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ORGANIC CARBON SUPPLEMENTATION OF STERILIZED MUNICIPAL WASTEWATER IS ESSENTIAL FOR HETEROTROPHIC GROWTH AND REMOVING AMMONIUM BY THE MICROALGA *CHLORELLA VULGARIS*[1]

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Heterotrophic growth of the microalga *Chlorella vulgaris* Beij. in synthetic as well as sterilized municipal wastewater of a nonindustrialized city was measured. The city wastewater contained high levels of ammonium and nitrate, medium levels of phosphate, and low levels of nitrite and organic molecules and could not support heterotrophic growth of *C. vulgaris*. Evaluation of 11 known carbon sources for this microalga that were added to standard synthetic wastewater containing the same levels of nitrogen and phosphorus as the municipal wastewater revealed that the best carbon sources for heterotrophic growth were Na-acetate and D-glucose. These provided the highest growth rates and the largest removal of ammonium. Growth increased with concentration of the supplement to an optimum at 0.12 M Na-acetate. This carbon source was consumed completely within 10 d of incubation. Higher concentrations inhibited the growth of *C. vulgaris*. The microalgal populations under heterotrophic growth conditions were one level of magnitude higher than that under autotrophic growth conditions that served as a comparison. No growth occurred in the dark in the absence of a carbon source. Na-acetate was superior to D-glucose. In municipal wastewater, when Na-acetate or D-glucose was added, *C. vulgaris* significantly enhanced ammonium removal under heterotrophic conditions, and its capacity was equal to ammonium removal under autotrophic growth conditions. This study showed that sterilized wastewater can be treated by *C. vulgaris* under heterotrophic conditions if supplemented with the appropriate organic carbon source for the microalgae.

***Key index words:* *Chlorella*; heterotrophic growth; microalgae; municipal wastewater; wastewater treatment**

***Abbreviations:* ANOVA, analysis of variance; BOD, biological oxygen demand; EBPR, enhanced biological phosphorus removal; SE, standard error; SGM, synthetic wastewater medium**

Chlorella spp. are photosynthetic microalgae (de-Bashan et al. 2008) that have a capacity to grow heterotrophically, independent of light, if supplemented with carbon and energy from an organic carbon source (Chen 1996, Shi et al. 2000, Lee 2004, Chen and Chen 2006, Qiao et al. 2009). Autotrophic microalgae for tertiary wastewater treatment to remove nutrients have two major limiting factors when they are light dependent: (i) wastewater is treated in large volumes in bioreactors where penetration of light into a dense culture is limited (Apt and Behrens 1999, Behrens 2005), and (ii) the density of cells in the wastewater culture is low (Lee 2001, Valderrama et al. 2002).

Heterotrophic growth may solve these difficulties (Lee 2001, Chen and Chen 2006). It eliminates the need for light and may allow cultivation of denser concentrations of the microalgae (Chen 1996, Ogbonna et al. 1997). These major advantages notwithstanding, heterotrophic growth may possess several major limitations for microalgae. These include (i) low tolerance to high carbon concentrations; (ii) competition with other microorganisms, bacteria, and yeasts whose growth is enhanced in heterotrophic nonaxenic cultures; (iii) that not all species of microalgae can grow without light, and that some organic compounds inhibit growth; and (iv) lack of production of photosynthetic

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metabolites (Chen 1996). Only high densities of microalgae and high metabolic activity can justify the use of heterotrophic cultures of microalgae (Lee 2004, Behrens 2005).

Heterotrophic growth of microalgae was investigated for more than half a century by using *Chlorella pyrenoidosa* (Samejima and Myers 1958). Subsequent studies have built on these findings for investigating conditions relevant to wastewater treatment, such as mixing of microalgae and bacteria at different pH (Mayo and Noike 1994), mixotrophic growth (Endo et al. 1977), and microaerobic growth (Qiao et al. 2009). In some heterotrophic cultures, growth rate of *Chlorella* is much higher than in autotrophic cultures, which mainly depends on the species and strain (Shi et al. 2000). *C. vulgaris* is able to clean wastewaters by consuming the waste nitrogen and phosphorus (Gonzalez et al. 1997, Valderrama et al. 2002, Olguín 2003).

Municipal and domestic wastewater treatment is routinely carried out by large treatment plants specialized in removal of particulate organic matter by digestion (Henze et al. 2002). Some small operations use wetlands (Kivaisi 2001) and bioreactors of many types and designs (Aiyuk et al. 2004). Removal of nutrients and other pollutants may be accomplished by chemical methods. Nutrient removal with biological technology, although employing plants for enhanced biological phosphorus removal (EBPR), has the smallest impact on the industry (de-Bashan and Bashan 2004, 2010).

Domestic and municipal wastewaters are a very complex mixture of suspended and dissolved materials (Almeida et al. 1999). Most municipal and domestic wastewater, after microbial secondary treatment, contains large amounts of dissolved organic matter. Although carbon sources are available for bacteria, most are not available as a carbon source for microalgae (Henze et al. 2002). Wastewater contains a complex mixture of small and large molecules and polymers from <500 to >5,000 Da that are part of hydrophilic and hydrophobic acid fractions of dissolved organic carbon (Imai et al. 2002). These compounds include humic material, polysaccharides, polyphenols, peptides, proteins, lipids, large fatty acids, synthetic detergents (Qualls and Haines 1991), and chemical derivatives of many of those compounds that the microalgae cannot metabolize. Microalgae, as unicellular plants, can use only relatively simple molecules, such as nitrogen compounds, sugars, organic and amino acids, and several aromatic compounds (Bollman and Robinson 1977, Kaplan et al. 1986, Semple et al. 1999, Lee 2004).

