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Removal of dissolved organic carbon and nutrients from urban wastewaters by *Galdieria sulphuraria*: Laboratory to field scale demonstration

S.M. Henkanatte-Gedera [a], T. Selvaratnam [b], M. Karbakhshravari [a], M. Myint [a], N. Nirmalakhandan [a,*], W. Van Voorhies [c], Peter J. Lammers [b]

[a] Civil Engineering Department, New Mexico State University, Las Cruces, NM 88003, United States
[b] School of Sustainable Engineering and the Built Environment, Arizona State University, Mesa, AZ 85212, United States
[c] Molecular Biology Program, New Mexico State University, Las Cruces, NM 88003, United States

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ABSTRACT

Previous laboratory studies have demonstrated the ability of microalgae *Galdieria sulphuraria* (*G. sulphuraria*) in removing organic carbon and nutrients from filtered primary-settled urban wastewater via mixotrophic metabolism. An advantage of mixotrophic cultivation of *G. sulphuraria* over heterotrophic conditions is higher biomass yield that can potentially translate into higher energy recovery from the biomass. This study recorded a yield of 0.63 g biomass/g glucose under mixotrophic conditions while that under heterotrophic conditions was 0.42 g biomass/g glucose. These laboratory studies were extended to cultivate *G. sulphuraria* under field conditions in a 700 L photobioreactor (PBR) fed with primary-settled wastewater. Biomass growth and removal of dissolved organic carbon and nutrients in this PBR under batch mode were monitored over a range of influent and operating conditions. This field study confirmed that *G. sulphuraria* was able to grow well in primary-settled wastewater and reduce organic carbon (measured as BOD_5), ammoniacal nitrogen, and phosphate levels to below the respective discharge standards; corresponding 3-day removal efficiencies ranged 46–72%; 63–89%; and 71–95%.

1. Introduction

Galdieria sulphuraria (abbreviated as *G. sulphuraria*) is unicellular red alga that has the flexibility to grow autotrophically, mixotrophically and heterotrophically [1,2]. This metabolic versatility makes it an ideal choice for removing dissolved organic carbon and nutrients from liquid waste streams such as urban wastewater (UWW), with the potential for treating the wastewater to mandated discharge levels. As such, *G. sulphuraria* is a promising strain for single-step urban wastewater treatment instead of the current two-step practices of secondary treatment followed by tertiary treatment. Current secondary treatment by activated sludge organisms converts nearly 50% of the dissolved organic carbon (DOC) in the wastewater to carbon dioxide [3]. An algal system can theoretically oxidize the DOC in wastewater to CO_2 and recapture the metabolic CO_2 via photosynthesis. Thus, algal wastewater treatment would produce significantly more energy-rich biomass than current methods. Technologies are currently available to generate fuels from algal biomass [4–6]. Mixotrophically derived CO_2 from respiration and O_2 from photosynthesis would also significantly reduce the total metabolic gas supply requirements relative to current secondary and tertiary processes. Based on the above, it is hypothesized that mixotrophic wastewater treatment using *G. sulphuraria* could be employed for energy-positive urban wastewater treatment.

Our previous reports on *G. sulphuraria* have demonstrated the following under laboratory conditions: i) growth rate of *G. sulphuraria* in filter-sterilized primary effluent was comparable to that in the standard growth medium confirming its adaptability to wastewater [7]; ii) biomass yield per unit ammoniacal-nitrogen of *G. sulphuraria* is about 10% higher than the average reported in the literature for other algal strains [7,8]; iii) removal of dissolved organic carbon (measured as biochemical oxygen demand, BOD) from primary effluent by *G. sulphuraria* is comparable to that reported in the literature [7–9]. Even though the above findings were based on laboratory experiments using filter-sterilized primary effluent, they support the premise that *G. sulphuraria* offers good potential for energy-positive, single-step urban wastewater

* Corresponding author.
E-mail address: nkhandan@nmsu.edu (N. Nirmalakhandan).

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Table 1
Summary of test conditions.

Test	Lab/field	Growth medium[a]	Test volume	Initial pH
I	Lab	MCM in DI water	6 mL	1.0, 2.5, 4.0
II	Lab	MCM in DI water	6 mL	2.5
		Glucose	6 mL	2.5
		Sucrose	6 mL	2.5
III	Field	MCM in UWW	700 L	2.5
IV	Field	MCM in UWW	700 L	2.5, 4.0
V	Field	MCM in UWW	700 L	2.5
		UWW	700 L	2.5

[a] MCM- modified Cyanidium medium; DI- deionized water; UWW- primary-settled urban wastewater.

treatment. The current study was undertaken to extend the preliminary laboratory findings and validate the above premise by evaluating growth of *G. sulphuraria* on primary effluent at pilot scale, under field conditions.

