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Chlorella minutissima—A Promising Fuel Alga for Cultivation in Municipal Wastewaters

Ashish Bhatnagar · Monica Bhatnagar · Senthil Chinnasamy · K. C. Das

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Abstract It is imperative to slash the cost of algal oil to less than \$50 bbl^{-1} for successful algal biofuel production. Use of municipal wastewater for algal cultivation could obviate the need for freshwater and the nutrients—N and P. It would also add CO_2 through bacterial activity. *Chlorella minutissima* Fott et Nova dominated the entire phycoflora year around and through each stage of the wastewater treatment at the oxidation pond system of Wazirabad (Delhi) in India. The ability to grow so profusely in such varied and contrasting situations made this alga unique. Besides pollution tolerance, it grew heterotrophically in dark under acidic conditions and as a mixotroph in presence of light over a range of organic C substrates. It could utilize both ammoniacal and nitrate nitrogen, survived anaerobicity, 5% NaCl and −10 bar of osmotic stress. *C. minutissima* grew at pH 4–11 and raised the pH set initially by 1 to 3 units in 7.5 h. It showed gigantism and largely kept afloat in presence of utilizable organic carbon, while flocculated in mineral medium and on aging. The alga also possessed potential for biofuel production. The studied parameters indicate why *C. minutissima* was a potential biomass builder in municipal sewage and could be used to determine which other alga(e) may serve the purpose.

Keywords Anaerobiosis · Biofuel · *Chlorella minutissima* · Mixotrophy · Wastewater

Introduction

Algae are considered potent feedstock candidates for biofuels as they may be produced locally on non-arable lands. However, their high cost of production needs to be lowered by ~20–25-folds to ensure economic viability. Apart from sunlight and CO_2, water, nitrogen,

A. Bhatnagar (✉) · S. Chinnasamy · K. C. Das
Biorefining and Carbon Cycling Program, Department of Biological and Agricultural Engineering,
The University of Georgia, Athens, GA 30602, USA
e-mail: bhatnagarashis@gmail.com

M. Bhatnagar
Satellite Centre for Microalgal Biodiversity in Arid Zones of Rajasthan, Department of Microbiology,
Maharshi Dayanand Saraswati University, Ajmer 305 009, India

and phosphorus are the three major inputs for algae cultivation. Major nutrients such as N and P alone contribute to ~10–20% of production cost of algae biomass [1]. Recent hike in fertilizer prices would have further increased this share. On the other hand, depleting freshwater resources are drifting nations to the brink of war.

Human beings generate ~3 billion ton of domestic wastewater every year [2]. One person can produce as much nutrients as necessary for his/her food needs [3]. Yet, continuous application of municipal wastes for agriculture is not possible due to health concerns and over the period of time, soils become toxic to the crops themselves. Many algae thrive in municipal wastewaters that are an integral part of all cities of the world having sewer systems. Use of municipal wastewater to grow algae might obviate the need for freshwater and would also curtail or eliminate fertilizer requirement for the purpose. Not only this, continuous bacterial activity in the wastewater could supply CO_2, in addition to what is available through atmosphere. And besides valuable algal biomass, the process would churn out reusable water free from pollutants. Therefore, as a near-term solution, algae cultivation for biofuels needs to focus itself on the utilization of municipal wastewaters to reduce the cost.

However, not all algae can grow in municipal wastewaters. At the inlet of a treatment system, this wastewater shows excess of nutrients, dissolved salts and toxicants, and negligible to nil oxygen and light penetration. Fluctuations in the quality and quantity further aggravate the environmental harshness. The only advantage this habitat provides is the reduction of competition. Therefore, the organisms that tolerate such conditions are able to dominate the scenario. It is, therefore, essential to look for algae that possess a kitty of special attributes to grow in municipal wastewaters.

Chlorella minutissima Fott et Nova was reported contributing 45.6% (raw sewage) to 65% (treated water) to the total phytoplankton count throughout the year at Nehru Vihar Oxidation Pond System (OPS) at Delhi, India [4], and removed 75% biochemical oxygen demand $(BOD)_5$, 41% N, 30% P, and 30% S. The treated water had 1.3 mg O_2 L^{-1}, with 83% increase in Sechhi disk transparency (SDT). BOD_5, N, P, and S showed strong negative correlation and dissolved oxygen and SDT showed strong positive correlation with *C. minutissima* indicating its plausible role in wastewater remediation [5]. Considering this, the present study was undertaken to determine the attributes that imparted this alga such a high degree of versatility and to evaluate its potential as a biofuel feedstock.

Materials and Methods

Microorganism, Medium, and Culture Conditions

Chlorella minutissima Fott et Nova AZAR 5105 was obtained from the Satellite Centre for Microalgal Biodiversity in Arid Zones of Rajasthan, Maharshi Dayanad Saraswati University, Ajmer (India). It was freed from bacteria using penta antibiotic (benzyl penicillin sulfate, streptomycin sulfate, chloramphenicol, neomycin, and actidione (cycloheximide)) treatment after Droop [6], and absence of bacteria was determined by streaking over nutrient agar medium. *C. minutissima* are solitary, unicellular spherical cells ranging from 2–4 μm in diameter in BG 11 medium and 2–8 μm when medium was supplemented with organic carbon. Single pyrenoid could be seen in large cells. It was maintained by frequent subculturing in BG 11 medium [7] fortified with 0.15% sodium nitrate and incubated in BOD incubator at 8±1 °C temperature and 7 μE $m^{-2}s^{-1}$ light intensity.