Our hypothesis was that the municipal wastewater of a medium-size, nonindustrialized city does not contain sufficient available carbon sources for tertiary wastewater treatment (removal of nutrients) by the microalgae *C. vulgaris* growing in complete darkness. Therefore, an appropriate and available organic carbon source should be selected as a supplement. This analysis was done by initially analyzing the composition of the municipal wastewater in detail, evaluating several potential carbon sources dissolved in these wastewaters, designing the composition of synthetic wastewater resembling these municipal wastewaters, in terms of nutrient concentration, and performing ammonium removal using a microalgal suspension in the synthetic and municipal wastewaters. All of this has been done as an initial, but essential, step in devising new heterotrophic approaches to tertiary wastewater treatment.

MATERIALS AND METHODS

Microalga and growth conditions. The unicellular microalga *C. vulgaris* (UTEX 2714, Austin, TX) was used in all experiments. This strain is an efficient wastewater treatment agent (Gonzalez et al. 1997, de-Bashan et al. 2002, 2004, Valderrama et al. 2002). Most experiments were done under heterotrophic conditions in total darkness in an incubator shaker (Innova 4340; New Brunswick, Edison, NJ, USA) in 500 mL Erlenmeyer flasks or in 50 mL test tubes. The microalga was grown in modified synthetic wastewater medium (SGM) (de-Bashan et al. 2002), where the ammonium and phosphate concentrations were adjusted to the concentrations found in municipal wastewater, which is described in the Results section. This synthetic wastewater contained the following ingredients ($mg \cdot L^{-1}$): NaCl, 7; $CaCl_2$, 4; $MgSO_4 \cdot 7H_2O$, 2; K_2HPO_4, 21.7; KH_2PO_4, 8.5; Na_2HPO_4, 25; and NH_4Cl, 191, supplemented with 10 $g \cdot L^{-1}$ of various carbon sources at pH 6.7 at 28 ± 1°C under constant 120 rpm shaking. The supplemented carbon sources were: D-glucose, L-arabinose, D-fructose, Na-acetate, Ca-acetate, Na-citrate, DL-malic acid, DL-lactic acid, acetic acid, peptone, and urea (all from Sigma, St. Louis, MO, USA), commercial sucrose, fulvic acid (Grupo Bioquímico Mexicano, Saltillo, Mexico), and 5% commercial vinegar (Sabormex, Mexico City, Mexico). When autotrophic growth was performed in synthetic wastewater, light intensity was adjusted to continuous 60 μmol photon $\cdot m^2 \cdot s^{-1}$ using conventional fluorescent lamps that are used in similar bioreactors, and no carbon source was added. The specific medium C30 for *Chlorella* sp. (Gonzalez et al. 1997), nutrient broth (Sigma), and saline solution (0.85% NaCl) were used as controls. Municipal wastewater samples (described later) were used as the substrate. The wastewater was filtered twice through filter paper (#1, Whatman, Maidstone, UK), sterilized with sterile cellulose nitrate membranes (Whatman) of 0.45 and 0.2 μm pore size, and then used for heterotrophic and autotrophic growth trials, as it is, or supplemented with a carbon source. Natural wastewater was sterilized to measure nutrient supplementation requirements of microalgae and avoid competition with other microorganisms in the wastewater that may consume the same supplements.

Wastewater sampling. Wastewater was collected nine times on 3 d during 1 week (for details, Fig. 1) at the municipal wastewater treatment facility of the city of La Paz, Baja California Sur, Mexico. The city has a population of ~250,000 with tourism, public administration (state capital), and higher education as the dominant income sources. There are very few industrial plants. Samples were collected from a stream of wastewater after the secondary aerobically activated sludge treatment but before the final chlorination step. Samples were immediately transported to the laboratory. Sampling was conducted on the following schedule: samples were collected at 06:00–07:00, 13:00–14:00, and 21:00–22:00 h, with three wastewater samples (5 L each) taken at each sampling time. Each sample was used for analysis of nutrients, biological oxygen demand for 5 d (BOD_5), and dissolved