1.1. Algal systems for wastewater treatment

Previous studies have reported on cultivation of algae in urban and agricultural wastewaters utilizing the traditional open raceway-type pond configuration [10,11]. While the raceway configuration has served well for growing biomass for producing high-value end products, its application to urban wastewater treatment is limited due to the following. Traditional raceways are driven by paddlewheels to maintain the cultures in suspension; and sparged with CO_2-enriched air (CEA) to provide the carbon needs to the cultures. To ensure adequate sunlight penetration in such raceways, the culture depth has to be shallow (<0.4 m) and achievable cell density is limited by light availability to values typically <0.8 g L^{-1} [12,13]. Both of these constraints have adverse implications. Shallow depths translate to larger footprint, resulting in excessive water loss by evaporation. Low biomass densities translate to inefficient downstream solids-liquid separation and higher costs of recovery. Other drawbacks of open raceways include high potential for contamination, predation by invaders, and release of offensive odors.

In this study, an enclosed photobioreactor (PBR) configuration is proposed that is specifically designed for cultivating *G. sulphuraria* in warm-humid and warm-arid environments. By taking advantage of solar heat gain in the enclosed photobioreactors, temperatures in the photobioreactors fall within the range of values tolerated by *G. sulphuraria* cultures [9]. Strains of *G. sulphuraria* with lower temperature optima than strain CCMEE 5587.1 used here could theoretically be used for cooler seasons or locations [2]. The enclosed PBR design circumvents most of the limitations noted above: minimizes evaporative water loss, odor emissions, and potential for contamination and invasion; and maximizes CO_2 utilization. Since *G. sulphuraria* is capable of mixotrophic metabolism, greater culture depths and higher biomass densities than those in the traditional raceways can be maintained to minimize footprint and improve separation of solids.

A unique feature of *G. sulphuraria* is that it is an acidophilic strain that grows well in the pH range of 1.0 to 4.0. This is can be beneficial in urban wastewater treatment, controlling pathogens in the feed while minimizing contamination and invasion by common parasites. However, this requires the pH of the feed to the PBR be lowered to maintain the optimal pH in the PBR. Oesterhelt et al. [14] had demonstrated *G. sulphuraria*'s ability to naturally acidify its growth medium under both heterotrophic and phototrophic conditions. Here we report additional laboratory and field results on the mixotrophic growth of *G. sulphuraria* on simple sugars, in settled primary wastewater, and its ability to remove organic carbon and nutrients as a function of initial pH.

2. Materials and methods

The growth rate of *G. sulphuraria* was first evaluated in the laboratory under varied temperature, pH and metabolic substrate conditions.