Attributes of *C. minutissima* were evaluated on the basis of various experiments set in triplicates of treatments in BG 11 medium. For all experiments, 5% inoculum was added from 10-day-old cultures and grown at 27 μE $m^{-2}s^{-1}$ light in 8/16 h L/D cycle using 40 W cool Phillips fluorescent lamps in a growth room maintained at 27±1 °C temperature.

Utilization of Organic Carbon Substrates and Heterotrophic Growth

The growth response of alga was evaluated in BG 11 medium supplemented with various sources of organic carbon (glucose, sucrose, sodium acetate, sodium propionate, and sodium citrate at 0.01 g mL^{-1}). Dichloromethyl urea (DCMU; final concentration in medium 10 μM) treated replicates were kept to confirm heterotrophic growth on glucose along with unamended BG 11 medium as control. Other than chlorophyll [8] and biomass, cell count was determined using Merck Neubauer double ruling hemocytometer.

Effect of Glucose on Carbohydrates, Proteins, and Lipids

Effect of glucose on cellular lipids, carbohydrates, and proteins was determined in presence of glucose under light and dark conditions. After a 10-day incubation period, suitable aliquots were withdrawn and centrifuged. Pellets were washed thrice with tap water before analysis to remove adhering material. Chlorophyll *a*, cellular protein [9], carbohydrates [10], lipids [11], and biomass [12] were determined.

Glucose Uptake Rate

Five milliliter aliquot of 10-day-old culture grown on BG 11 amended with 0.01 g mL^{-1} of glucose was centrifuged, and the supernatant was separated to a test tube. Pellet was washed thrice in 3 mL distilled water by resuspension and recentrifugation. Washings were pooled with the supernatant and the volume was made up to 15 mL. Glucose was estimated spectrophotometrically after Somogyi [13].

^{14}C Fixation Rate

^{14}C assay was carried out with or without glucose amendments using Beckman scintillation counter after Kumar et al. [14] and Larslandner [15]. A known amount of algal suspension (3.01 μg Chl *a* mL^{-1}) preincubated in dark for 2 h was supplemented with 100 mL of $NaH^{14}CO_3$ (specific radioactivity 0.003 μCi.μmol^{-1}) in BG 11 medium with or without glucose and DCMU (10 μM). One set of the cultures was incubated in saturating light intensities for 30 min, and the other, in dark. The reaction was terminated by adding 37% formaldehyde. After centrifugation, the excess radioactivity was driven off by adding 1 mL concentrated acetic acid. Pellet was dried at 60 °C, suspended in 10 mL of scintillation cocktail, and read in the counter.

Effect of Light Intensity

Response of *C. minutissima* to low light intensities varying from compete darkness to 30 μE m^{-2} s^{-1} was studied in BG 11 medium amended with or without glucose in triplicate. Every second day, biomass was determined [12]. Data, thus, generated was used to calculate generation time.

Anaerobiosis

A preliminary test was performed to confirm if *C. minutissima* could grow in anaerobic BG 11 agar (amended with 0.5% sodium sulfite, 1% glucose, and 2% agar) stabs. Sterilized medium was cooled to 40–45 °C and poured into test tubes containing 2 mL culture followed by vortex mixing. On solidification, 1 in. thick layer of mineral oil was poured over it and the tube was capped with suba seal. On observing visible green flakes of growth after 25 days of incubation, growth response was observed in liquid medium. Five milliliter culture was placed in presterilized test tube. Anaerobic BG 11 was added until brimful and the tube was capped with suba seal. A 10-mL syringe was pricked through the seal with its piston pressed down so as to allow liberation of gasses during growth. In the growth room, one set of triplicates was placed in light and the other was kept wrapped in aluminium foil to block light. After 10 days, cells were counted, and chlorophyll *a*, extracellular protein, and free fatty acids liberated in the medium [16] were determined.

Nitrogen Source Utilization and Tolerance to Salt, Osmotic Pressure, and pH

Sodium nitrate, urea, and ammonium chloride were added to BG11 medium at 250 μg N mL^{-1} to determine the source of N preferred by *C. minutissima*. Halotolerance was determined using graded concentrations of sodium chloride (0–5%). Osmotic stress (0 to −15 bar) was generated using a nonelectrolytic osmoticum- polyethylene glycol 6000 after Michael and Kaufmann [17] to assess osmotolerance. For all these experiments, chlorophyll *a* was measured as a parameter for growth. The alga was also grown over various pH regimes (4 to 12) under unbuffered conditions and final pH and chlorophyll *a* were monitored after 10 days of incubation.

Autosedimentation

Six test tubes, each of cultures with or without supplementation of glucose, were incubated for 1 and 3 weeks. Cultures were vortexed for 2 min, and cells were counted. Tubes were left undisturbed for 30 min, and 3-mL aliquot was separated slowly from the upper layer of culture to count cells again.

Optimum Wastewater Concentration for Growth

Simple tap water was added with varying quantities of wastewater obtained from the inlet channel to the Nehru Vihar OPS at Delhi. It had 145 mg L^{-1} BOD_5, 95.9 mg L^{-1} total N, 7 mg L^{-1} total P, and a pH of 7.12. Wastewater was either mixed directly without filtration or after filtration using Whatman No. 52 filter paper. Autoclaved flasks were incubated for 15 days in the growth room. Growth was measured in terms of chlorophyll *a*.

