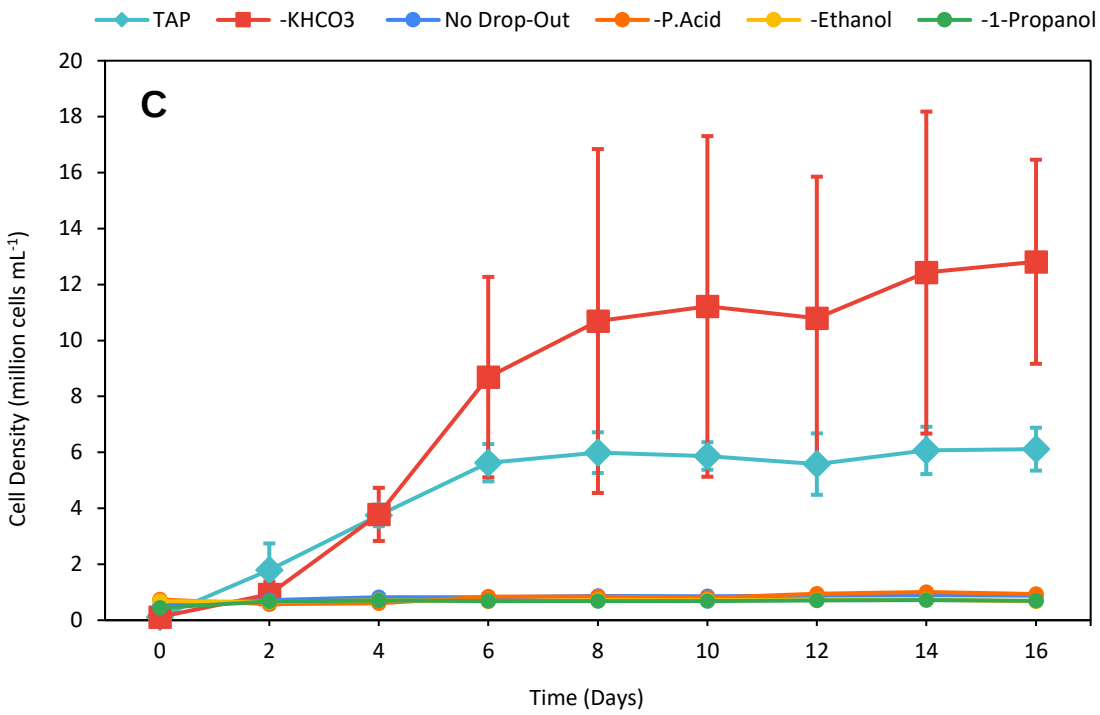


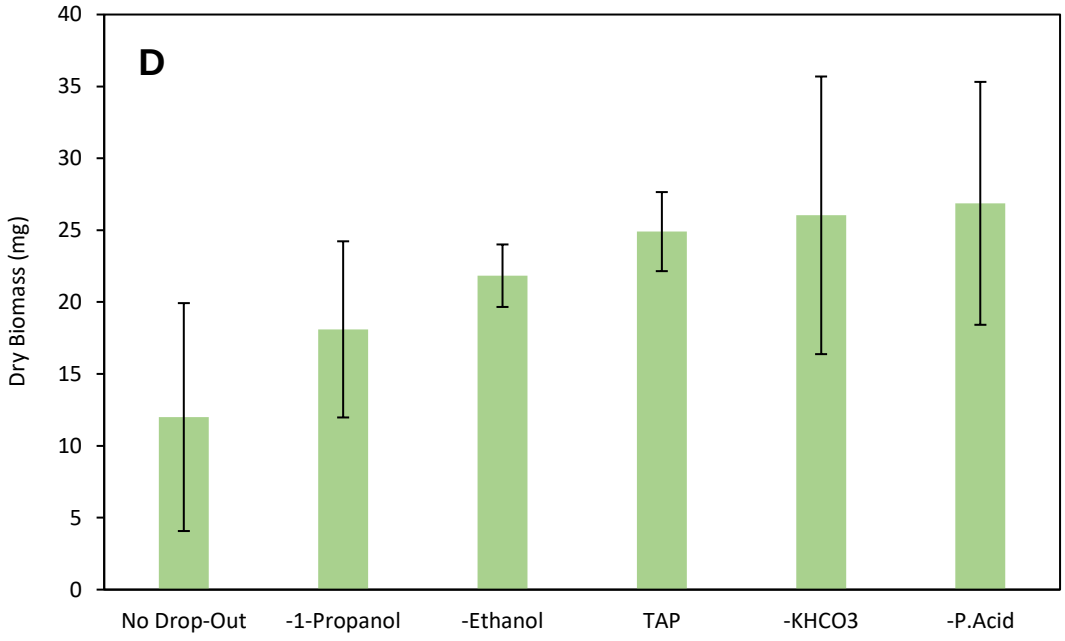
Fig.1 Algae grown on effluent, no growth in even light conditions, with even worse performance when grown in the dark.

Chlamydomonas grow in the light and in the dark, with and without acetate sourced from simulated electrocatalytic effluents (table 1) after 7 days (A) images (B) cell counts.