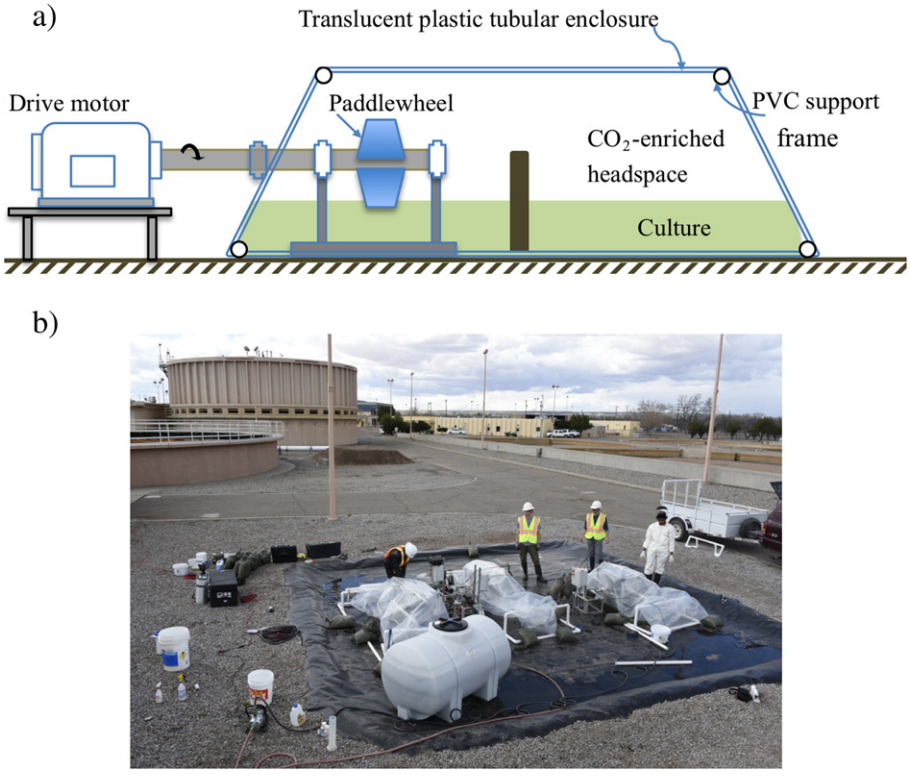

Fig. 1. a. Schematic of enclosed photobioreactor developed for field demonstration b. Field installation of 3 × 700 L enclosed photobioreactors at the Las Cruces Wastewater Treatment Plant, fed with primary effluent.

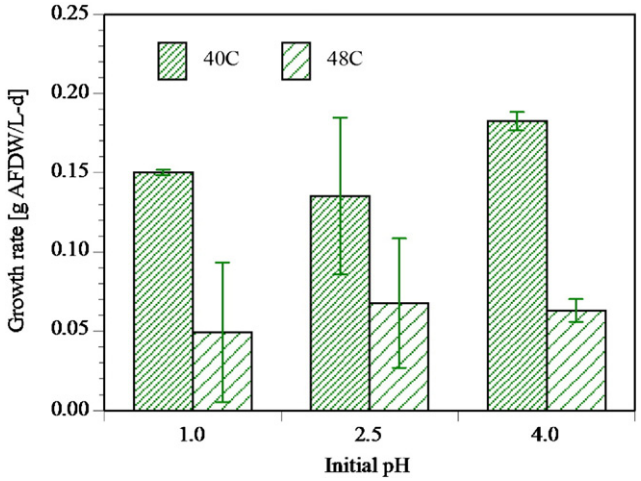

Fig. 2. Results of Test I: growth rate of *Galdieria sulphuraria* under laboratory conditions in modified Cyanidium medium as a function of initial pH (of 1.0, 2.5, and 4.0) and cultivation temperature (of 40 °C and 48 °C).

For these experiments *Galdieria sulphuraria* (CCMEE 5587.1) obtained from Culture Collection of Microorganisms from Extreme Environments (University of Oregon) was grown in 16 mm glass tubes. Algal growth was analyzed based on optical density measurements at 750 nm (OD 750) with a Beckman DU-530 UV/Vis spectrophotometer. Over the course of the experiment the optical density of the algal cultures increased beyond the linear range of the spectrometer. A high OD conversion factor was generated by measuring the OD in dense tube cultures and then diluting the culture in this tube down to achieve an OD of <0.5. This was considered the actual OD of the tube. The relationship between high OD and actual OD was approximated by a 3rd order polynomial ($r^2 > 0.99$). These conversion equations were applied when OD750 readings were >0.7. Five different tests were conducted in this study as summarized in Table 1 and detailed below. Triplicate samples were analyzed for OD, BOD, N, and P in each test, from which the standard deviation was calculated and included in the plots as error bars.

2.1. Test I — effect of initial pH and temperature on growth of G. sulphuraria

The goal of Test I was to assess the growth of *G. sulphuraria*, under laboratory conditions, at three initial pH values (of 1.0, 2.5, and 4.0) and at two temperatures (of 40 °C and 48 °C) using three replicates per treatment. This test was conducted with the standard Cyanidium medium [15] was modified as follows: ammonium sulfate and potassium phosphate concentrations were increased 2-fold relative to the standard medium; the medium was supplemented with the vitamin component of f/2 algal medium [16]. The initial pH of the Cyanidium medium was 2.5; to set the test pH values of 1.0 and 4.0, the pH of the modified CM medium was adjusted with 10 N H_2SO_4 or 1 M NaOH. The final composition of the modified CM used to grow the stock cultures was as follows: $(NH_4)_2SO_4$, 2.64 g L^{-1}; KH_2PO_4, 0.27 g L^{-1}; NaCl, 0.12 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.25 g L^{-1}; $CaCl_2 \cdot 2H_2O$, 0.07 g L^{-1}; Nitch's Trace Element Solution, 0.5 mL; $FeCl_3$ (solution = 0.29 g L^{-1}), 1.0 mL L^{-1}.