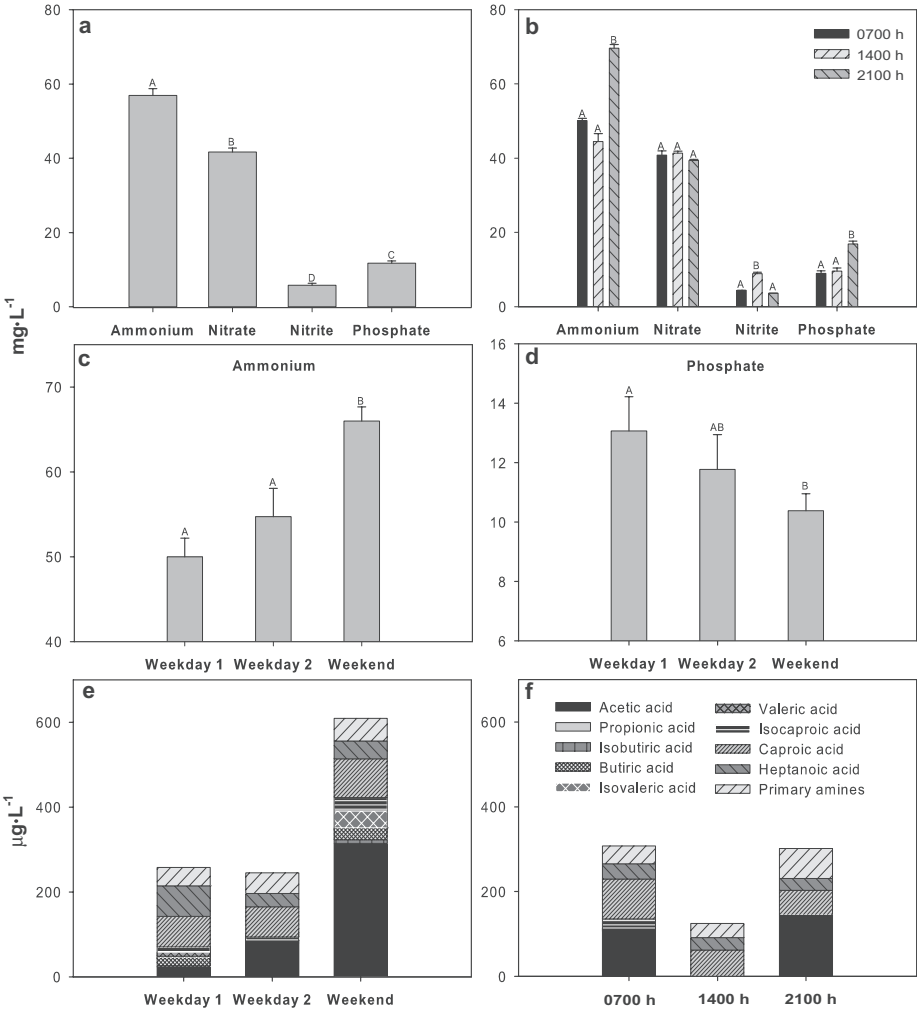

FIG. 1. Chemical analyses of the municipal wastewater treatment after secondary treatment at the wastewater facility of La Paz, B.C.S., Mexico. (a) Average levels of ammonium, nitrate, nitrite, and phosphate; (b) levels of ammonium, nitrate, nitrite, and phosphate per hour of sampling; (c) levels of ammonium per day; (d) level of phosphate per day; and level of organic acids and primary amines per day (e) and per hour of sampling of weekday 2 (f). Columns denoted by a different letter in each subfigure (a, c, d) differ significantly at $P < 0.05$ by one-way analysis of variance (ANOVA) and Tukey's analyses. Groups of three columns (b) denoted by a different letter differ significantly at $P < 0.05$ by one-way ANOVA and Tukey's analyses. Bars represent standard error.

organic compounds. Four replicates were taken from each wastewater batch for individual analysis. If necessary, debris in the wastewater was filtered through a gauze-cotton filter lining a funnel. All wastewater was analyzed at the time of delivery to the laboratory. If necessary, wastewater was stored at –80°C.

Wastewater analyses. In synthetic and municipal wastewater used for cultivation of the microalgae, ammonium was measured by the phenate colorimetric method (Solorzano 1969) adapted to a microplate reader (Versa Max tunable microplate reader; Molecular Devices, Sunnyvale, CA, USA) (Hernández-López and Vargas-Albores 2003). In addition, analyses of the wastewater content were done by the Analytical Service Unit at CIBNOR using the following analytical methods: ammonium (as above), nitrate and nitrite (by cadmium reduction assays), and orthophosphate (by molybdate assay). For these assays, an analyzer (Flow Injection Analysis, Lachat QuikChem series 8000; FIAS+, Loveland, CO, USA) was used according to the manufacturer's manuals (APHA, AWWA, WEF 2005). BOD_5 was assayed by incubation in Winkler bottles with dissolved oxygen assay by the iodine titration method (APHA, AWWA, WEF 2005). Primary amines were quantified by the *o*-phthalaldehyde colorimetric method (Roth 1971) adapted to the microplate with a fluoraldehyde reagent solution (26025; Pierce, Rockford, IL, USA). Small-chain organic acids were extracted from wastewater samples with ether and quantified by injecting 1 µL samples into a gas chromatograph equipped with a flame ionization detector (Model 5890 Series II; Hewlett Packard, Palo Alto, CA, USA) equipped with a Omegawax Supelco 24136 30 × 0.35 mm column (Supelco, St. Louis, MO, USA) under the following conditions: initial temperature of 80°C for 2 min, temperature increase change rate of $8°C \cdot min^{-1}$, and final temperature of 140°C (APHA, AWWA, WEF 2005). The concentration of Na-acetate in the growth medium was quantified, using a kit for acetic acid (K-ACETRM 06/07; Megazyme International Ireland, Bray, Ireland) employing the acetate kinase-phosphotransacetylase enzymatic method (Beutler 1988). The following analyses have been made in situ at the sample site: water flow (OCM-III; Siemens Multitronics, Munich, Germany), pH, salinity, temperature, and conductivity with a multiparametric instrument (U-10; Horiba, Kyoto, Japan).

Growth measurement of the microorganisms. Proliferation of *C. vulgaris* was measured by taking samples every 24 or 48 h and

counting the number of cells under a light microscope (Olympus BX41, Olympus, Tokyo, Japan) with a Neubauer Hematocytometer (Gonzalez and Bashan 2000) connected to an image analyzer that automatically quantified the population (Image ProPlus 4.5; Media Cybernetics, Bethesda, MD, USA). Growth rate (μ) was determined by the formula:

$$\mu = (\ln N_{t_1} - \ln N_{t_0})/(t_1 - t_0) \quad (1)$$

where N_{t_1} is the number of the cells at sampling, and N_{t_0} is the initial number of cells at the beginning of the experiment (Oh-Hama and Miyachi 1992).