A
TAP
TP
No Drop-Out
-KHCO3
-Propanoic Acid
-Ethanol
-1-Propanol


TAP
-KHCO3
No Drop-Out
-P.Acid
-Ethanol
-1-Propanol
B
OD750 (AU)
Time (Days)
0
0.5
1
1.5
2
2.5
3
0
2
4
6
8
10
12
14
16


TAP
-KHCO3
No Drop-Out
-P.Acid
-Ethanol
-1-Propanol
C
Cell Density (million cells mL-1)
Time (Days)
0
2
4
6
8
10
12
14
16
18
20


D
Dry Biomass (mg)
No Drop-Out
-1-Propanol
-Ethanol
TAP
-KHCO3
-P.Acid
0
5
10
15
20
25
30
35
40

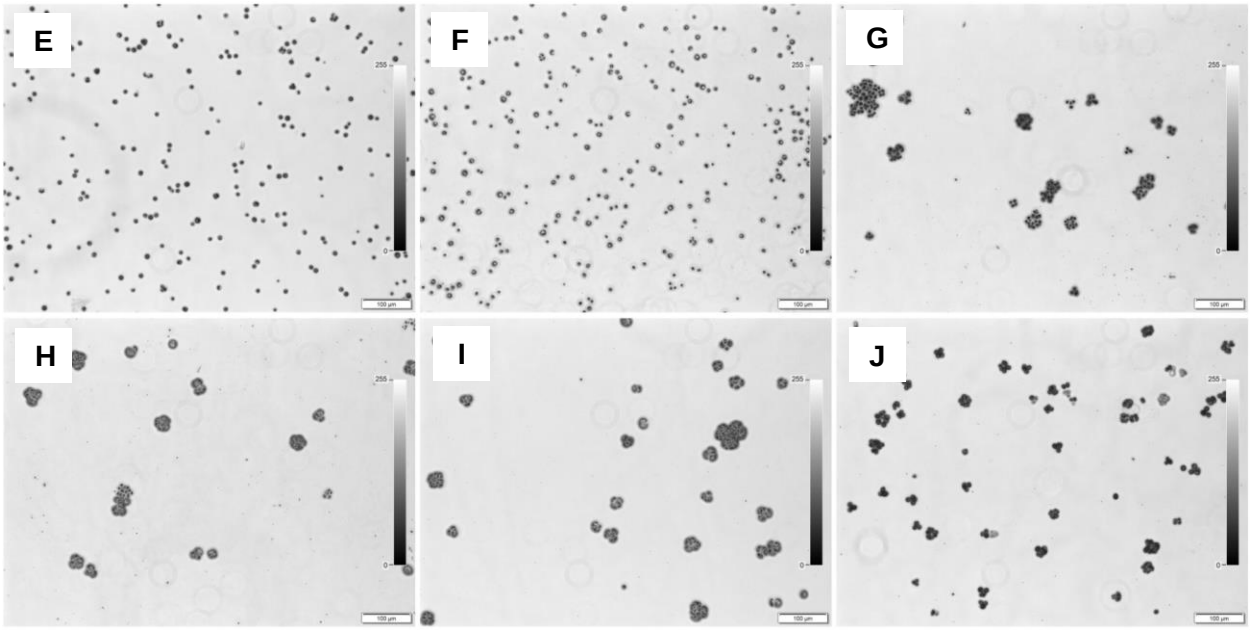
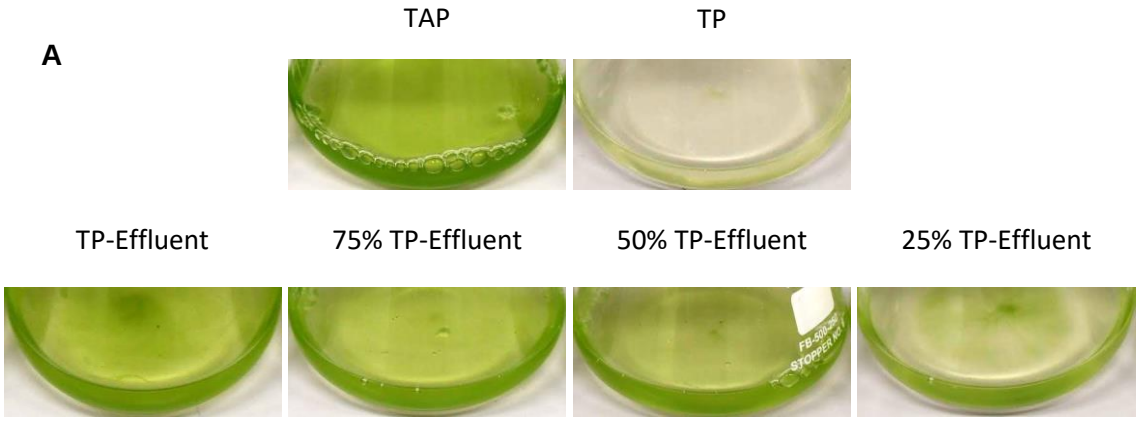
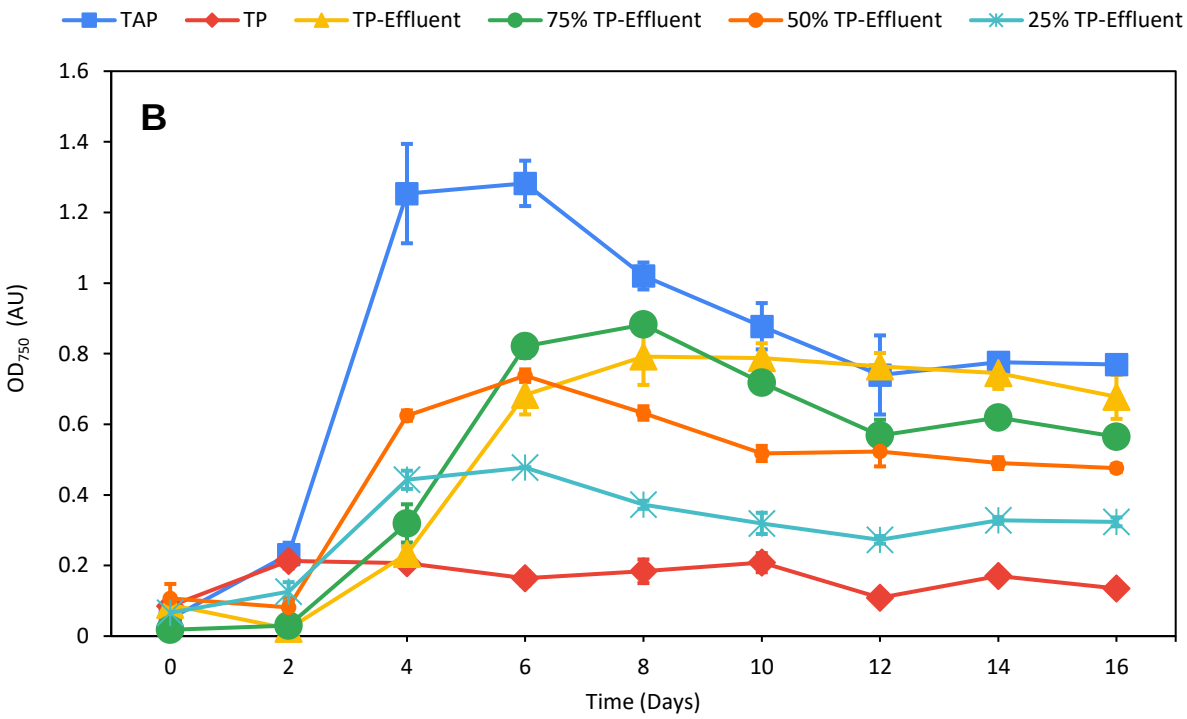
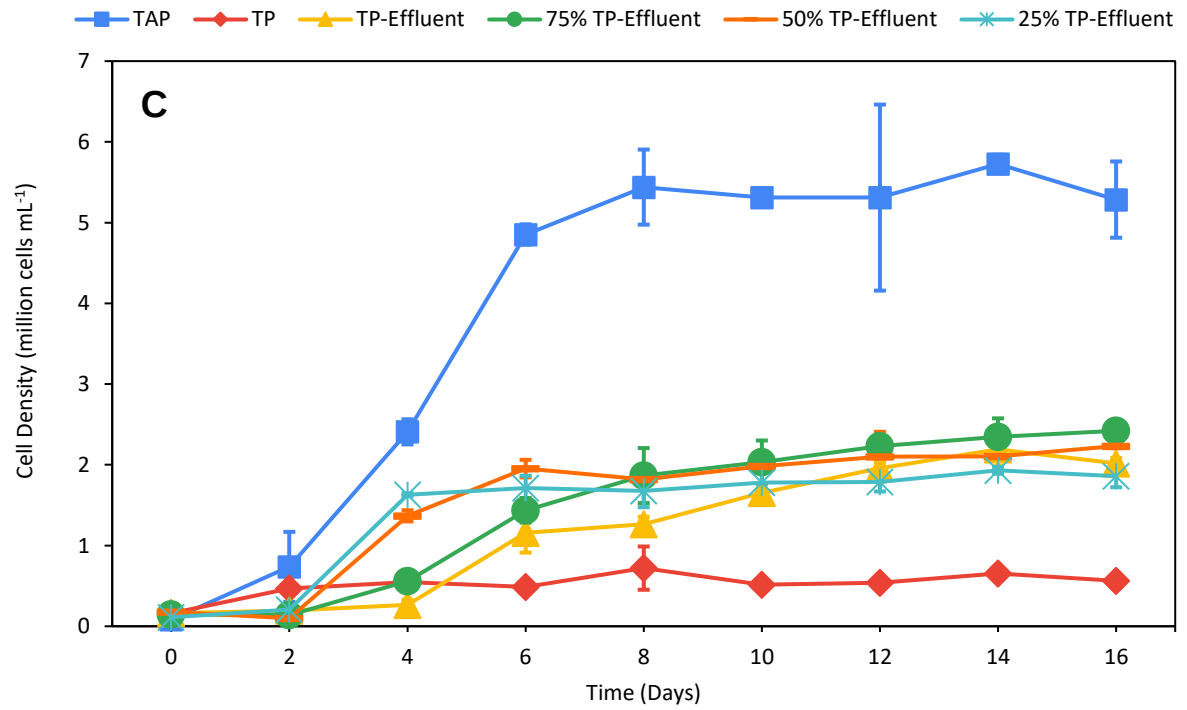