Cultures were grown in 500 mL flasks on a rotary shaker at 40 °C prior to beginning the growth measurements. To ensure that the cultures were in log growth phase at the start of the growth experiments the cultures were diluted to an OD750 of ~0.5 two days prior to starting the experiments and diluted again the day before starting the growth experiment. At the start of the growth measurements the flask culture was diluted down 10-fold with medium at pH 2.5 or 4. A portion of this culture (5.5 mL) was then added to 16 mm glass tubes, which were then capped and parafilmed. Three tube cultures were started for each strain at each temperature and pH. Tube cultures placed in outer ring of rollordrum (New Brunswick Scientific) set to 10 rpm. Cultures were grown in separate Percival incubators at either 40 or 48 °C with constant illumination. Incubator temperatures were monitored with a 15-minute resolution with temperature data loggers (Onset Hobo). CO_2 levels in the incubators were controlled between 1.5 and 2% by flowing in a controlled volume of CO_2 and monitoring levels with Licor 6251 CO_2 analyzer. The cultures were illuminated with fluorescent lights with a PAR intensity of ~150 μmol s^{-1} m^{-2} at the edge of the culture tubes.

2.2. Test II — effect of growth medium on growth of G. sulphuraria

Since all the previous studies were conducted with modified Cyanidium medium, this test was run, under laboratory conditions, to verify if *G. sulphuraria* could be cultivated on carbon-rich media mimicking urban wastewaters. Three different growth media were evaluated in this test: 1) Cyanidium medium enriched 2× in N and P with F/2 vitamins. 2) Glucose: Amresco Biotechnology grade lot # 1393B24-added at 4.5 g L^{-1}, final pH = 2.48; 3) Sucrose: MP Biochemicals lot # R19184 added at 4.5 g L^{-1} final pH = 2.58. These tests also evaluated

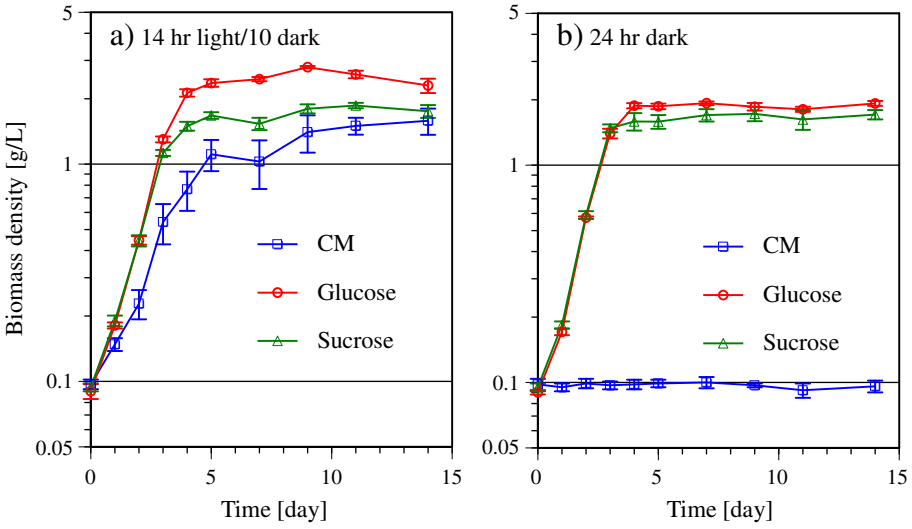

Fig. 3. Results of Test II: growth of *Galdieria sulphuraria* under laboratory conditions as a function of growth substrate (Cyanidium medium (CM), glucose, and sucrose). a) 14 h light/10 h dark cycle; b) 24 h dark.