Calculations of nitrogen mass balance. We assumed that for each day of incubation the remaining nitrogen in the culture medium plus the nitrogen in the generated biomass is equal to the total nitrogen in the growth system (the flask). Consequently, nitrogen mass balance was calculated using the following four equations:

$$N_m + N_b = N_t \quad (2)$$

where N_m is the remaining nitrogen in synthetic wastewater (in $mg \cdot L^{-1}$ by direct measurements), N_b is the nitrogen in biomass per liter of culture ($mg \cdot L^{-1}$, calculated by eq. 4), N_t is the total nitrogen in the system (in $mg \cdot L^{-1}$, calculated), and N_E is the error nitrogen, lack or excess of nitrogen in the flask (in $mg \cdot L^{-1}$).

$$N_E = N_t - N_{tt} \quad (3)$$

where N_{tt} is the theoretical total nitrogen in the flask (the initial concentration of nitrogen in the medium, 50 $mg \cdot L^{-1}$, a constant value). N_b was calculated according to equation 4.

$$N_b = (\%N_b \times X)/100 \quad (4)$$

where $\%N_b$ is the fraction of nitrogen in biomass (constant value between 6.6% and 10.5%, Mandalam and Palsson 1998, Boyle and Morgan 2009) and X is the biomass dry weight in culture ($mg \cdot L^{-1}$, calculated value by eq. 5).

$$X = N \times X_c \quad (5)$$

where N is the number of cells per liter of culture ($cells \cdot L^{-1}$ by direct measurements), and X_c is the biomass dry weight per cell (27.5 $pg \cdot cell^{-1}$, a constant value, de-Bashan et al. 2005).

General index. The general index of the performance of the microalgae (growth and removal of ammonium), using different carbon source supplements, employed a scale of 1–5, where *C. vulgaris* population scores ($cells \cdot mL^{-1}$) are 5 ($>11.0 \times 10^6$); 4 ($>8.0 \times 10^6$); 3 ($>5.0 \times 10^6$); 2 ($>2.5 \times 10^6$); and 1 ($>1 \times 10^6$). Residual ammonium scores ($mg \cdot L^{-1}$) are 4 (>22.0); 3 (>32.0); 2 (>42.0); 1 (>49.00).

Experimental design and statistical analysis. The experiments were performed in 50 mL glass tubes and 500 mL Erlenmeyer flasks, mostly in the dark, unless otherwise specified. Each experiment was performed in triplicate, where one flask or tube served as a replicate. The setup was of batch cultures. Three 1 mL samples were taken for each medium for analysis at each sampling time. Each experiment was repeated two times. Results were analyzed first by one-way analysis of variance (ANOVA) and then by Tukey's post hoc analysis or by Student's *t*-test, with significance set at $P < 0.05$ for both analyses with statistical software (Statistica v. 6.0; StatSoft, Tulsa, OK, USA).

RESULTS

Analysis of municipal wastewater. Most of the ecophysical parameters of the water varied only slightly, regardless of the sampling date: water flow was 331.7 ± 19.84 $L \cdot s^{-1}$; water temperature, 24.01 ± 0.22°C; pH, 7.37 ± 0.02; salinity, 1.08 psu; conductivity, 2.27 ± 0 $mS \cdot cm^{-1}$; turbidity, 3.38 ± 0.23 NTU; and BOD_5, 22.7 ± 2.04 mg O_2. Nutrients in the wastewater were analyzed on the basis of average total amount and their variation per day and hour of sampling. On average, we detected high levels of ammonium and nitrate, medium levels of phosphate, and low levels of nitrite (Fig. 1a) comparable to samples taken from this wastewater treatment plant before (de-Bashan et al. 2002, 2004, Hernandez et al. 2006). Ammonium and phosphate were higher only in the night samples, nitrate levels did not change, and nitrite increased in the midday samples (Fig. 1b). Samples taken on the weekend contained more ammonium and less phosphorus (Fig. 1, c and d), yet nitrates did not change (data not shown). The quantity of organic molecules was relatively low and increased only on the weekend (Fig. 1e). Hourly analyses showed only small differences (Fig. 1f). The main organic compounds, regardless of the day or the hour of sampling, were acetic acid, followed by caproic acid, and primary amines in third position. Small quantities of seven other organic acids were detected.

Heterotrophic growth of C. vulgaris *in municipal wastewater. C. vulgaris* was heterotrophically grown in complete darkness for 8 d on three different samples of municipal wastewater. As controls, it was also grown on saline solution, nutrient broth, and C30 medium. *C. vulgaris* did not significantly grow on any medium apart from the control nutrient broth and minimal growth occurred in the municipal wastewater cultures (Fig. 2), but the cells did not die in any of the media tested (data not shown).

Design of synthetic growth medium for heterotrophic growth of C. vulgaris. Growth of the microalgae for 10 d was further measured in the basic SGM (de-Bashan et al. 2002) modified with supplements of ammonium and phosphate concentrations found in the municipal wastewater samples, separately, with 11 carbon sources known to be used by *C. vulgaris* (Bollman and Robinson 1977, Lee 2004). SGM medium without a carbon source or solutions without microalgae served as controls. Almost no growth occurred without a carbon source; all carbon sources supported growth of *C. vulgaris* to some degree. The best carbon sources affecting high rates of growth were Na-acetate and D-glucose (Fig. 3a) and provided the highest removal of ammonium from the medium (Fig. 3b). L-arabinose, DL-lactic acid, and citrate failed to remove significant amounts of ammonium. Measurements of ammonium were not possible in synthetic wastewater with urea and peptone because of very high values of the chromogenic reaction in the analysis (Fig. 3b). General evaluation of the effect of supplementations with carbon sources on growth and ammonium removal indicates that Na-acetate and D-glucose supported the highest production. Urea was the least effective (Table 1). Nitrogen mass-balance calculations, after