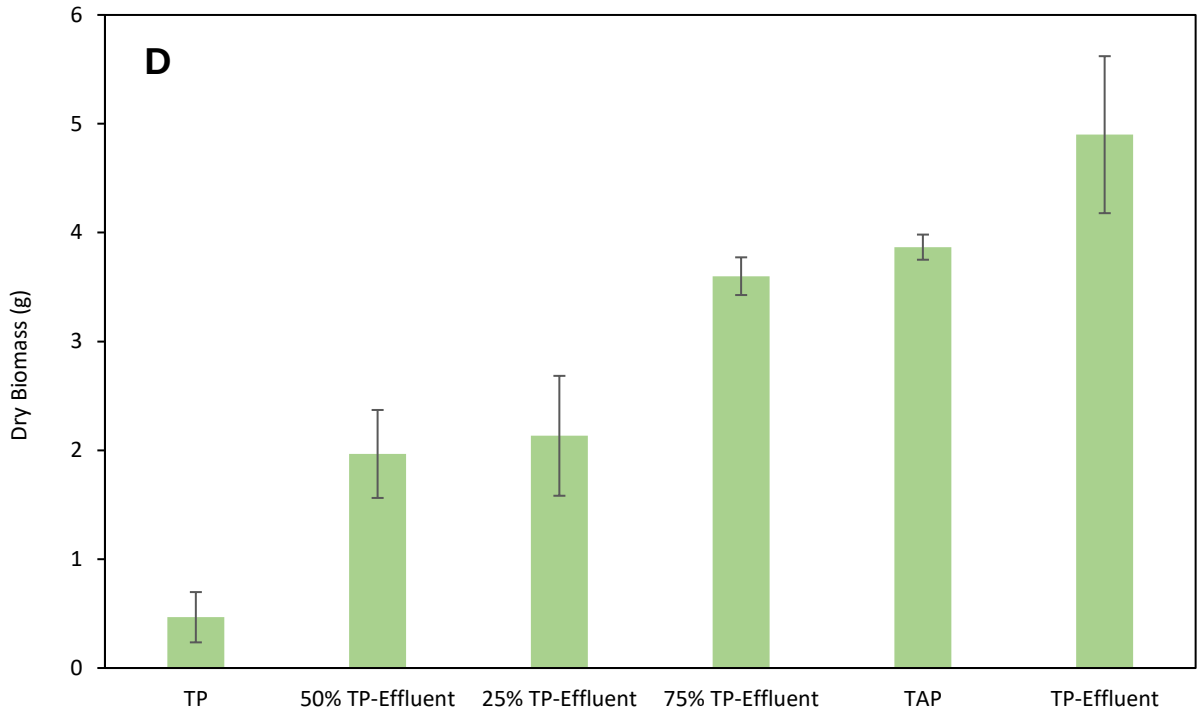
Fig.2 Potassium Bicarbonate ($KHCO_3$) is the primary cytotoxic component.

Chlamydomonas grown in the light of effluents with drop out treatment (A) images, (B) optical density (OD_{750}), (C) cell counts, (D) dry biomass throughout 16 days of growth. Error bars indicate standard deviation between triplicates. (E-J) Microscopic images taken to examine cell clumping and morphology; TAP (E), -$KHCO_3$ (F), No Drop-Out (G), -Ethanol (H), -1-Propanol (I), -Propanoic Acid (J).




TAP
TP
TP-Effluent
75% TP-Effluent
50% TP-Effluent
25% TP-Effluent
B
OD750 (AU)
Time (Days)


TAP
TP
TP-Effluent
75% TP-Effluent
50% TP-Effluent
25% TP-Effluent
C
Cell Density (million cells mL-1)
Time (Days)


D
Dry Biomass (g)
0
1
2
3
4
5
6
TP
50% TP-Effluent
25% TP-Effluent
75% TP-Effluent
TAP
TP-Effluent