Antibiotic Sensitivity

Hi Media (India) antibiotic disks containing 25 μg of ampicillin, 10 μg each of gentamicin, amikacin, amoxycillin, cephaloridine, ciprofolxacin, and streptomycin; 10 units of bacitracin; 15 μg of erythromycin; 25 μg of cotrimazole, 30 μg each of cephalothin, kanamycin, novobiocin, oxytetracycline, tetracycline, and vancomycin; 100 μg carbenicillin, and 300 μg each of nitrofurantoin and triple sulphas were used for antibiotic sensitivity test.

Results

Organic Carbon Substrate Utilization

Abundance of *C. minutissima* in the first pond of the OPS, containing dark black sewage, suggested that it utilized organic carbon substrates heterotrophically. To confirm, it was grown over glucose, sucrose, and sodium salts of propionate, citrate, and acetate in presence and absence of light creating environment for chemoheterotrophic and mixotrophic (photoheterotrophic) growth, respectively. This was compared with photoautotrophic control (BG11 medium in presence of light). Propionate was not utilized and therefore alga could not grow in dark (Table 1). Even in presence of light, it showed an inhibitory role reducing chlorophyll *a* and biomass by 87% and cell count by 75% over BG 11. Acetate under dark conditions allowed growth. It improved in presence of light, yet could not reach anywhere near that of photoautotrophic growth in unamended BG 11 medium in light. In case of other sugars, growth in dark was significantly less than that in presence of light. Citrate showed 12% more biomass, but 25% less cells in presence of light over BG 11 that were significantly reduced under dark incubation. Only glucose showed synergistic effect on growth in presence of light, while in case of sucrose, dark growth was at par with phototrophic control but presence of light showed slight improvement over it; suggesting that presence of glucose or sucrose was not inhibitory to photosynthesis and the alga was able to utilize both organic and inorganic carbon source simultaneously. Heterotrophy over glucose was confirmed by inhibiting photosynthesis using DCMU, wherein growth of alga was slightly more than in absence of light with glucose and DCMU, and slightly lower than in BG 11 in presence of light. The differences were, however, statistically nonsignificant. Sum of growth under heterotrophic condition (glucose + DCMU or dark) and phototrophic (BG 11 in light) was 50% less than under mixotrophic condition (glucose + light), indicating a stimulatory role of glucose on growth in presence of light.

Cell counts were maximum under photoautotrophic conditions. The profile in terms of cell count was BG11>sucrose>acetate>glucose>citrate>glucose+DCMU>propionate. In the dark, it was glucose>sucrose>glucose+DCMU>citrate>acetate. Distribution pattern of cell sizes (2–4 μm in unamended BG 11 and 2–8 μm in presence of organic carbon substrates—data not shown) showed that organic substrates increased proportion of larger cells. Propionate and acetate were exceptions to this as cell size composition was at par and chlorophyll content was lower than the control, indicating suppression of photosynthetic biosynthesis.

Effect of Glucose on Carbohydrate, Protein, and Lipid

Effect on biomass, chlorophyll, cellular lipids, carbohydrates, and proteins was determined in presence of glucose under light and dark conditions after 10 days of incubation. Lipid accumulation in cells followed the pattern of biomass (Table 2). It was maximum under light incubated glucose-supplemented conditions (14.9%) and minimum in BG 11 medium without glucose (5.4%). Contribution of protein to biomass was lower in BG 11 supplemented with glucose under both light and dark incubation (~39%) than in BG11 medium (56%), while carbohydrates contributed maximum over glucose on dark incubation (22.2%), followed by light-incubated glucose supplemented BG11 (17.8%) and BG 11 medium without glucose (13.1%).

Table 1 Growth of *Chlorella minutissima* on various carbon sources in BG 11 medium in presence or absence of light (18/6 h L/D cycle) after 15 days of incubation.

Treatment	Growth parameter	BG 11	BG 11 with					
			Propionate	Acetate	Citrate	Sucrose	Glucose	Glucose+DCMU
Light	Chl a (mg L^{-1})	13.41 (2.146)	1.72 (0.06)	9.4 (1.41)	15.26 (1.02)	17.63 (1.476)	37.21 (6.512)	12.02 (1.683)
	Chl b (mg L^{-1})	7.89 (1.262)	0.98 (0.034)	5.7 (0.855)	9.54 (0.64)	10.25 (1.277)	21.89 (3.831)	6.68 (0.935)
	Biomass (mg L^{-1})	899 (47.65)	120 (21.8)	630 (60.79)	1,007 (191)	1,164 (152)	2,456 (515)	805 (43.35)
	Cell Count ($\times 10^5$ mL^{-1})	2.01 (0.292)	0.51 (0.032)	1.89 (0.138)	1.5 (0.12)	1.93 (0.27)	1.76 (0.317)	1.11 (0.189)
Dark	Chl a (mg L^{-1})	No growth		5.59 (0.064)	5.13 (0.15)	10.09 (1.998)	11.39 (2.48)	11.22 (2.23)
	Chl b(mg L^{-1})			3.11 (0.358)	3.02 (0.09)	5.94 (1.176)	6.51 (0.846)	7.01 (1.402)
	Biomass (mg L^{-1})			364 (43.08)	328 (24.6)	656 (78.24)	740 (43.78)	726 (64.41)
	Cell Count ($\times 10^5$ mL^{-1})			0.02 (0.001)	0.51 (0.1)	1.28 (0.205)	1.36 (0.031)	0.98 (0.076)

The amount of Chl *a* in initial inoculum was 0.034±0.0013 mg L^{-1}. Values are mean of triplicates. Parentheses show value of standard deviation

Table 2 Effect of glucose over biomass and major cell constituents in presence and absence of light after 10 d of growth.