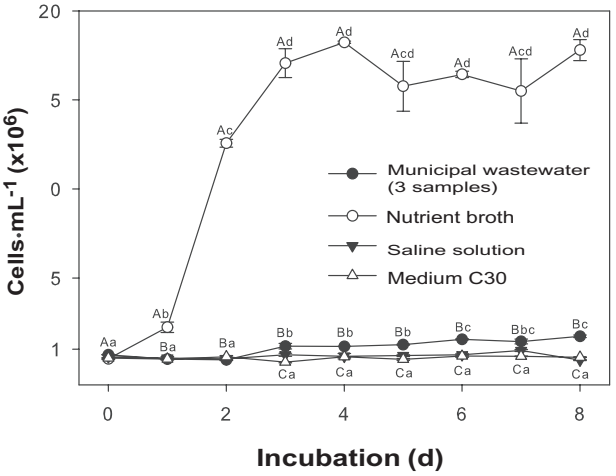

FIG. 2. Heterotrophic growth of *Chlorella vulgaris* in sterilized municipal wastewater, nutrient broth, saline solution, and microalgal medium C30 for 8 d in conical bioreactors. Points on curves denoted by different lowercase letters differ significantly at $P < 0.05$ by one-way analysis of variance (ANOVA) and Tukey's analyses. Points in each day denoted by different capital letters differ significantly at $P < 0.05$ by one-way ANOVA and Tukey's analyses. Bars represent standard error.

incubation for 5 d, indicated that the highest biomass was obtained by cultivating the microalgae with Na-acetate, less biomass with D-glucose, and far less with other substrates; variations in total nitrogen in each cultivation type (apart from cultivation on peptone and urea) were small (Table 2).

From comparisons of heterotrophic growth of *C. vulgaris* in synthetic and municipal wastewaters with different concentrations and sources of acetate and glucose, we determined that Na-acetate and D-glucose in synthetic wastewater supported higher population development than autotrophic growth (serving as the control). Supplementation with Ca-acetate, sucrose, and vinegar support meager multiplication of *C. vulgaris*, and absence of a carbon source completely inhibited multiplication (Fig. 4a), but the microalgae did not die in any treatment (data not shown). The consumption of Na-acetate in synthetic wastewater by *C. vulgaris* under heterotrophic conditions showed a significant decline in the concentration of Na-acetate from the first day, followed by a gradual decline to approximately half in 3–5 d, reaching the minimal level after 10 d of incubation (Fig. 4b). At the same time, the microalgae multiplied. Increasing the concentration of Na-acetate in synthetic wastewater yielded increased growth of *C. vulgaris* up to a concentration of 0.12 M. Excess acetate inhibited growth (Fig. 4c). The positive effect of 0.12 M Na-acetate also occurred in municipal wastewater, but to a smaller extent (Fig. 4c). Similarly, increasing the concentration of D-glucose increased the population of *C. vulgaris*, but the populations were smaller than what occurred with Na-acetate; compare Figure 4, c and d, at a concentration of 120 mM.

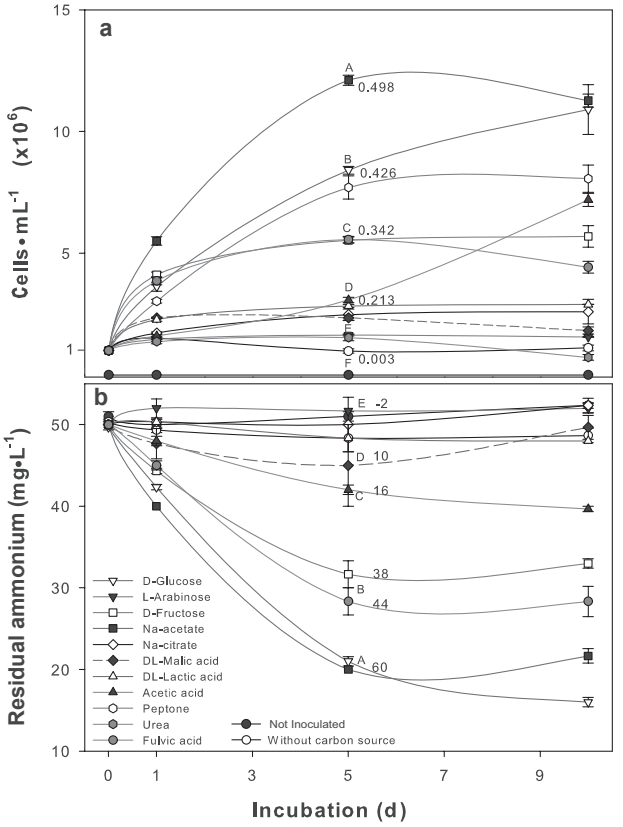

FIG. 3. (a) Growth curves and growth rates of *Chlorella vulgaris* growing under heterotrophic conditions and (b) residual ammonium after *C. vulgaris* heterotrophic growth in synthetic wastewater supplemented separately with 11 different carbon sources. Numbers indicate the growth rate per day of the microalgae measured after 5 d of incubation (a) and the level of ammonium removed at that date by percentage (b). Values denoted by a different letter at each subfigure differ significantly at $P < 0.05$ by one-way analysis of variance (ANOVA) and Tukey's analyses. Bars represent standard error.

Heterotrophic ammonium removal from wastewater by C. vulgaris. Removal of ammonium by *C. vulgaris* from synthetic and municipal wastewaters was enhanced by supplements of 0.12 M Na-acetate or D-glucose when grown heterotrophically, compared to heterotrophic growth in the absence of a carbon source, and this was statistically equal to removal of ammonium under autotrophic conditions (Fig. 5). Removal was greater in synthetic wastewater, using the same concentration of 0.12 M Na-acetate. Increasing glucose concentration up to 0.18 M slightly improved removal of ammonium. Increasing Na-acetate to this concentration did not increase removal of ammonium, compared to the 0.12 M treatment (Fig. 5).

