Biotechnol Lett (2009) 31:1043–1049
DOI 10.1007/s10529-009-9975-7

ORIGINAL RESEARCH PAPER

Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions

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Received: 4 February 2009 / Revised: 4 March 2009 / Accepted: 9 March 2009 / Published online: 27 March 2009

Abstract Biomass and lipid productivities of *Chlorella vulgaris* under different growth conditions were investigated. While autotrophic growth did provide higher cellular lipid content (38%), the lipid productivity was much lower compared with those from heterotrophic growth with acetate, glucose, or glycerol. Optimal cell growth (2 g l^{-1}) and lipid productivity (54 mg l^{-1} day^{-1}) were attained using glucose at 1% (w/v) whereas higher concentrations were inhibitory. Growth of *C. vulgaris* on glycerol had a similar dose effects as those from glucose. Overall, *C. vulgaris* is mixotrophic.

Keywords Algal fuels · Autotrophic · Biodiesel · *Chlorella vulgaris* · Glycerol · Heterotrophic · Lipid productivity

Introduction

Utilizing microalgal lipids has been of interest since Harder and von Witsch (1942) proposed the mass cultivation of diatoms to produce urgently needed fat in World War II. From 1978 to 1996, US Department of Energy (DOE) dedicated $25 million to the Aquatic Species Program (ASP) to identify high lipid yielding strains and to develop technologies for producing an algal-derived liquid fuel (Sheehan et al. 1998). This program was terminated due to the finding that producing fuel from microalgae was feasible, but not cost-effective. It has been commonly observed that under nitrogen deficiency, microalgae would increase cellular lipid level from 20–30% to 60–70%, but the growth rate was greatly reduced resulting in the overall lipid productivity being unchanged. Hence, low lipid productivity was the primary concern and bottleneck for large-scale biodiesel production (Benemann and Oswald 1996; Chisti 2007).

The ASP program only investigated the autotrophic growth mode of microalgae. Autotrophic growth does provide several advantages. For example, (1) microalgae can harvest the radiant energy from the sun into valuable products at the expense of inexpensive natural resources (e.g. CO_2 and H_2O) (Carvalho et al. 2006), which contributes to global CO_2 reduction; (2) microalgae can bloom at places where salty water, excessive sun exposure, and lack of vital nutrients inhibit other crops to grow (Chisti 2007, 2008). The idea of autotrophic growth of microalgae for biodiesel production is interesting, tempting, and technically feasible. This culture mode, however, is difficult to reach a high density of microalgae biomass since light penetration is inversely proportional to the cell concentration (Chen and Johns 1991, 1995). Additionally, mutual shading of cells can also cause light insufficiency, which leads to a very low algal lipid

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productivity resulting from low biomass productivity (Martinez and Orus 1991). Furthermore, low biomass concentration also increases the biomass harvesting cost (Benemann and Oswald 1996; Wen and Chen 2003).

To develop cost-effective algal oil production, microalgae can be cultured in heterotrophic conditions where organic carbons, such as sugars and organic acids, serve as carbon sources. This mode of culture eliminates the requirement for light and therefore, offers the possibility of greatly increased cell density and productivity (Chen 1996). Some microalgae can grow rapidly heterotrophically (Vazhappilly and Chen 1998a, b; Miao and Wu 2004, 2006; Xu et al. 2006). Heterotrophic algal cultivation has been reported to provide not only a high algal biomass productivity, but high cellular oil content as well (Miao and Wu 2004; Xu et al. 2006; Li et al. 2007). In the case of *Chlorella protothecoides*, heterotrophic growth on corn powder hydrolysate results in a 3.4 times higher biomass yield than that from autotrophic growth while the lipid content is increased 4.2 times.

However, even though the biomass and lipid productivities are significantly higher compared with those from autotrophic growth, the cost of the organic carbon sources (usually in the form of glucose or acetate) is high when compared against all other added nutrients. To overcome this high carbon cost, a cheap resource must be found. Crude glycerol, which is derived from biodiesel production processes, is capable of providing such a supply.