Growth Parameters (mg L^{-1})	BG 11	BG 11+Glucose	
	Light	Light	Dark
Biomass	73.03 (8.76)	379.22 (32.75)	140.43 (17.63)
Chl *a*	1.09 (0.093)	6.79 (0.906)	2.396 (0.314)
Chl *b*	0.61 (0.052)	3.02 (0.483)	1.079 (0.162)
Total Chl	1.70 (0.119)	9.81 (1.46)	3.475 (0.432)
Proteins	41.12 (2.535)	148.24 (11.19)	55.36 (4.827)
Carbohydrates	9.60 (1.83)	67.51 (13.28)	31.13 (2.49)
Lipids	3.94 (0.559)	56.44 (16.49)	16.55 (2.90)

Values are mean of triplicates. Figures in parentheses are values of standard deviation

Glucose Uptake and ^{14}C Fixation

Glucose uptake (Table 3) was not photosensitive as it was induced to the same extent in light and dark. Maximum ^{14}C uptake could be observed in presence of light and absence of DCMU. Addition of glucose showed slight reduction in ^{14}C uptake but the difference was statistically non-significant.

Effect of Light Intensity

While there was no growth in dark, presence of organic carbon as glucose supported growth, albeit at a very slow rate, with a generation time of 27 h 28 min. A doubling time almost at par (27 h 49 min) was also achieved in BG 11 with just 2 μE $m^{-2}s^{-1}$ light intensity. Increase in light intensities beyond this decreased the generation time in absence of glucose to 19 h 12 min at 30 μE $m^{-2}s^{-1}$ light, while much faster growth was achieved in presence of glucose (11 h 44 min) at the same light intensity. Presence of glucose at 2 μE $m^{-2}s^{-1}$ light could yield almost as high growth as at 30 μE $m^{-2}s^{-1}$ in absence of

Table 3 ^{14}C and glucose utilization by *Chlorella minutissima* under various nutritional regimes in BG 11 medium.

Medium	+ DCMU	− DCMU	
	Light[a]	Light[a]	Dark
^{14}C utilization (μmol $NaH_{14}CO_3$ mg chl^{-1} h^{-1})			
BG11 − Glucose	362.79 (4.64)	5042.25 (679.47)	NA
BG 11+Glucose	500.19 (29.21)	4338.50 (189.25)	187.39 (20.12)
Glucose uptake (n mole glucose. mg DW^{-1} h^{-1})			
BG 11+Glucose	13.32 (0.683)	13.43 (0.898)	13.73 (1.093)

No reducing sugar was found liberated in medium under light+DCMU-Glucose condition. All values are mean of triplicates and parentheses show value of standard deviation

NA Not analyzed

[a] Light: 6/18 h L/D cycle

glucose. Statistically significant regression equations, thus, generated from the data could be used to predict generation time of the *C. minutissima* based on light intensity (Fig. 1).

Anaerobiosis

C. minutissima showed an average count of 7×10^3 cells mL^{-1} in dark colored, turbid sewage having nil dissolved oxygen indicating survival and/or growth in anaerobic condition. When grown in reduced solid agar stabs under mineral oil, green flakes of growth were observed in 25 days at various positions of the stab. In liquid cultures grown anaerobically, 15.7 (±2.59) mg and 11.2 (±1.74) mg of free fatty acid equivalents were found liberated under light and dark conditions, respectively. Acid production albeit in low amount was also observed in presence of glucose in absence of sodium sulfite [21(±3.68) and 84 (±15.12) mg in light and dark, respectively]. But no free fatty acids were detected in the medium without glucose and sodium sulfite. Despite liberation of free fatty acids, pH of the medium invariably raised from initial 7 to 8.11–8.46 in anaerobic medium and glucose supplemented BG 11 and to 9.91 in unamended BG 11 medium.

There were extracellular liberations of protein in unamended BG 11 medium (120 mg L^{-1}) that increased to 380 (±49) and 580 (±51) mg L^{-1} in presence of glucose and 2,060 (±169) and 2,780 (±269) mg L^{-1} in anaerobic medium in light and dark, respectively.

Nitrogen Source Utilization

C. minutissima generated 16.5 mg chlorophyll *a* L^{-1} in 10 days on ammonium-N as against 4.3 mg L^{-1} on nitrate-N. Growth on ammonium-N was rapid and reached the peak between 10–15 d, while that on nitrate-N did not show decline even on 20th day of incubation (Fig. 2). Urea however could not support growth.