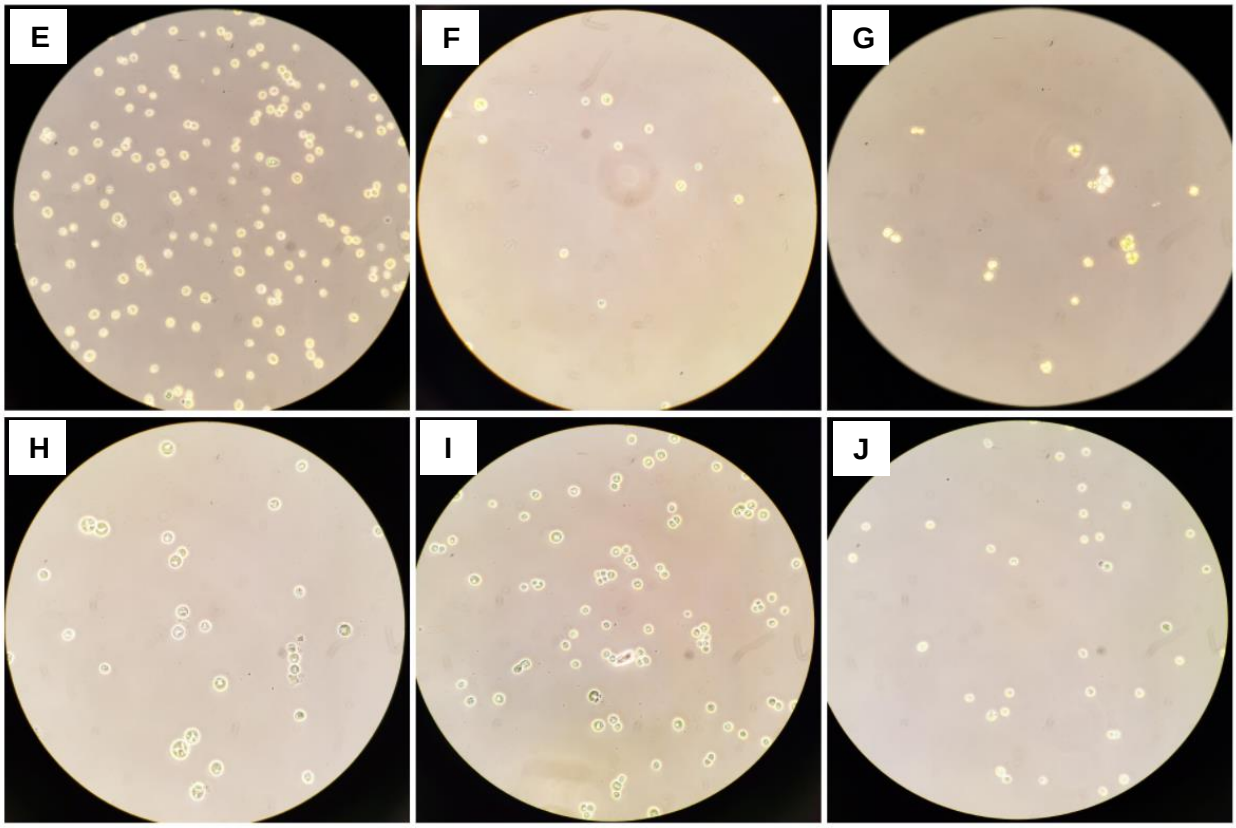
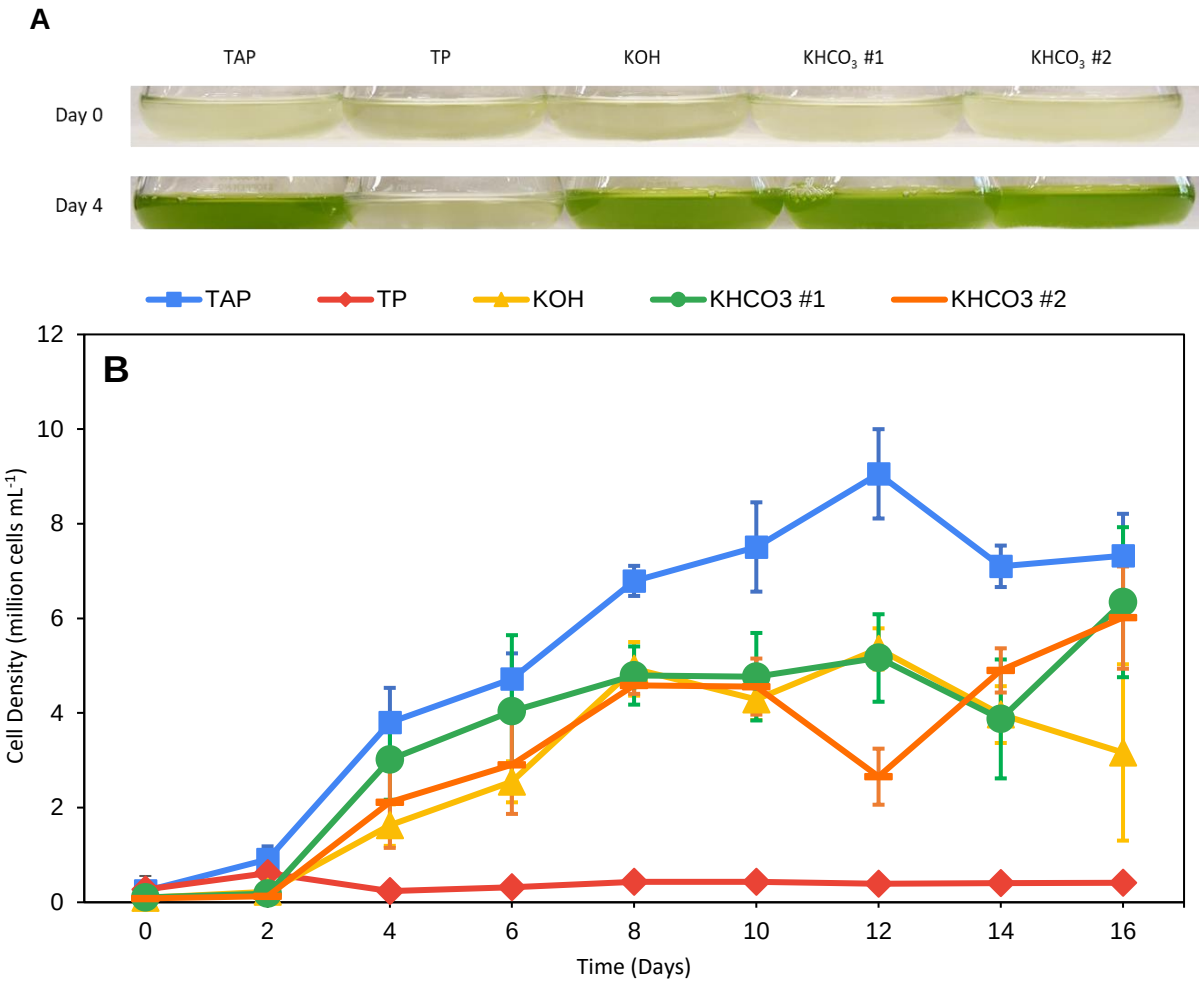