As biodiesel production continues to increase, the market is being flooded with crude glycerol (Thompson and He 2006). Crude glycerol prices have dropped from \$0.25/lb in 2004 to \$0.025–0.05/lb in 2006 (Johnson and Taconi 2007). The increased supply and low demand for crude glycerol have pushed biodiesel producers into eagerly seeking ways to dispose this by-product.

Chlorella has been the oldest commercial application of microalgae. Green algae have the bulk of their fatty acids as saturated and unsaturated $C18_s$, a composition similar to that of vegetable oils (Benemann and Oswald 1996). *C. vulgaris*, a freshwater, fast-growing green alga, has different lipid production capabilities (30–40% of dry weight) under natural conditions (Pratt and Johnson 1963; Nichols et al. 1967; Harris and James 1969; Podojil et al. 1978). Its heterotrophic growth mode with the presence of glucose or acetate has been studied in the 1960s and 1970s. However, at that time, the focus was not on lipid production (Tanner 1969; Komor and Tanner 1971, 1974; Haass and Tanner 1974). In this study, the cell growth and lipid production under autotrophic and heterotrophic conditions were compared. The uptake of three carbon sources, glucose, acetate, and glycerol were evaluated. This is also the first study to investigate the *C. vulgaris* growth on glycerol.

Materials and methods

Strain and growth medium

Chlorella vulgaris #259 was purchased from Culture Collection of Alga at the University of Texas (UTEX, Austin, TX). The cells were maintained in a medium containing (per liter): 0.25 g $NaNO_3$, 0.025 g $CaCl_2 \cdot 2H_2O$, 0.075 g $MgSO_4 \cdot 7H_2O$, 0.075 g K_2HPO_4 0.175 g KH_2PO_4, 0.025 g NaCl, and 1 g proteose peptone (Difco). The cells were grown in 250-ml Erlenmeyer flasks on an orbital shaker set at 150 rpm at room temperature.

Growth conditions

For autotrophic growth, cells were grown in 1,000 ml polycarbonate bottles with an air flow rate of 200 ml min^{-1} under constant fluorescent light. Medium with or without $NaNO_3$ was tested. In terms of heterotrophic growth, three carbon sources, acetate, glucose, and glycerol were adopted. Culture under autotrophic growth conditions served as the initial inoculum. After three subcultures, the cells were fully adapted to the supplemented carbon sources as demonstrated by rapid and repeatable growth rates. These cells were used for studying the heterotrophic growth under different conditions. Cells grown with acetate, glucose, or glycerol were evaluated regarding the effects from nitrate, from light and different glucose concentrations, and from different glycerol concentrations, respectively. Cell growth was monitored by measuring the OD_{680}.

Sampling and analyses

Cell dry weight (DW) was determined after drying the cell pellet in an oven at 80°C overnight. Lipid

extraction was performed in a way that was similar to the procedures reported by Zhu et al. (2002) and Shen et al. (2008). Briefly, 0.05 g dried cells was transferred to a 7 ml chamber of a bead-beater (BioSpec Products, Bartlesville, OK). This chamber was filled with 1 mm glass beads to approx 5 ml. Methanol was then added to fill the rest of the chamber. After cells were disrupted by bead-beating for 2 min, the entire content was transferred to a 50 ml glass centrifuge tube. The chamber was washed twice using methanol (total 10 ml) to collect the algal residue. Chloroform was then added to the tube to make the chloroform/methanol (2:1, v/v). The tube was vortexed for 5 min and was allowed to stand for 24 h. After that, the tube was centrifuged at 4,000*g* for 15 min to remove the glass bead and algal solids. The supernatant was collected and the solvent was vaporized using Rotovap. Oil left in the flask without solvent was weighed to calculate oil content.

The carbohydrate content was analyzed based on the procedure published by Miao and Wu (2004). In short, 0.1 g dried algal pellet was acidified by adding 20 ml 2.5 M HCl. The acidified solution was then hydrolyzed at 100°C for 30 min and neutralized to pH 7. The volume was adjusted to 100 ml. The filtered sample was subjected to 3,5-dinitrosalicylic acid (DNS) assay (Miao et al. 2003). The ash content was determined as the residue after ignition at 550°C overnight. The protein content was then calculated by subtraction.