Tolerance to Salt, Osmotic Stress, and pH

Domestic sewage is known to have high concentrations of sodium especially due to detergents and soaps, therefore, organisms living in municipal wastewater must have halotolerance. *C. minutissima* showed halotolerance up to 3% NaCl (1.66 (±0.17) mg chl a L^{-1}) but optimum growth was observed at 1% NaCl (3.46 (±0.67) mg chl a L^{-1}). There was a 7.3%

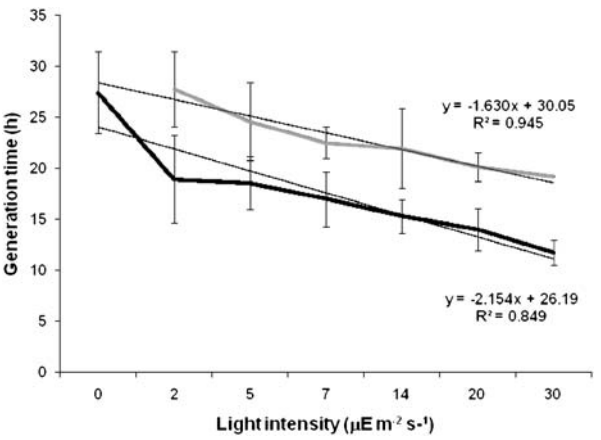

Fig. 1 Effect of low light intensities over generation time of *C. minutissima* as affected by the supplementation of glucose. Changes in generation time are depicted by *gray line* on BG 11 medium without glucose supplementation and with *black line* for glucose amended BG 11 medium. Respective *thin lines* for each of them depict the trend lines along with regression equations and coefficients of determination

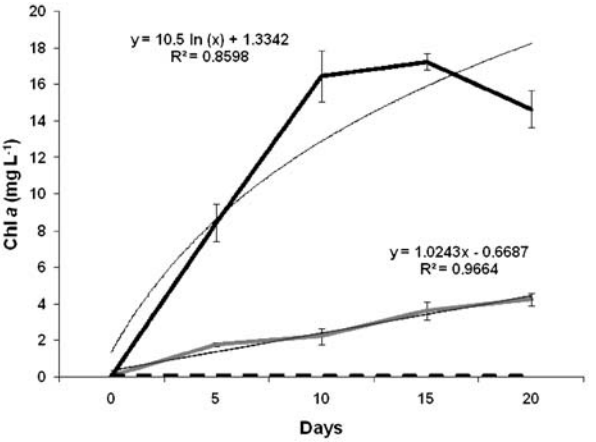

Fig. 2 Growth dynamics of *C. minutissima* over different nitrogen sources in BG 11 medium. *Bars* indicate standard deviation. *Solid black line* indicates growth in terms of chlorophyll *a* on ammonium chloride, and *solid gray line* indicates growth on sodium nitrate, while *dashed line* represents urea. Respective *thin lines* for ammonium chloride and sodium nitrate depict the trend lines along with regression equations and coefficients of determination

and 64% reduction in chlorophyll *a* at −5 and −10 bar osmotic stress generated by adding polyethylene glycol 6000 as against unamended BG 11.

C. minutissima could grow at all pH values from 4 to 12, but optimum growth was observed at pH 7.0 (Fig. 3). Growth was severely restricted at pH 4 and 5. Alkaline pH range however did not show much variation. As a result of growth of the alga, the initially set pH from 6 to 11 changed to ~8, while that set to 4 and 5 had risen to ~6 and 7, respectively.

Autosedimentation

C. minutissima showed 80% sedimentation within 30 min after vigorous shaking of 7-day-old culture grown in BG 11, as could be seen through the cell counts in the supernatant, while those grown in BG 11 supplemented with glucose showed only 53% sedimentation (Fig. 4). Sedimentation increased to 99% and 67%, respectively in 21-day-old cultures.

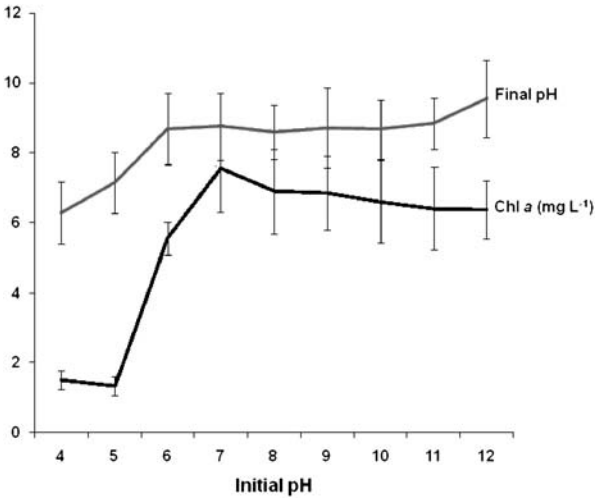

Fig. 3 Effect of pH on Chlorphyll *a* content of *C. minutissima* and pH of the medium after 10 days of incubation. *Bars* indicate standard deviation

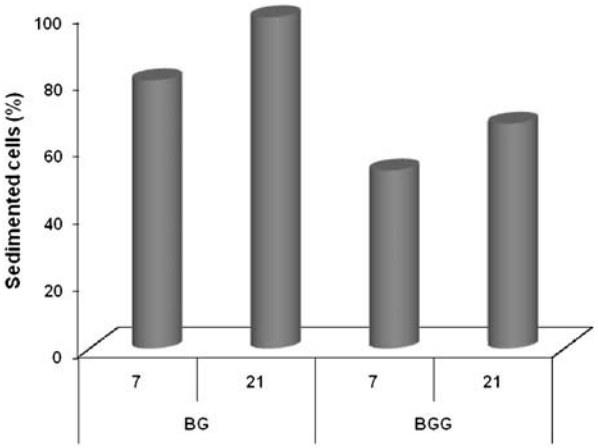

Fig. 4 Autosedimentation of *C. minutissima* in glucose supplemented (BGG) and unamended BG 11 (BG) medium after 7 and 21 days of growth. *Cylinders* depict percentage of cells that settled after 30 min of initial vortexing