DISCUSSION

Heterotrophic growth of microalgae has significant economic advantage over autotrophic growth for mass production of microalgae, provided the

TABLE 1. General index of the effect of different carbon source supplements on heterotrophic growth and on ammonium removal by *Chlorella vulgaris* in synthetic wastewater.

	Scores		
	C. vulgaris population	Residual ammonium	Total
Na-acetate	5	4	9
D-glucose	4	4	8
D-fructose	3	3	6
Fulvic acids	3	3	6
Peptone	4	NM	4
Acetic acid	2	2	4
Malic acid	2	1	3
Lactic acid	2	1	3
Na-citrate	2	1	3
L-arabinose	1	1	2
Without organic carbon	1	1	2
Urea	1	NM	1
Not inoculated	ND	1	1

C. vulgaris population scores (cells · mL^{-1}): 5 (>11.0×10^6); 4 (>8.0×10^6); 3 (>5.0×10^6); 2 (>2.5×10^6); 1 (>1×10^6). Residual ammonium scores (mg · L^{-1}): 4 (>22.0); 3 (>32.0); 2 (>42.0); 1 (>49.00). ND, not detected; NM, not measurable.

TABLE 2. Nitrogen mass balance of heterotrophic cultures of *Chlorella vulgaris* growing on different substrates after 5 d of incubation (calculated per liter of culture in mg · L^{-1}).

Substrate	N_m	N_b	N_t	N_{tt}	N_E
Na-acetate	20.2	34.7	54.9	50.0	+4.9
D-glucose	22.1	24.3	46.4	50.0	−3.6
D-fructose	31.8	15.8	47.6	50.0	−2.4
Fulvic acids	28.3	15.6	43.9	50.0	−6.1
Peptone	188.7	22.5	211.2	50.0	+161.2
Acetic acid	42.5	8.9	51.5	50.0	+1.5
Malic acid	45.0	4.1	49.1	50.0	−0.9
Lactic acid	48.3	4.9	53.2	50.0	+3.2
Na-citrate	50.5	4.4	54.9	50.0	+4.9
L-arabinose	51.7	2.8	54.5	50.0	+4.5
Without organic carbon	48.0	1.7	49.8	50.0	−0.2
Urea	178.2	2.6	180.8	50.0	+130.8
Not inoculated	50.0	0.0	50.0	50.0	0.0

N_m, remaining nitrogen in synthetic wastewater; N_b, nitrogen in biomass per liter of culture; N_t, total nitrogen in the flask, initial concentration of nitrogen in the medium; N_{tt}, theoretical total nitrogen in the flask; N_E, error nitrogen, lack or excess of nitrogen in the flask.

Specific equations are listed in Materials and Methods.

appropriate microalgae are chosen and grown under appropriate conditions (Chen and Chen 2006). Most applications of microalgae use light because microalgae are very efficient solar energy converters; they can produce a great variety of metabolites (Lebeau and Robert 2006) and can efficiently clean natural wastewater (de-Bashan et al. 2002, 2004, Hernandez et al. 2006). The main difficulty facing commercialization of new microalgae and microalgal products is the need for closed, capital-intensive systems. The high cost of these processing systems relates to the need for light and the relatively slow growth rate of these algae. Consequently, autotrophic closed systems are justified only when a fine, high-value chemical is the goal (Borowitzka 1999, Lebeau and Robert 2006). Thus, under standard wastewater treatment, high-volume domestic wastewater treatment cannot economically support costly operations. Heterotrophic treatment may open possibilities for cost reduction (Chen 1996, Lee 2001).

Our study showed that the municipal wastewater generated by a medium-sized city without significant industrial wastes contained large amounts of nitrogenous compounds, but insufficient small carbon molecules capable of supporting very large growth of *C. vulgaris*. The wastewater of La Paz after secondary treatment resembles, in composition, gray water of other cities (Eriksson et al. 2002), only enriched by nitrogen. The practice of adding untreated wastewater to enhance carbon concentration essential for tertiary wastewater treatment is not practiced in the La Paz wastewater treatment plant. Consequently, for tertiary treatment by heterotrophic microalgae, a source of available organic carbon should be supplied.

This study demonstrated that it is possible to heterotrophically grow dense populations (>10^7 CFU · mL^{-1}) of *C. vulgaris* in filtered-sterilized wastewater if certain carbon sources are added. These densities are higher than normal autotrophic populations of this species by at least one order of magnitude (Lau et al. 1995, Gonzalez and Bashan 2000). The population differences between autotrophic and heterotrophic could be explained by a better ATP economy. Normally, autotrophic carbon fixation by the Calvin cycle consumes ~70% of the ATP present in the cells; an ATP yield of 3.11 g biomass · $mmol^{-1}$ ATP produced, compared to 19.3 g biomass · $mmol^{-1}$ ATP yields in heterotrophic cells (Yang et al. 2000). Generally, higher populations of *C. vulgaris* in wastewater seemed to be more beneficial because these treatments achieve satisfactory nutrient removal in less time (de-Bashan et al. 2004, Hernandez et al. 2006). This is also true for some other applications of microalgae, where, with higher densities, the better the results (Chen 1996). Therefore, a treatment that enhances the microalgal population is a useful goal to enhance the value of the treatment with by-products.