Results

The relationship between DW and OD is best described by a power regression with a R^2 close to 1 within a broad OD range from 0 to 2.8. Based on this relationship, all the OD values were converted to biomass (g l^{-1}) in the following sections.

Bubbling air into the *C. vulgaris* culture exerted a positive effect on cell growth whereas the presence or absence of nitrate did not cause a significant difference. However, even with air at 200 ml min^{-1}, the growth rates under autotrophic conditions were much lower compared with those from heterotrophic growth (Fig. 1). With the presence of glucose or acetate (both 1%, w/v) in dark, cell growth rates were increased 9 or 7 times in 12 days, respectively. Between glucose and

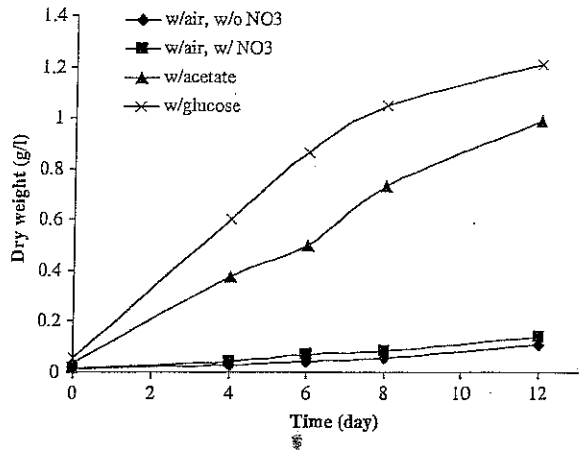

Fig. 1 Biomass dry weight changes with time under autotrophic and heterotrophic growth conditions. Under heterotrophic growth modes, cultures were incubated in dark. ◆, with air, without nitrate in the medium; ■, with air, with nitrate in the medium; ▲, with acetate (w/v, 1%); ×, with glucose (w/v, 1%)

acetate, glucose addition produced more significant effect.

The lipid productivity, a product of biomass productivity and lipid content, under different growth conditions were compared and summarized in Table 1. The maximum biomass density (2 g l^{-1}) was obtained when cells were grown with 1% (w/v) glucose with light in 6 days. The highest lipid content was attained in autotrophic growth mode without nitrate presence (38%). The highest lipid productivity (54 mg l^{-1} day^{-1}) was achieved when cells were grown with 1% glucose in light.

Different glucose and glycerol concentrations had different effects on cell growth (Figs. 2, 3 and Table 2). When compared with autotrophic growth, all the doses used showed faster growth rates. But the low dose additions of 1% and 2% for both glucose and glycerol improved biomass production significantly compared to high doses of 5% and 10%. In terms of doses of 1% and 2%, there were no statistically significant difference between them for both glucose and glycerol. However, with regard to glucose, cell growth with 5% addition was significantly higher than that from 10%. But in the case of glycerol, 5% and 10% supplements did not have a significant difference.

The cellular compositions of cells grown with glucose, acetate, or glycerol were compared in Table 3. The ash contents were basically the same among cultures from these supplements. But the other

Table 1 Biomass and lipid productivities of *Chlorella vulgaris* grown on CO_2, acetate, glucose, or glycerol

Culture conditions		Max. biomass density (mg l^{-1})	Max. biomass productivity (mg l^{-1} day^{-1})	Lipid content (%)	Lipid productivity (mg l^{-1} day^{-1})
With air[a]	With NO_3	250 ± 7	10 ± 0	38 ± 2	4 ± 0
	Without NO_3	315 ± 5	13 ± 0	33 ± 1	4 ± 0
With 1% acetate[b]	With NO_3	987 ± 9	87 ± 1	31 ± 1	27 ± 1
	Without NO_3	898 ± 16	79 ± 3	36 ± 1	29 ± 4
With 1% glucose[c]	In dark	1206 ± 20	151 ± 3	23 ± 2	35 ± 6
	With light	1696 ± 16	254 ± 3	21 ± 1	54 ± 2
With glycerol[c]	At 1%	722 ± 12	102 ± 0	22 ± 4	22 ± 5
	At 2%	656 ± 15	91 ± 1	34 ± 4	31 ± 4