Optimum Wastewater Concentration for Growth

C. minutissima showed a significant increase in chlorophyll *a* when grown in tap water amended with sterilized filtered wastewater (Fig. 5). Chlorophyll *a* content was 10.37 mg L^{-1} in the presence of 25% filtered wastewater which was ~32% more than in BG 11 medium, whereas ~145% increase in chlorophyll *a* was observed in 50% unfiltered wastewater. Further concentration of wastewater reduced the growth but even at 75% concentration, it was ~16% and 24% more than BG 11 of filtered and unfiltered wastewater, respectively. However, 100% wastewater reduced the growth of *C. minutissima*.

Antibiotic Sensitivity

C. minutissima was resistant to 10 μg of amikacin, amoxycillin, cephaloridine, ciprofolxacin, and streptomycin; 10 units of bacitracin; 15 μg of erythromycin; 25 μg of cotrimazole, 30 μg of cephalothin, kanamycin, novobiocin, oxytetracycline, tetracycline, and vancomycin; 100 μg carbenicillin and 300 μg of nitrofurantoin and triple sulphas. However, ampicillin (25 μg) and gentamicin (10 μg) formed a zone of inhibition of the size 2 mm and 1 mm, respectively.

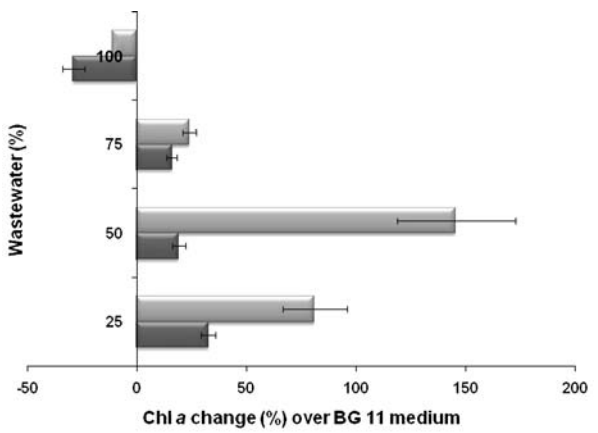

Fig. 5 Change in growth of *C. minutissima* in presence of graded concentrations of filtered (*dark gray bars*) and unfiltered (*light gray bars*) sterilized wastewater with reference to BG 11 medium after 15 days of incubation. *Bars over the column* indicate standard deviation

Discussion

The observations suggested that *C. minutissima* tolerated absence of oxygen and could grow at varying pH and light intensities and probably utilized variety of organic substances during facultative chemoorganotrophic mode of nutrition. Such ability has been observed in a number of green algae [18]. Importance of this phenomenon in raceway-type high-rate algal bacterial pond system for wastewater remediation was highlighted by Abeliovich and Weismann [19]. They claimed that 25–50% of algal carbon could be derived directly from the organic matter that has not been oxidized to carbon dioxide by bacteria. It is also known that algae utilizing fixed carbon sources grow in greater densities than that associated with autotrophic growth [20]. Recently, Andrade and Costa [21] advocated cultivation of *Spirulina platensis* over molasses exploiting its mixotrophic behavior.

Our studies showed that propionate and acetate were inhibitory as compared to the control. Growth over acetate in absence of light indicated facultative heterotrophy but it inhibited photosynthesis to certain extent. Citrate<sucrose<glucose supported mixotrophic growth in *C. minutissima*. Under mixotrophic conditions, growth was more than the sum of heterotrophic and autotrophic growth as was also supported by ^{14}C studies and glucose uptake. Mixotrophy has been reported in many algae (viz. *Chlorella vulgaris, C. pyrenoidosa, S. platensis* [5], *Cochlodinium polykrikoides* [22], *Ochromonas tuberculata* and *Cryptomonas* sp. [23], and *Chlamydomonas acidophila* [24]).

Although cell count did not vary significantly in response to organic carbon, increase in the cell size and contribution of such large cells translated into higher productivity during mixotrophic or chemotrophic growth, which is in confirmation with the observations on biomass. Orus et al. [25] observed that glucose promoted formation of polyploid giant cells along with delay in cell division. Photohetreotrophy and chemoheterotrophy also presented polyploid nuclei, but the increase in cellular size was delayed with respect to mixotrophic cells. However, we could not observe such polyploidy.

DCMU adversely affected ^{14}C uptake in presence of light, however, presence of glucose somewhat improved the uptake. The small amount of the observed ^{14}C fixation may be attributed to the common light independent CO_2 fixation reactions such as β-carboxylation of 3C precursors by PEP carboxykinase, presence of carbamoyl phosphate synthetase [26] and other carboxylases and catalytic role of CO_2 in lipid synthesis and electron transport [27]. ^{14}C and glucose uptake studies showed slight repression of the rate of photosynthesis in presence of glucose but it was statistically nonsignificant, whereas presence of light had no adverse effect on glucose consumption. Ogawa and Aiba [18] reported glucose sensitivity of photosynthesis and attributed it to low chlorophyll synthesis. Glucose uptake system and mitochondrial respiration were reportedly not photosensitive in *C. vulgaris* UAM 101 [28] unlike others in which light-inhibited glucose uptake by affecting G6P dehydrogenase [29]. Kamiya and Kowallik [30] showed induction of a carrier protein in cell membranes of a *C. vulgaris* mutant in presence of glucose that was inhibited by blue light. On the other hand, Bagchi et al. [31] reported light stimulation of glucose uptake in *Phormidium uncinatum*. Relative independence of both photosynthesis and oxidative assimilation of carbon source is reported by Ogawa and Aiba [18] and Martinez and Orus [28].