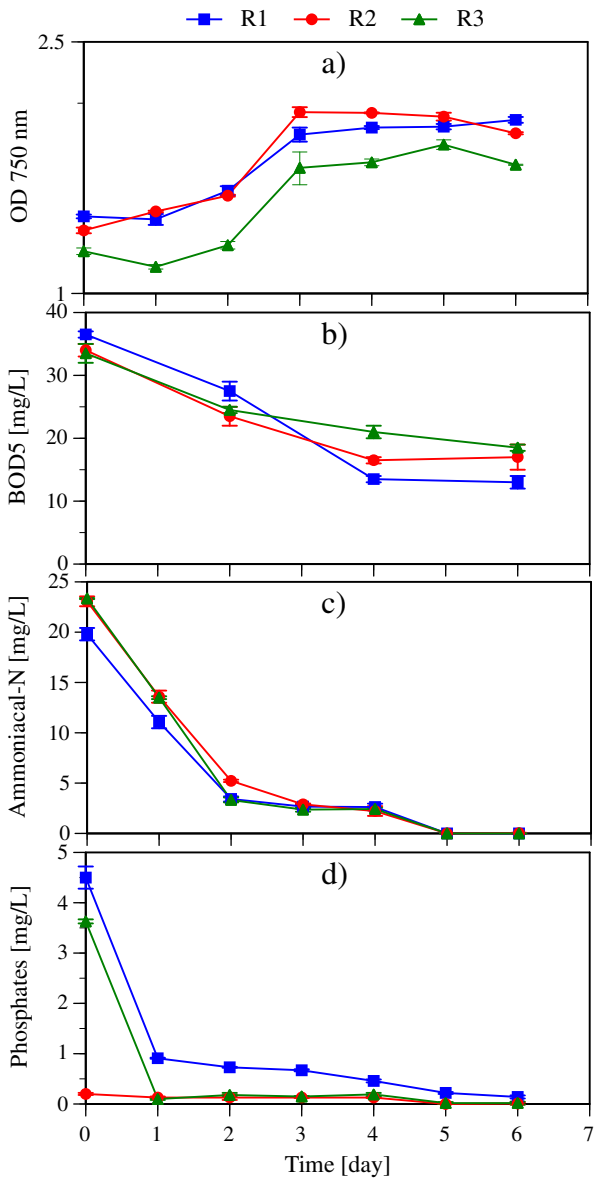

Fig. 4. Results of Test III: cultivation of *G. sulphuraria* under field conditions in primary-settled wastewater supplemented with modified Cyanidium media components. Temporal profiles of: a) biomass; b) BOD_5; c) nitrogen; and d) phosphates.

the viability of *G. sulphuraria* in the above three media under both 14 h light/10 h dark cycle (mimicking outdoor conditions) and dark conditions to check heterotrophic metabolic capability of *G. sulphuraria*.

Two roller drums (New Brunswick) were placed in Percival incubators set at 40 °C; one set was illuminated on a 14 h light/10 h dark cycle, while the other set was run under dark conditions. CO_2 levels in the lighted incubator were adjusted by running in a controlled flow of CO_2 enriched air. CO_2 levels were analyzed continuously with a CO_2 analyzer and kept between 1.5 and 2%. Percival incubators had 4 side lights on and 2 light fixtures (2 bulbs each) were suspended above the roller drum. The measured light was ~300 μmol s^{-1} m^{-2} at the edge of the tubes on both the tops and sides of the drum. The dark set did not receive any CO_2 enrichment.

2.3. Test III — feasibility of cultivating G. sulphuraria under field conditions

The goal of Test III was to verify the ability of *G. sulphuraria* to grow in a primary effluent of UWW and assess its BOD, N, and P removal capabilities under field conditions. The *G. sulphuraria* culture used for the field experiments was started from axenic colonies grown on agar plates. Colonies were transferred to Cyanidium growth medium in 100 mL flasks. This culture was then serially scaled up to 250 mL, 2 L and 20 L volumes. The 20 L culture was used to inoculate the outdoor PBRs containing Cyanidium growth medium mixed with potable municipal water. After the cultures grew to a biomass density of approximately 1 g L^{-1}, these cultures were added to wastewater medium and pre-adapted for 5–7 days before starting the experimental runs. Five serial batch culture runs were conducted under outdoor conditions for Tests III–V.

Test III was conducted in three identical enclosed PBRs (R1, R2, and R3) deployed at the Las Cruces Wastewater Treatment Plant in Southern New Mexico. These PBRs were fabricated out of translucent polyethylene tubes laid horizontally with a central berm forming a closed raceway configuration, supported internally by a skeleton fabricated of PVC pipes as shown schematically in Fig. 1a. A motor-driven paddlewheel installed in the middle of each bag on one side of the berm provided circulation of the cultures. The operational batch-volume was 700 L, initiated with 400 L of wastewater and 300 L of pre-adapted cultures. The headspace was filled with 2% CO_2-enriched air. Fig. 1b shows the PBRs installed at the Las Cruces Wastewater Treatment Plant.

The standard Cyanidium recipe for *G. sulphuraria* [7] was modified in this test as follows: deionized water of the standard growth medium was replaced with raw primary effluent; and, ammonium sulfate and mono-potassium phosphate from the standard recipe were excluded as wastewater contained adequate N and P for biomass growth. The pH level of the media was adjusted to 2.5 by adding 10 N sulfuric acid.