[a] Data from 23-day cell growth were used for calculation

[b] Data from 12-day cell growth were used for calculation

[c] Data from 6-day cell growth were used for calculation

Data are reported as means ± standard deviation of triplicates

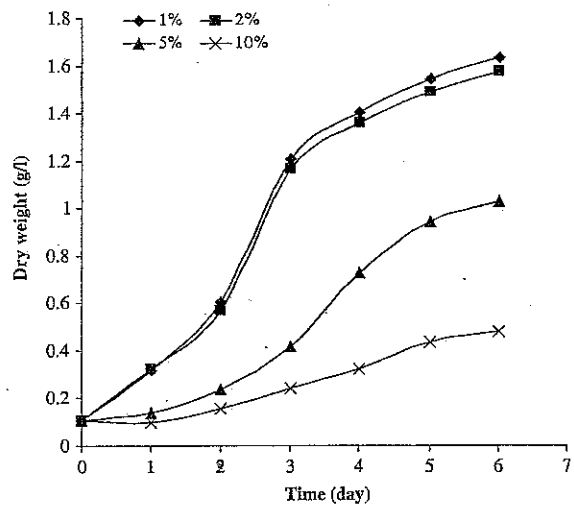

Fig. 2 Cell growth (dry weight, g l^{-1}) with time for different glucose doses (w/v) in light. ◆, 1%; ■, 2%; ▲, 5%; ×, 10%

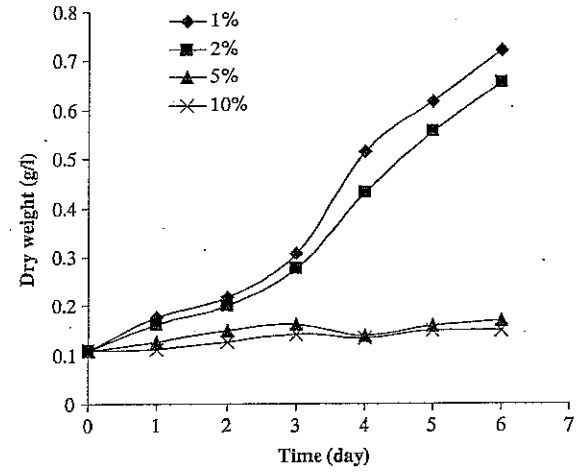

Fig. 3 Cell growth (dry weight, g l^{-1}) with time for different glycerol doses (v/v) in light. ◆, 1%; ■, 2%; ▲, 5%; ×, 10%

cellular components were different for different substrates. The culture with 2% (v/v) glycerol had a higher cellular lipid content than that from 1% (v/v) glycerol.

Discussion

Nitrogen has a prominent effect on microalgal lipid production. In this study, when no nitrate was added to the medium, growth of *C. vulgaris* was decreased but not to a significant extent when compared with

Table 2 Biomass productivity of *Chlorella vulgaris* grown with glucose (w/v) or glycerol (v/v) at different doses from 1 to 10%

Dose (%)	Dry weight (mg l^{-1} day^{-1})	
	Glucose	Glycerol
1	254 ± 3	102 ± 0
2	244 ± 2	91 ± 1
5	153 ± 1	10 ± 0
10	62 ± 0	7 ± 0

Data are reported as means ± standard deviation of triplicates

Table 3 Cellular compositions of *Chlorella vulgaris* grown with different carbon sources including glucose, acetate, or glycerol

Composition (%)	With 1% glucose	With 1% acetate	With 1% glycerol	With 2% glycerol
Carbohydrate	44 ± 0	23 ± 0	29 ± 3	34 ± 4
Lipid	21 ± 1	31 ± 1	22 ± 4	32 ± 2
Protein	32	42	45	30
Ash	3	4	4	3

Data are reported as means ± standard deviation of triplicates

that with nitrate presence (Fig. 1). The cellular lipid content was increased from 33% to 38% due to nitrogen limitation. The small, but not large increase of lipid content could be due to the presence of peptone in the medium.

Under autotrophic growth conditions, both biomass and lipid productivities were low compared with those from heterotrophic growth as shown in Fig. 1 and Table 1. *C. vulgaris* can grow on acetate in the dark and in the light with acetate being directly converted to fatty acids (Nichols et al. 1967; Harris and James 1969).