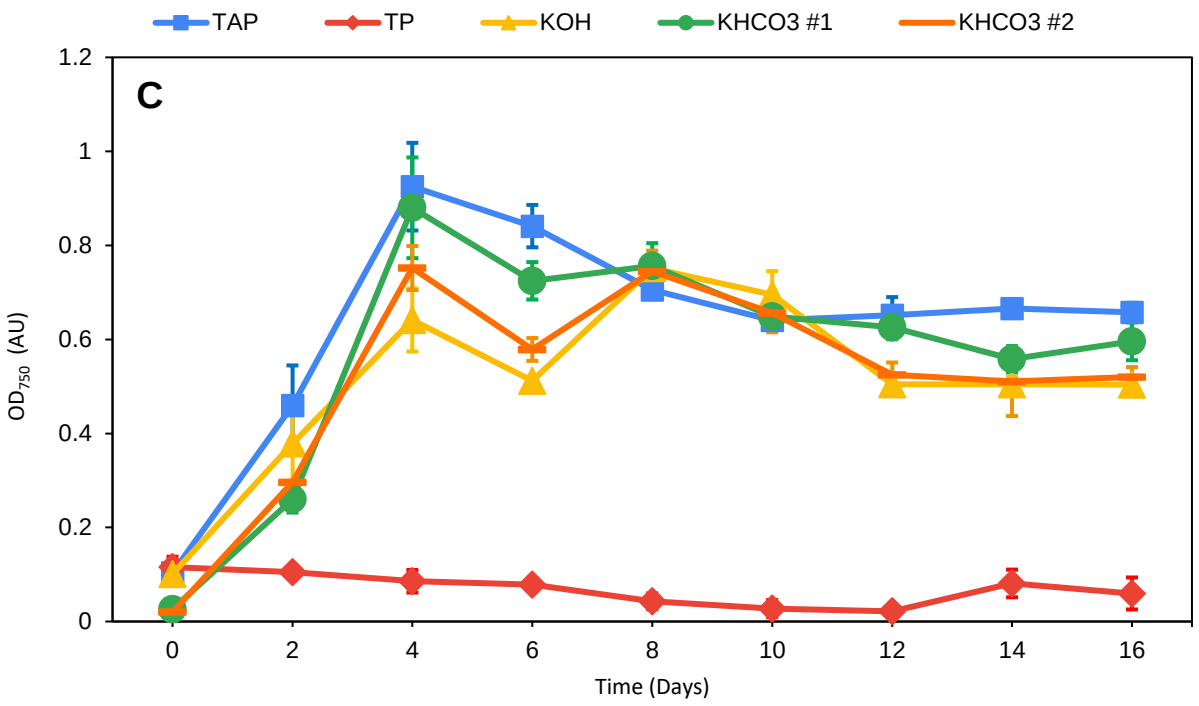