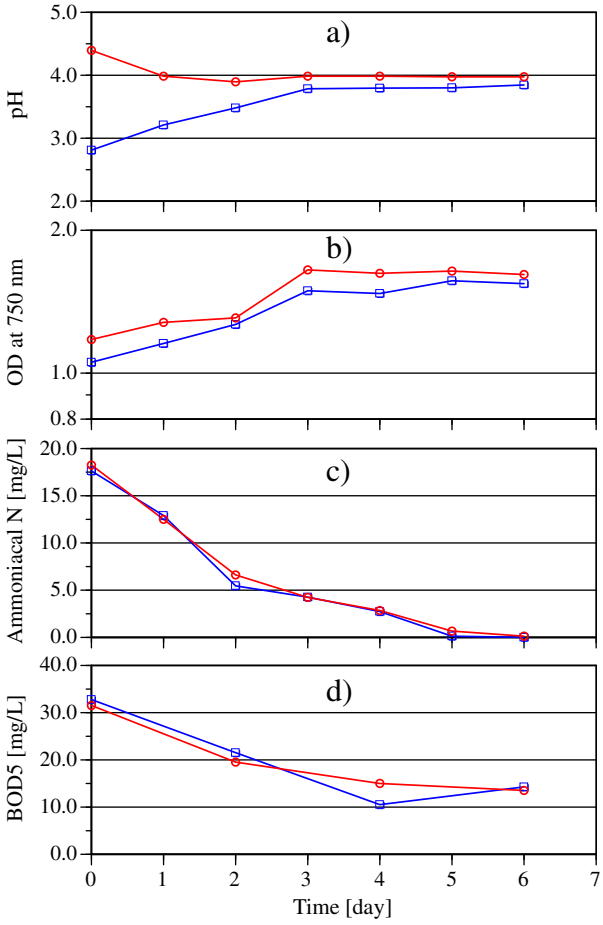

Fig. 5. Results of Test IV: effect of initial pH of 2.8 (□) and initial pH of 4.5 (○) on the performance of *G. sulphuraria* cultivated under field conditions in primary-settled wastewater supplemented with modified Cyanidium media components.

Cultures preadapted to primary effluent were added to three 700 L PBR's filled with primary effluent mixed with modified Cyanidium media in 3:4 volume-to-volume ratio.

Daily samples drawn over a period of six days were centrifuged and the supernatant was analyzed for ammoniacal-nitrogen, phosphates, and dissolved BOD_5. Ammoniacal nitrogen and phosphates were measured with HACH DR 6000 spectrophotometer with salicylate TNT method 10031 and Phosver 3 method 8048. Dissolved BOD_5 was measured following Standard Method 5210 B. Field measurements of temperature and dissolved oxygen were logged at 15 minute resolution with Onset HOBO data loggers (UA -002-64) and YSI DO probe ProODO DO_2 optical sensor, respectively.

2.4. Test IV — effect of initial pH on biomass growth and BOD_5/nitrogen removal

The goal of Test IV was to assess the effect of initial pH on biomass growth and nutrient removal capabilities of *G. sulphuraria* under field conditions. Based on the initial laboratory tests conducted over a range of initial pH values, it was envisaged that *G. sulphuraria* could lower pH levels from 4.0 to 2.5 under field conditions as well. To verify this, one reactor was initiated at a pH of 4.0 while another reactor was initiated with pH of 2.5. This test lasted six days. Samples were analyzed for ammoniacal-nitrogen, and phosphates every day; and for BOD_5 every two days.