Similar acetate uptake was also reported for *Nannochloropsis* sp. (Hu and Gao 2003) and *C. protothecoides* (Xu et al. 2006). In terms of the strain investigated in this study, acetate utilization especially when nitrogen was limited, provided higher lipid content compared with those from growth on glucose. But the overall lipid productivity was not high.

C. vulgaris has been demonstrated to possess a hexose transport system which can be induced by glucose and its analogues 3-*O*-methylglucose or 6-deoxyglucose (Tanner 1969; Komor and Tanner 1971; Haass and Tanner 1974). The current study confirmed that *C. vulgaris* did grow on glucose and this growth mode produced a 14 times higher lipid productivity compared with that from autotrophic growth with nitrogen limitation. Growth on glucose can be affected by light. As reported, some strains of *C. vulgaris* can only grow in the dark (Haass and Tanner 1974) while others can only grow in the light (Karlander and Krauss 1966). Regarding the strain tested in this study, it can grow under both conditions as what have been published for another *C. vulgaris* strain (Estep and Hoering 1981). Moreover, light stimulated its growth as indicated by higher biomass and lipid productivities. Based on these evidences, the tested *C. vulgaris* is mixotrophic. This is also in agreement with the work of Patino et al. (2007). As a mixotrophile, *C. vulgaris* has the potential to reach the highest lipid productivity in large scale if shallow surface or enough light penetration is provided.

Obligate phototrophy is common in the algae world in nature (Wetherell 1958). But some microalgae can grow faster on organic carbon sources in laboratories in the dark (Vazhappilly and Chen 1998a, b; Miao and Wu 2004, 2006; Xu et al. 2006). To our knowledge, only few microalgae are mixotrophic. Freshwater flagellate *Haematococcus pluvialis* (Margalith 1999), *C. protothecoides* (Yang et al. 2000), and *Ochromonas minima* (Flöder et al. 2006) are examples. *Nannochloropsis* sp. (Hu and Gao 2003) is also mixotrophic, but it cannot uptake glucose. One strain of *C. protothecoides* can grow on glucose, acetate, and other organic compounds in the dark but it is unknown whether it can grow on organic carbon sources with the presence of light (Xu et al. 2006). As discussed above, *C. vulgaris* #259 is very versatile on this aspect, which may allow its broad uses in various applications.

To date, the dose effects from glucose on microalgal growth have not been intensively explored. In this study, under mixotrophic growth conditions, 1% and 2% glucose improved cell growth significantly compared with those at 5% and 10%. The highest glucose addition (10%) was exert inhibitory. This phenomenon was also observed for *C. protothecoides* when it was fed with glucose within the range between 1.5 and 6%. However, the negative influence of higher glucose concentration only appeared during the first 3 days of culture. After that time, higher glucose concentration produced higher biomass productivity in 9 days (Xiong et al. 2008). Hence, the substrate inhibition effect is strain-dependent and needs to be evaluated for individual study strain.

Glycerol uptake by some microalgal species has been described. For example, lipids and pigments

have been produced from glycerol by cyanobacterium *Spirulina platensis* (Narayan et al. 2005). Docosahexaenoic acid (DHA) and other lipids were generated from a marine microalga, *Schizochytrium limacinum* SR21 grown on glycerol (Pyle et al. 2008). However, glycerol utilization has not been reported for *Chlorella* species. In this study, high glycerol concentration had an inhibitory effect as what have been seen with glucose. A fed-batch process will be essentially necessary with regard to glycerol utilization if maximum rate of glycerol consumption is desired.

In summary, the investigated *C. vulgaris* strain is mixotrophic. Growing on glucose with light produced the highest lipid productivity compared with other growth modes. Both glucose and glycerol had inhibitory effects at high concentrations. Concentrations of 1% provided the fastest growth rate. With a 2% glycerol concentration, both the cellular lipid content and lipid productivity were higher than that from 1% concentration. This capability is attractive in light of the fact that large amount of crude glycerol is being generated and more will come from the biodiesel production industry in the future.

Acknowledgments This research is supported by Southern Illinois University Carbondale (SIUC)'s new faculty start-up funds. Financial support from SIUC Material Technology Center is also greatly acknowledged.

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