The best supplements for enhancing the microalgal population in wastewater were Na-acetate and D-glucose. These compounds were well known as basic nutrients of *Chlorella* sp. in autotrophic culture medium (Kaplan et al. 1986) and are used for heterotrophic cultivation of microalgae for other objectives (Chen and Johns 1996). The relatively simple metabolism of these compounds and its link to assimilating ammonium can explain why our system worked. Under dark aerobic conditions, as in this study, microalgae take up acetate with a

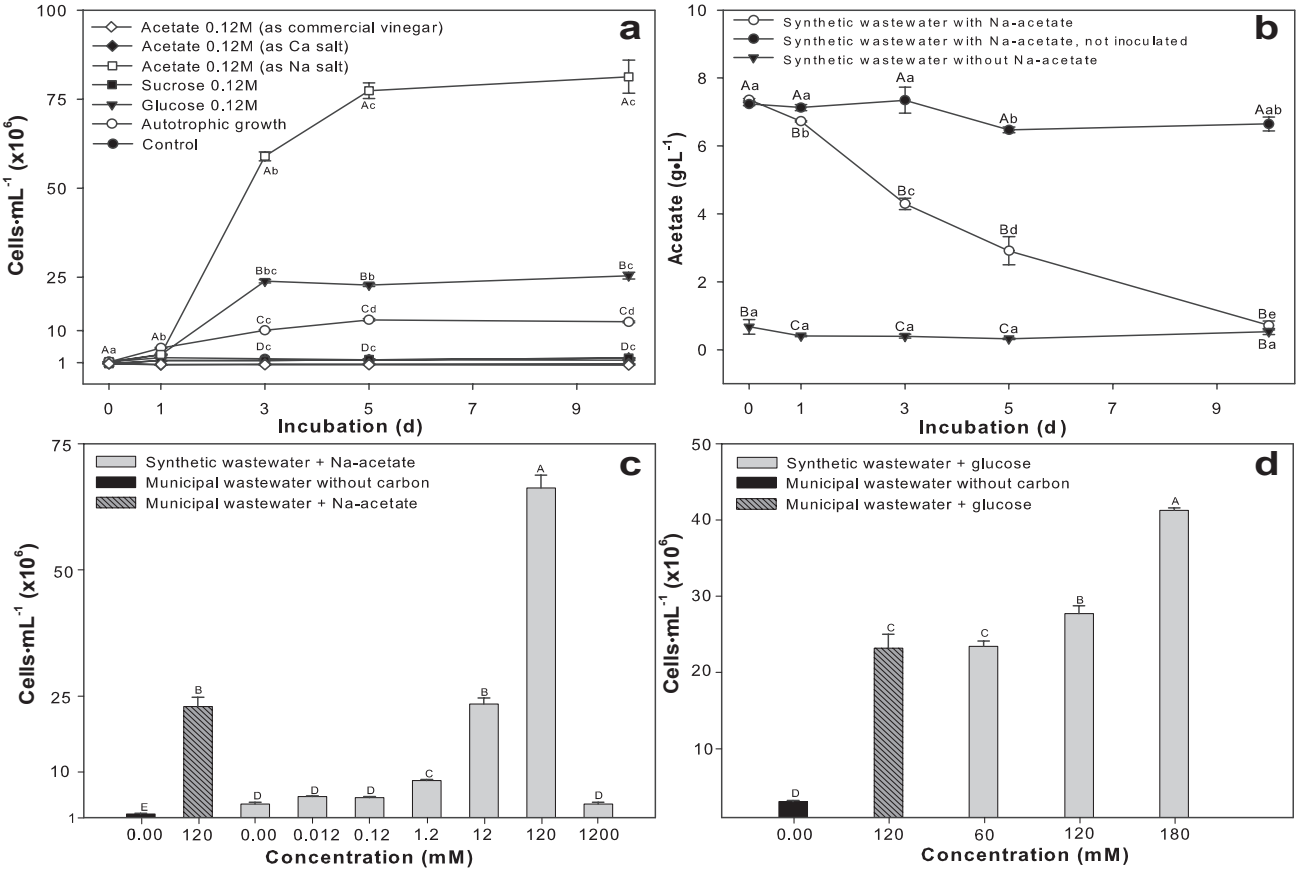

FIG. 4. Heterotrophic growth of *Chlorella vulgaris* on synthetic and municipal wastewater supplemented with different carbon sources and the consumption of acetate. (a) Growth in synthetic wastewater medium supplemented with glucose, sucrose, Na, Ca-acetate, and acetate as commercial vinegar. (b) Acetate consumption in synthetic wastewater. (c) Growth yield in synthetic and municipal wastewater supplemented with increasing levels of Na-acetate. (d) Growth yield in synthetic and municipal wastewater supplemented with increasing levels of glucose. Data for (c) and (d) are after 5 d of incubation. Curves denoted with different lowercase letters in each subfigure differ significantly at $P < 0.05$ by one-way analysis of variance (ANOVA) and Tukey's analyses. In (a) values of incubation day denoted with capital letters differ significantly at $P < 0.05$ by one-way ANOVA and Tukey's analyses. Bars represent standard error (SE). Absence of a bar indicates negligible SE.