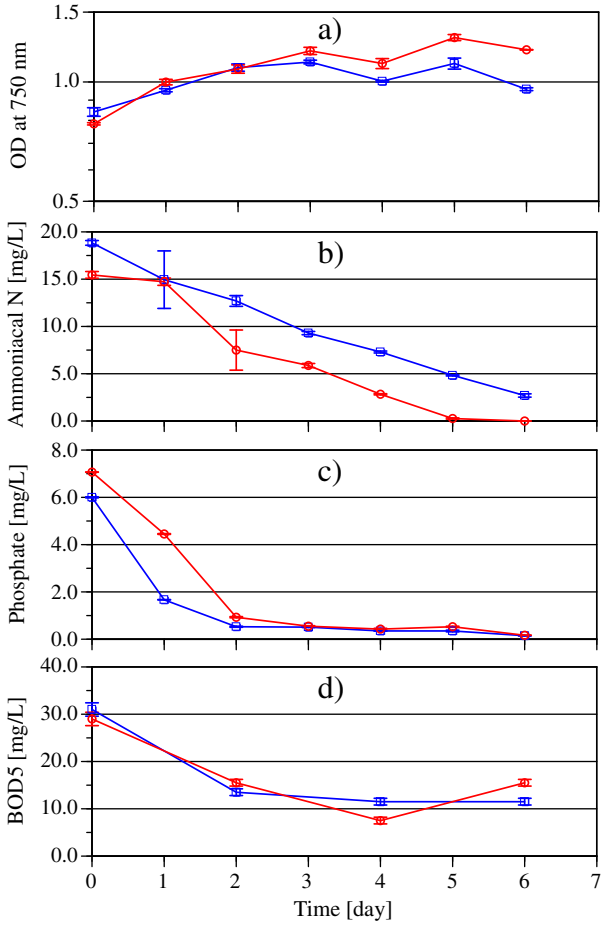

Fig. 6. Results of Test V: performance of *G. sulphuraria* under field conditions, in raw primary-settled wastewater (□) vs. in primary wastewater supplemented with modified Cyanidium media components (○).

2.5. Test V — growth of G. sulphuraria in raw primary effluent

All of the previous tests had utilized the standard Cyanidium medium with suitable modifications. The goal of Test V was to evaluate the growth of *G. sulphuraria* in raw primary effluent without addition of any supplements and assess its removal capabilities against those in modified Cyanidium medium prepared with primary effluent, excluding the N and P components of the recipe. In both cases, the initial pH was adjusted to 2.5 by adding 10 N sulfuric acid.

3. Results and discussion

3.1. Test I — effect of initial pH and temperature on growth of G. sulphuraria

Five-day growth rates as a function of initial pH and cultivation temperature were determined from the observed biomass growth profiles. A liner regression procedure was applied for each replicate for the linear phase (days 2 through 6) to determine the growth rates; R^2 values of these regressions ranged 0.93–0.99. Based on the comparison of the averaged growth rates (shown in Fig. 2), it is concluded that the initial pH does not impact the growth rate significantly. This conclusion has practical implications in minimizing cost of pre-adjustment of pH of the feed to the cultivation system. Irrespective of initial pH, the 5-day growth rates at 40 °C are found to be higher than those at 48 °C.

3.2. Test II — effect of growth medium on growth of G. sulphuraria

Test II compares the growth of *G. sulphuraria* with glucose and sucrose as substrates relative to photoautotrophic control cultures grown on the modified Cyanidium medium.

Glucose and sucrose were selected in this study following a previous study [17] that had reported on another *G. sulphuraria* strain, 074G, that could utilize sucrose; as such, the intent of this test was to ascertain if strain CCMEE 5587.1 would as well because, if so, this would enable large-scale outdoor mixotrophic growth trials using less expensive sucrose.

Results of Test II recorded under 14 h light/10 h dark conditions are shown in Fig. 3a; results recorded under dark conditions are shown in Fig. 3b. Fig. 3a shows enhanced growth of *G. sulphuraria* with organic substrates over the modified Cyanidium medium under autotrophic conditions (0.45 and 0.32 g L^{-1} day^{-1} with glucose and sucrose, respectively, vs. 0.02 g L^{-1} day^{-1} with Cyanidium medium). While no growth was noted with Cyanidium medium in the absence of light as expected, slightly higher mixotrophic growth rate was noted with glucose than with sucrose (0.36 vs. 0.30 g L^{-1} day^{-1}), resulting in a lower final cell density with sucrose. While further study is required to explain this observation, it clearly suggests that glucose is a preferred carbon source relative to sucrose. A hierarchy of substrate preferences has been established for *G. sulphuraria* that also demonstrated the need for induction of alternative carbon source utilization pathways [18]. Biological oxygen demand in urban wastewater is the sum of many different carbon compounds. The potential for oxidation of the full range of organic molecules present in wastewater BOD by *G. sulphuraria* will require additional studies [19].

The mixotrophic conditions used here led to higher yields than pure heterotrophic growth. This is best illustrated by substrate yields, g-biomass/g-glucose, derived from the data in Fig. 3. The day 9 mixotrophic biomass yield (= biomass produced/substrate consumed) was nearly 50% higher over heterotrophic yield (0.63 vs. 0.42). An identical heterotrophic substrate yield was reported by Graverholt et al. for *G. sulphuraria* grown in airlift fermentation system [20]. In contrast, two published reports suggest near complete catabolic repression of photosynthesis by glucose in *G. sulphuraria* cultures. [14,21]. Those reports were carried out under low light, low cell density and room temperature conditions. Our experiment was carried out at the near optimal temperature of 40–48 °C for the *G. sulphuraria* strain used in this study

with sufficient light to achieve a photoautotrophic cell density of 1.5 g ash-free dry weight per liter. The importance of these results lies in the demonstration that *G. sulphuraria* is able to oxidize organic carbon and reduce CO_2 via photosynthesis in the same culture. The catabolic repression of photosynthesis in *G. sulphuraria* needs further investigation to identify the underlying mechanism. It will be important to identify conditions that allow for maximal BOD oxidation coupled to photosynthetic CO_2 fixation in order to maximize net energy yields from algal wastewater treatment.