Glucose assimilation in *C. minutissima* was related to lower protein productivity and higher accumulation of lipids and carbohydrates as has been reported earlier [18, 28]. Per hectare productivity [32] in open raceway ponds (200 ponds of 400 L capacity per acre) for *C. minutissima* was approximately 4 ton lipids ha^{-1} $year^{-1}$ under mixotrophic conditions (Table 4) against the desired value of 20–30 tons ha^{-1} $year^{-1}$ [33]. But this was achieved at 30 μmol $m^{-2}s^{-1}$ light intensity and at ambient CO_2 concentration. Higher light and CO_2

Table 4 Estimated productivity of biomass, proteins and carbohydrates of *C. minutissima* under ambient CO_2 concentration in the headspace and 30 μE $m^{-2}s^{-1}$ light intensity under phototrophic (unamended BG 11 medium incubated in 6/18 L/D cycle), mixotrophic (BG 11 medium supplemented with glucose incubated in 6/18 L/D cycle), and heterotrophic (BG 11 medium supplemented with glucose and incubated in dark) conditions.

Growth Parameters	Phototrophic	Mixotrophic	Heterotrophic
	t ha^{-1} $year^{-1}$		
Biomass	5.3	27.7	10.3
Proteins	3	11	4
Carbohydrates	0.7	4.9	2.3
Lipids	0.29	4.1	1.2

concentration are known to increase algal productivity [12] besides addition of 1% salt could also enhance productivity of *C. minutissima*. Therefore, the low lipid content of *C. minutissima* could be compensated by its rapid and high biomass generation ability, particularly in wastewaters containing organic carbon substrates that would allow it to perform mixotrophy. Thus, cheap cultivation is possible without freshwater and fertilizer requirements.

C. minutissima showed linear growth stimulation over increasing light intensities both in presence and absence of glucose. Light saturation at 10 klx was observed by Ogawa and Aiba [18] for *C. vulgaris*. Glucose stimulation effect took place at lesser illumination only.

Anaerobic growth and fermentation by algae have been supposed to be insufficient for their maintenance of growth [34]. One has to distinguish between truly anaerobic conditions i.e., in the dark or with photosynthetic oxygen evolution inhibited in light and less strict anaerobiosis with at least some photosynthetic oxygen production in light. Extracellular liberation of protein in *C. minutissima* increased in the following pattern: BG11+light< BG11+dark<anaerobic BG11+light<anaerobic BG 11+dark. Protein liberation enhanced by 19–20 times under anoxic stress. There was a lowering of pH by 1 unit in presence of sugar, and under anoxic conditions as a result of liberation of free fatty acid equivalents in medium.

Diluted wastewater supported better growth of *C. minutissima* than the BG 11 medium and unfiltered wastewater was better than the filtered. Since the wastewater was sterilized, the possibility of wastewater-borne contribution to chlorophyll content was ruled out. Thus, the increase in growth under unfiltered condition was perhaps due to the dissolved organic matter rich in proteins, carbohydrates, and lipids, which might have degraded during autoclaving. This presumably provided nutrients and also served as the substrate for the heterotrophic growth of the alga. Differential response of the algae to the filtered and unfiltered wastewater indicated once again at the complexity of forces asserting influence over its growth.

Conclusion

The study revealed that municipal wastewater (50%) supported 146% more growth than the standard BG 11 medium. Thus, it was possible to grow *C. minutissima* without addition of any nutrients and in half the amount of freshwater that would be usually required. Ability to

tolerate environmental fluctuations was well-established by its dominance through all stages of treatment and seasons. This versatility could be imparted to the following attributes of *C. minutissima*: [1] Tolerance to a wide range of pH (4–12), anaerobicity, and very low illumination (2 μE $m^{-2}s^{-1}$) in absence of glucose, [2] Wide substrate choice for carbon and nitrogen [3] Mixotrophic nature and heterotrophy in dark and [4] Tolerance to NaCl, osmotic stress, and a number of antibiotics. Besides, its tendency to autosediment in clearer water can significantly reduce the harvesting cost. Therefore, if municipal wastewaters are to be used for the production of algae for biofuel and bioenergy applications, then *C. minutissima* and other such native forms would be potent candidates.

For the purpose of biodiesel feedstock, low lipid content (5%) of *C. minutissima* was found improved to ~15% under mixotrophic conditions. Besides, high biomass build up could give an estimate of up to 4 tons lipids h^{-1} $year^{-1}$ under laboratory conditions. Even this appears very small against the achievable target of 20–30 ton lipids ha^{-1} $year^{-1}$, but the light (30 μE $m^{-2}s^{-1}$) was limiting, and no CO_2 was added. Therefore, better productivity may be expected in sunlight with CO_2 supplementation. Our results also show 1% NaCl as the requirement to achieve optimum growth of *C. minutissima* in BG 11 medium, indicating at the possibility to manipulate wastewater nutrients to further increase the productivity.