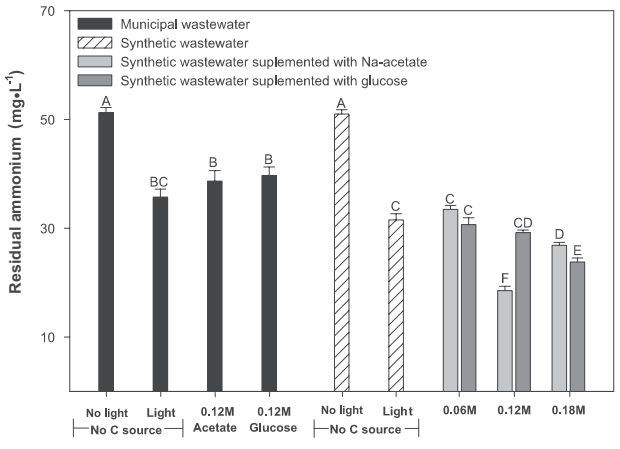

FIG. 5. Residual ammonium in the medium under heterotrophic growth conditions of *Chlorella vulgaris* in sterilized municipal wastewater and synthetic wastewater supplemented with Na-acetate or glucose. Columns denoted by different letters differ significantly at $P < 0.05$ by one-way analysis of variance (ANOVA) and Tukey's analyses. Bars represent standard error.

monocarboxylic/proton symport (a protein that aids transport of monocarboxylic molecules across the membrane). Once inside the cell, acetate is assimilated as part of the acetyl-coenzyme A (acetyl-CoA) metabolism in a single-step reaction catalyzed by acetyl-CoA synthetase (de Swaaf et al. 2003, Boyle and Morgan 2009). Acetate is generally oxidized metabolically through the glyoxylate cycle to malate in glyoxysomes and through the tricarboxylic acid cycle (TCA) to citrate in the mitochondria, providing carbon skeletons, energy, and reducing power; many of the intermediates of both cycles are the same metabolites (Neilson and Lewin 1974, Ahmad and Hellebust 1990, Boyle and Morgan 2009). Glucose is consumed via an active hexose/proton symport system regulated by the concentration of glucose and H^+ (Komor et al. 1985) and assimilated mainly through the pentose phosphate pathway followed by the TCA cycle (Yang et al. 2000). Carbon and nitrogen metabolism are linked because they must share organic carbon and energy supplied

directly from respiration of fixed (autotrophic) and assimilated (heterotrophic) carbon, the TCA cycle, and the mitochondrial electron transport chain. The primary assimilation of inorganic nitrogen (ammonium) into amino acids requires carbon skeletons in the form of keto acids (2-oxaloglutarate) and energy in the form of ATP. In both autotrophic and heterotrophic cells, the keto acids are intermediates of the TCA cycle (Inokuchi et al. 2002).

We observed that Na-acetate was a significantly better substrate for growth than Ca-acetate. This may be a consequence of some cations (Ca^{2+}, Mg^{2+}, and K) varying from deficiency to toxicity levels for growth of *Chlorella* sp.; Ca^{2+} can inhibit the normal growth of *C. vulgaris* at 40 mM (Trelease and Selsam 1939) and of *Chlorella sorokiniana* at 50 mM (MacCarthy and Patterson 1974). Our study employed higher concentrations of Ca-acetate (60 and 120 mM). The carbon supplements Na-acetate and glucose allowed the microalga to remove ammonium from synthetic and municipal wastewater under heterotrophic growth conditions at a level equivalent to ammonium removal by illuminated *C. vulgaris* (de-Bashan et al. 2002, 2004, 2008).

Our strategy of adding organic carbon to wastewater that already contains large amounts of carbon compounds was previously tested to enhance performance of a biological agent used in decontamination of wastewater. Kalogo et al. (2001) used a water extract from seeds of the horseradish tree *Moringa oleifera* to enhance the start-up of a self-inoculated up-flow anaerobic sludge blanket reactor treating raw domestic wastewater, which also enhanced microbial activity. Wastewater plants employing an EBPR scheme used carbon supplements, mainly acetate, as part of the process of removing phosphate (Lemos et al. 1998, Ahn et al. 2001). For EBPR processes, glucose was experimentally tested (Wang et al. 2002), and supplementation of different ratios of propionic to acetic acid in real wastewater supplemented with volatile fatty acids was also investigated (Chen et al. 2004).

So far, supplementation with acetate was the most economical material for wastewater treatment. The substrate, at industrial quality, is inexpensive (de-Bashan and Bashan 2004). Supplementation with glucose and acetate was also used for industrial-scale processes for heterotrophic production of microalgal aquaculture feed (Day et al. 1991, Tsavalos and Day 1994). For practical purposes of wastewater treatment, take-up of ~50% of the added Na-acetate in 3 d is a reasonable retention time. Furthermore, it is advantageous if more retention time is needed for treatment of specialized wastewater. This occurs because a single application of Na-acetate can support the heterotrophic growth of the microalgae for up to 10 d without a second application.

One reservation should be mentioned. Practical systems of wastewater treatment are unlikely to be based on a pure species, unless such a species can be protected from competing and faster-growing bacteria in the mixed-population environment common to wastewater treatment systems. Such protective systems were previously developed, based on immobilization of microalgae in an assortment of polymer matrices (Lau et al. 1997, Tam and Wong 2000, de-Bashan and Bashan 2004, Hernandez et al. 2006), and are currently being tested for this system, as well (Perez-Garcia et al. 2010; S. A. Covarrubias, L. E. de-Bashan, and Y. Bashan, unpublished data).

In summary, our study demonstrated that supplementing municipal wastewater with acetate or glucose performs well for removing excess ammonium when using *C. vulgaris* as the agent; refinements of the experimental procedure as well as the actual application of the procedure are pending.

CONCLUSIONS

To allow *C. vulgaris* to grow heterotrophically on effluent from a secondary wastewater treatment plant without industrial waste products, the wastewater needs to be supplemented with an organic carbon source. Although several carbon sources support heterotrophic growth of *C. vulgaris*, acetate and glucose are the most efficient supplements for heterotrophic removal of ammonium from municipal wastewater. Heterotrophic growth has a sounder potential than autotrophic growth to produce large microalgal populations; the population density of *C. vulgaris* under heterotrophic growth is one order of magnitude higher than that under autotrophic culturing. Efficiency of removing ammonium under heterotrophic culturing is equal to autotrophic culturing.

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