Fig.3 In short time frames, growing algae on lower effluent concentrations yields similar growth to higher concentrations.

Chlamydomonas grown in the dark after 16 days at different effluent and acetate concentrations (A) images, (B) optical density (OD_{750}), (C) cell counts, (D) dry biomass after 16 days of growth. Error bars indicate standard deviation between triplicates. (E-J) Microscopic images taken to examine cell clumping and morphology; TAP (E), TP (F), TP-Effluent (G), 75% TP-Effluent (H), 50% TP-Effluent (I), 25% TP-Effluent (J).


A
TAP
TP
KOH
KHCO3 #1
KHCO3 #2
Day 0
Day 4
TAP
TP
KOH
KHCO3 #1
KHCO3 #2
B
Cell Density (million cells mL-1)
Time (Days)
0
2
4
6
8
10
12
14
16


TAP
TP
KOH
KHCO3 #1
KHCO3 #2
C
1.2
1
0.8
0.6
0.4
0.2
0
OD750 (AU)
0
2
4
6
8
10
12
14
16
Time (Days)

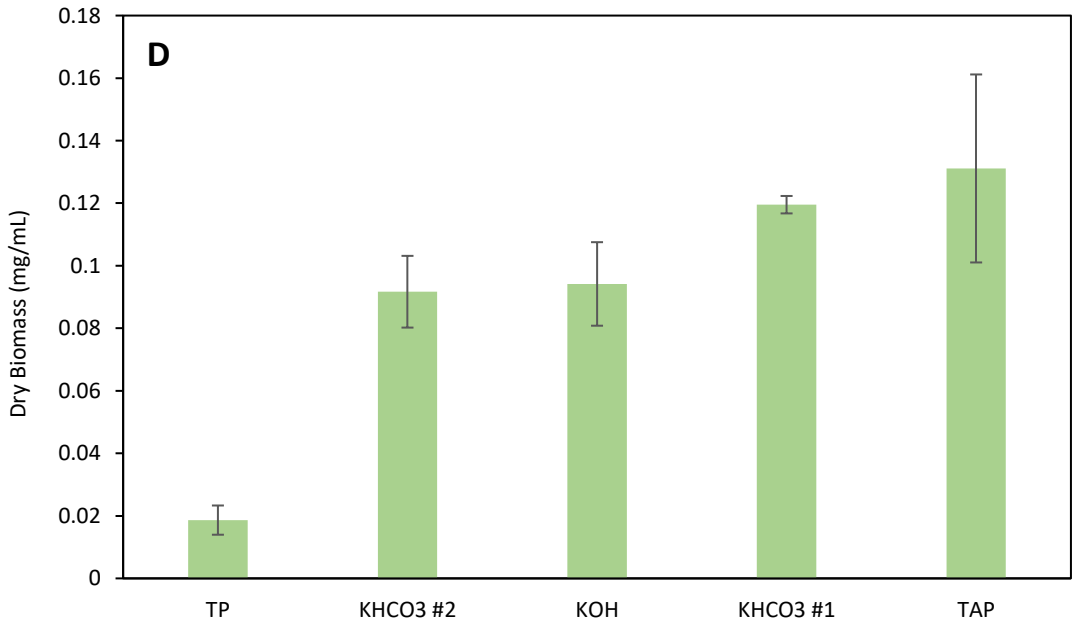

D
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Dry Biomass (mg/mL)
TP
KHCO3 #2
KOH
KHCO3 #1
TAP

Fig.4 Algae can grow heterotrophically with effluent as the sole carbon and energy source Chlamydomonas grown in the dark on various sample effluents produced, (A) images taken on day 0 and day 4, (B) optical density (OD_{750}) (C) cell counts (D) dry biomass after 16 days of growth. Error bars indicate standard deviation between triplicates.

DISCUSSION

The first growth of the algae with effluent proved to be unconvincing, with poor growth even in traditional photosynthetic conditions. There was also noticeable clumping of the cells inside the effluent flasks, suggesting cell stress. This indicated that there was a strong cytotoxic component in the media, and therefore in the simulated effluent as well.

In the drop-out experiment, after 16 days of growth, the results strongly suggested that the cytotoxic component in the effluent was potassium bicarbonate, or $KHCO_3$. In Figure 2A, the appearance of the culture grown in media with $KHCO_3$ omitted was similar to the positive control, TAP. This growth appeared to be lush and dark green, while the other flasks had relatively patchy growth. This could be, however, the result of inadequate mixing. The OD_{750} and cell count assays demonstrated similar findings, with the TAP and -$KHCO_3$, cultures growing considerably better than the other experimental cultures. For cell density, the results even suggested that the -$KHCO_3$ cultures grew better than standard TAP, although the errors for this proved to be very large. Dry biomass collected at the end of the growth period showed that the TAP, -$KHCO_3$ and -propanoic acid cultures grew best. This was unexpected considering the OD and cell count measurements. By analyzing microscopic images, the TAP and -$KHCO_3$ (Figures 2E-F) cells were dispersed and not clumped. All $KHCO_3$ containing cultures (Figures

2G-J) had both lower cell density and cell clusters. This reflected what was observed in the initial growth experiment.

For the media optimization experiment, it was found that for medias containing TP-Effluent, 75% TP-Effluent and 50% TP-Effluent, there were not significant differences in cell viability over the course of a 16-week growth period, shown in Figures 3B and 3C. The 25% TP-Effluent showed weaker growth, possibly because the cells were too quickly depleted of a carbon source, limiting their growth even when the presence of cytotoxic chemicals was reduced. The dry biomass measurement (Figure 3D) showed highly unexpected results, with the TP-Effluent having more biomass accumulation than even TAP. One explanation for this result is that the residual chemicals in the effluent were not properly washed away or evaporated during the dry biomass collection process. $KHCO_3$ is a salt that could have been retained in the dry biomass, which could explain the relatively large dry biomass measurement for the TP-Effluent cultures. Microscopic analysis showed that 75% TP-Effluent and 50% TP-Effluent cultures had the highest cell densities (Figures 3H and 3I). The TP-Effluent and 25% TP-Effluent (Figures 3G and 3J) cultures more closely resembled the negative control TP cultures, with poor growth relative to the other cultures. Over longer growth periods, there could be a larger difference between the different acetate concentration, but for the purposes of this experiment, it was discovered that using smaller doses of effluent was practical and could increase the use-efficiency of the costly effluent. This experiment in conjunction with the Drop-Out experiment also helped our collaborators at the University of Delaware understand how to optimize the chemical composition of the effluent.