The cost of production may also be seen in view of the wastewater remediation potential of the alga. It was reported earlier that the oxidation pond system, Nehru Vihar (Delhi), India, the original habitat of the test alga, could reduce an average of 41% of total N and 30% of total P along with 75% of BOD_5. As the contribution of *C. minutissima* to the total phytoplankton population in these ponds was approximately 60% throughout the year, it may be assumed that it was responsible for most of the activity in the OPS.

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References

1. Benemann, J., & Oswald, W. (1996). *Final report to the US Department of Energy, Grant No. DE-FG22-93PC93204*. Pittsburgh Energy Technology Center.
2. Fahm, L. (1980). *The waste of nations: The economic utilisation of human waste in agriculture*. Montclair, New Jersey, USA: Allenhand, Osmun & Co. Pub, Inc.
3. Niemczynowicz, J. (1997). The water profession and agenda 21. Water Quality International, March/April: 9–11.
4. Bhatnagar, A. (1999). *Journal of Environmental Biology, 20*, 115–120.
5. Bhatnagar, A., & Bhatnagar, M. (2001). Innovative approaches in microbiology. In D. K. Maheshwari & R. C. Dubey (Eds.), India: Bishen Singh Mahendra Pal Singh, Dehra Dun, pp. 379–403.
6. Droop, M. R. (1967). *British Phycological Bulletin, 3*(2), 295–297.
7. Stanier, R. Y., Kunisawa, R., Mandel, M., & Cohen-Bazire, G. (1971). *Bacteriological Reviews, 35*, 171–205.
8. Porra, R. J., Thompson, A., & Friedelman, P. E. (1989). *Biochimica et Biophysica Acta, 975*, 384–394.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). *Journal of Biological Chemistry, 193*, 265–275.
10. Roe, J. H. (1955). *Journal of Biological Chemistry, 212*, 335–343.
11. Bligh, E. G., & Dyer, W. J. (1959). *Canadian Journal of Biochemistry and Physiology, 37*, 911–917.
12. Chinnasamy, S., Ramakrishnan, B., Bhatnagar, A., & Das, K. C. (2009). *International Journal of Molecular Sciences, 10*, 518–532.
13. Somogyi, M. (1945). *Journal of Biological Chemistry, 160*, 61–68.

14. Kumar, A., Tabita, F. R., & van Baalen, C. (1982). *Archives of Microbiology, 133*, 103–109.
15. Larslandner (1989). Advanced hazard assessment. In R. S. De Santo (Ed.), *Springer Ser. Environmental management* (pp. 223–225). Berlin, Germany: Springer-Verlag.
16. Cox, H. E., & Pearson, D. (1962). *The chemical analysis of foods*. New York, USA: Chemical Publishing Co. Inc.
17. Michael, B. E., & Kaufman, M. R. (1973). *Plant Physiology, 51*, 914–916.
18. Ogawa, T., & Aiba, S. (1981). *Biotechnology and Bioengineering, 23*, 1121–1132.
19. Abeliovich, A., & Weisman, D. (1978). *Applied and Environmental Microbiology, 35*(1), 32–37.
20. Day, J. D., Edwards, A. P., & Rodgers, G. A. (1991). *Bioresource Technology, 38*(2–3), 245–249.
21. Andrade, M. R., & Costa, J. A. V. (2007). *Aquaculture, 264*(1–4), 130–134.
22. Jeong, H. J., Du Yoo, Y., Kim, J. S., Kim, T. H., Kim, J. H., Kang, N. S., et al. (2004). *Journal of Eukaryotic Microbiology, 51*(5), 563–569.
23. Katechakis, A., Haseneder, T., Kling, R., & Stibor, H. (2005). *Limnology and Oceanography, 50*(4), 1290–1299.
24. Tittel, J., Bissinger, V., Gaedke, U., & Kamjunke, N. (2005). *Protist, 156*(1), 63–75.
25. Orus, M. L., Marco, E., & Martinez, F. (1991). *Bioresource Technology, 38*, 179–184.
26. Kremer, B. P. (1979). *Journal of Phycology, 15*, 244–247.
27. Raven, J. A., Johnson, A. M., & MacFarlane, J. J. (1990). Biology of the red algae. In: K. M. Cole and R. G. Sheath (Eds.), New York: Cambridge University Press, pp. 171–185.
28. Martinez, F., & Orus, M. I. (1991). *Plant Physiology, 95*, 1150–1155.
29. Turpin, D. H., Elrifi, I. R., Birch, D. G., Weger, H. G., & Holmes, J. J. (1988). *Canadian Journal of Botany, 66*, 2083–2097.
30. Kamiya, A., & Kowallik, W. (1987). *Pl Cell Physiol, 28*(4), 611–619.
31. Bagchi, S. N., Chauhan, V. S., & Palod, A. (1990). *Current Microbiology, 21*(1), 53–57.
32. Doucha, J., Straka, F., & Lívanský, K. (2005). *Journal of Applied Phycology, 17*, 403–412.
33. Rodolfi, L., Zittelli, G. C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., et al. (2009). *Biotechnology and Bioengineering, 102*, 100–112.
34. Ueno, Y., Kurano, N., & Miyachi, S. (1998). *Journal of Fermentation and Bioengineering, 86*(1), 38–43.