3.3. Test III — feasibility of cultivating G. sulphuraria under field conditions

Temporal variation of biomass growth, BOD_5, and nutrients recorded in Test III in the three PBRs under field conditions are shown in Fig. 4. During this test, culture temperature ranged 31–50 °C. The cultures grew with minimal lag phase, reaching a maximum density of 1.9 OD 750 nm in 6 days, by which time, both N and P had been reduced to negligible levels. On day 3, *G. sulphuraria* had achieved maximum growth rate of 0.3–0.5 OD 750 nm day^{-1} at a density of 1.7–1.9 OD 750 nm, by which time, BOD had been reduced from 36 to 13 mg L^{-1} (>64%), N from 23 to 2.6 mg L^{-1} (>86%), and P from 4.5 to 0.6 mg L^{-1} (>85%). These results are comparable to the previous laboratory results, validating the premise that *G. sulphuraria* could be a viable strain for UWW treatment under field conditions. Further BOD removal may require an extended adaptation period as noted above.

3.4. Test IV — effect of initial pH on biomass growth and BOD_5/nitrogen removal

Temporal profiles of pH, biomass growth, nitrogen, and BOD_5 recorded in Test IV are shown in Fig. 5. During these tests, the culture temperatures ranged 31–50 °C. It can be noted from Fig. 5(a) that *G. sulphuraria* was able to self-adjust the pH to a stable value of ~4 within 3 days. Fig. 5(b) shows that growth is not significantly impacted by the initial pH as was noted under laboratory conditions (Test I). Similarly, reduction of BOD and ammoniacal-N also are not impacted by the initial pH. These field results are in agreement with the report by Oesterhelt et al. [14] that doubling time of *G. sulphuraria* is not significantly impacted over an initial pH range of 1 to 5. This validation under field conditions is of significant utility value because of the potential savings in chemical costs associated with pH adjustment of real waste streams. The increase in pH observed in the test started at 2.8 is somewhat surprising. Oesterhelt et al. [14] has previously shown *G. sulphuraria* cultures decreasing pH in response to growth at various starting pH values. This can be interpreted as a result of ammonium ion uptake with subsequent stoichiometric release of one proton per ammonium ion assimilated [22]. The same authors show that nitrate assimilation will increase pH and it is possible the pH increase observed in Fig. 5 reflects a balance of ammonium versus nitrate assimilation; however, our study is not able to corroborate this since nitrate levels were not recorded in this study.

3.5. Test V — growth of G. sulphuraria in raw primary effluent

Temporal profiles of biomass growth, ammoniacal-N, phosphate, and BOD_5 recorded during the Test V are shown in Fig. 6. During these tests, culture temperature ranged 24–36 °C. The profiles in Fig. 6(a) show that growth in raw settled wastewater is comparable to that in the modified Cyanidium medium. Removal of nutrients and BOD_5 are also not significantly impacted when *G. sulphuraria* is cultivated in raw settled wastewater. This finding is also of significant utility value because of the high costs associated with media supplements. Considering that this process does not require oxygen input or any supplementary chemical input, and that this process meets the mandatory discharge standards for nutrients and BOD_5 in a single step, this process offers a more sustainable pathway for wastewater treatment than the current practice.

4. Conclusions

This study demonstrated the feasibility of removing organic carbon and nutrients from primary-settled urban wastewater by a mixotrophic strain, *Galdieria sulphuraria,* under field conditions. Guided by preliminary laboratory studies, a closed photobioreactor configuration was developed to cultivate *G. sulphuraria* and tested at a local urban wastewater treatment plant in batch mode, under varying influent and operating conditions. The field system was able to reduce organic carbon and nutrients in the feed to the required discharge standards in a batch process time of 2 days. Because of its mixotrophic metabolism, *G. sulphuraria* offers an alternate pathway for wastewater treatment that can conserve the energy used by the current aerobic process. Results of this study also demonstrated nearly 50% more biomass yield under mixotrophy than under pure heterotrophy; this can be of significant benefit in downstream biomass-to-biofuel conversions.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2016.08.001.

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