For the growth experiment where the effluent produced from the electrocatalytic process was procured and incorporated into the media, heterotrophic growth of algae was demonstrated

successfully. Figure 4A shows that all three cultures grown with effluent media exhibit clear growth after 4 days. This growth is comparable to TAP, as shown in Figures 4B-D. It was also found that performing cell counts through hemocytometry, although more labor intensive, significantly decreased the errors between triplicates. From this final experiment, the first instance of algal growth completely decoupled from photosynthesis was achieved.

For future continuation of this project, the next steps are to optimize the growing process by media treatment or to employ the use of highly controlled bioreactors. The use of other algal species or other strains of *Chlamydomonas reinhardtii* can be considered as well. A more robust strain could have higher resistances to salts present in the effluent. By doing so, there is an opportunity to develop a system that exceeds the efficiency of conventional photosynthetic systems and be applied to agriculture for food and biotechnology industries such as biofuel production.

This project was presented as an online presentation at the 2021 Undergraduate Research and Creative Activity Symposium at the University of California, Riverside (Le et al. 2021).

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SUPPLEMENTAL MATERIALS

TAP medium production protocol

(Tris-Acetate-Phosphate medium)

Gorman, D.S., and R.P. Levine (1965) *Proc. Natl. Acad. Sci. USA* **54**, 1665-1669.

Use: Freshwater medium used for the culture of *Chlamydomonas*. Contains acetate as a fixed carbon source which allows for fast growth of *Chlamydomonas* strains.

Prepare*:* Fill media bottle to ~80% of the final volume with DI water. Add the Tris base and then each stock solution. When adding Glacial Acetic Acid, wash pipette to ensure all is transferred. Adjust pH to 7.20 with HCl (Usually the pH should be close to 7.20 or slightly higher around ~7.3-7.4). Dilute to final volume. Autoclave.

Volume of TAP Medium desired: 1000 ml

Volume of TAP Desired	5000	4000	2000	1500	1000	500	100	ml
Tris base	12.1	9.68	4.84	3.63	2.42	1.21	0.242	g
TAP salts	125	100	50	37.5	25	12.5	2.5	ml
Phosphate Solution	1.875	1.5	0.75	0.5625	0.375	0.1875	0.0375	ml
Hutner's Trace Elements	5	4	2	1.5	1	0.5	0.1	ml
Glacial Acetic Acid	5	4	2	1.5	1	0.5	0.1	ml
Distilled water	4863	3891	1945	1459	973	486	97	ml
Agar (if needed 1%)	50	40	20	15	10	5	1	g

TAP Stock Solutions	grams needed		Stock Conc (g/l)	Media Conc (g/l)	Media Molarity (mmole/l)
TAP Salts	1000	ml			
NH_4Cl	15	g	15	0.375	7.01
$MgSO_4 \cdot 7H_2O$	4	g	4	0.1	0.41
$CaCl_2 \cdot 2H_2O$	2	gr	2	0.05	0.34
Phosphate Solution	100	ml			
K_2HPO_4	28.8	g	288	0.108	0.62
KH_2PO_4	14.4	g	144	0.054	0.40
Hutner's trace elements	1000	ml			
$ZnSO_4 \cdot 7H_2O$	22	g in 250 ml H2O	22	0.022	0.0765
H_3BO_3	11.4	g in 100 ml H2O	11.4	0.0114	0.1844
$MnCl_2 \cdot 4H_2O$	5.06	g in 200 ml H2O	5.06	0.00506	0.0256
$CoCl_2 \cdot 6H_2O$	1.61	g in 50 ml H2O	1.61	0.00161	0.0068
$CuSO_4 \cdot 5H_2O$	1.57	g in 50 ml H2O	1.57	0.00157	0.0063
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.1	g in 50 ml H2O	1.1	0.0011	0.00089
$FeSO_4 \cdot 7H_2O$	4.99	g in 50 ml H2O	4.99	0.00499	0.0179
Na_2 EDTA	50	g in 50 ml H2O	50	0.05	0.1487

To Make Hutner's

From Chlamy Sourcebook: Mix all solutions except EDTA. Boil mixture, add EDTA. Cool to 70°C. Adjust pH to 6.7 with hot KOH (20 wt%). Bring solution to 1 liter. Initial color of the solution is green. Solution will gradually turn purple and leave a brown precipitate which can be removed by filter paper.

Dry Biomass Protocol

1. Label weigh boats with replicate number(s)
2. Record weight of each boat using the 4 decimal places scale (sliding door)
3. Transfer remaining culture from flasks into 50 mL conical using 50 mL and record volume transferred for each.
4. pH each conical before spinning down
5. Centrifuge at 3500 rpm for 5 minutes
6. Remove supernatant and resuspend in 1.0 mL MQ Water pH supernatant
7. Centrifuge again at 3500 rpm for 5 minutes at 4°C
8. Remove supernatant and resuspend in 0.5 mL MQ Water
9. Transfer to corresponding weigh boat
10. Spray once with 70% Ethanol into the conical to remove cells stuck to walls, and transfer to corresponding weigh boat.
11. Dry in oven at 80°C overnight ~ 12 hours
12. Weigh boats and subtract weigh boat mass from total mass for dry biomass