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Molasses-based growth and production of oil and astaxanthin by *Chlorella zofingiensis*

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ABSTRACT

The aim of this study is to evaluate the industrial waste cane molasses as a carbon source for cell growth, lipid and astaxanthin production of *Chlorella zofingiensis*. Pretreated with cation exchange resin to remove the metal ions, cane molasses provided better productivities of biomass, lipid, and astaxanthin (1.55, 0.71 g L^{-1} day^{-1} and 1.7 mg L^{-1} day^{-1}, respectively) than glucose. Using a strategy of semi-continuous cultures coupled with feeding at a low concentration, molasses without pretreatment has the same effect as pretreated one on supporting the algal cell growth, lipid and astaxanthin production. The efficient metabolism of molasses triggered the up-regulation of genes involved in fatty acid and also astaxanthin biosynthesis, leading to the very high production of the two metabolites. This study highlights the possibility of using *C. zofingiensis* to deal with industrial wastes and to produce profitable biodiesel as well as the high-value astaxanthin.

1. Introduction

Biodiesel has been employed to solve the problems of energy and environment due to its sustainable and environmentally beneficial properties (Bozbas, 2008; Janaun and Ellis, 2010). Currently, biodiesel is mainly produced from plant oils, which can only meet a fraction of the soaring demand for transport fuels (Chisti, 2007). Microalgae are alternative feedstocks for biodiesel production in that they have faster growth rates and higher yields of oils (Del Campo et al., 2007; Chisti, 2008; Hu et al., 2008; Pruvost et al., 2009). Examples are the green microalgae *Chlorella* species, which have long been commercially cultured for human food and animal feed and more recently for biofuels using either photoautotrophic (open pound) or heterotrophic (fermentation) culture systems (Xu et al., 2006; Chiu et al., 2008; Liu et al., 2010a).

Heterotrophic cells of the microalga *Chlorella zofingiensis* were found to be superior to photoautotrophic one as feedstocks for biodiesel production (Liu et al., 2011). However the need of an organic carbon source (mostly glucose) for heterotrophic cultures makes it not as economically favorable as photoautotrophic cultures used for low-value biodiesel production. Cheap carbon sources like "cellulosic" materials or industrial waste sugars have been proposed to replace glucose (Xu et al., 2006; Cheng et al., 2009; Jiang et al., 2009). The most promising one may be cane molasses, which is a waste of the sugar industry, consisting of approximately 50% (w/w) total sugars (mainly sucrose, glucose and fructose), water, crude protein and fat, heavy metals, vitamins and others (Jiang et al., 2009). Cane molasses with proper treatment was found to be a suitable carbon source for the heterotrophic cultures of a number of microorganisms (Liu et al., 2008; Sharma et al., 2008; Jiang et al., 2009; Yan et al., 2011).

C. zofingiensis is a biotechnologically important microalga that is able to accumulate high amounts of both lipids and the high-value ketocarotenoid astaxanthin (Ip and Chen, 2005; Sun et al., 2008; Liu et al., 2010b). This study investigated the potential of *C. zofingiensis* fed with cane molasses for cell growth, lipid and astaxanthin production.

2. Methods

2.1. Algal strain and culture conditions

C. zofingiensis (ATCC 30412) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). This alga was maintained at 4 °C on an agar slant containing the Kuhl medium (Liu et al., 2010b) consisting of (per liter) 1.01 g KNO_3; 0.62 g

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$NaH_2PO_4{\cdot}H_2O$; 0.089 g $Na_2HPO_4{\cdot}2H_2O$; 0.247 g $MgSO_4{\cdot}7H_2O$; 14.7 mg $CaCl_2{\cdot}2H_2O$; 6.95 mg $FeSO4{\cdot}7H_2O$; 0.061 mg H_3BO_3; 0.169 mg $MnSO_4{\cdot}H_2O$; 0.287 mg $ZnSO_4{\cdot}7H_2O$; 0.0025 mg $CuSO_4{\cdot}5H_2O$; and 0.01235 mg $(NH_4)_6MO_7O_{24}{\cdot}4H_2O$. The pH of the medium was adjusted to pH 6.5 prior to autoclaving. Briefly, 10 mL of liquid Kuhl medium was inoculated with cells from slants and the alga was grown aerobically in flasks at 25 °C for 4 days with orbital shaking at 150 rpm and continuous illumination of 50 μmol photon m^{-2} s^{-1}. The cells were then inoculated at 10% (v/v) into a 250-mL Erlenmeyer flask containing 50 mL of the medium for growth. The algal cells at exponential growth phase were used as seed cells for the following cultures.

For heterotrophic batch cultivation, the dark-adapted seed cells were inoculated into 100 mL fresh medium in 500 mL flasks containing various sugars (glucose, fructose, sucrose, molasses), giving a starting cell density of 0.5 g/L and growing in the absence of light at 25 °C with orbital shaking of 150 rpm.

For semi-continuous cultivation, the seed cells were inoculated and grown in the 1-L bioreactor (Bioengineering Ag, Wald, Switzerland). The working volume of the bioreactor was 800 mL. The cultivation conditions in the bioreactor were controlled as follows: temperature 25 °C; agitation 300 rpm; air flow 1 v v^{-1} min^{-1}. No light was provided. When the batch cultures grew to the late exponential growth phase, semi-continuous cultivation was started by feeding fresh medium at different dilution rates (D), specifically 0.3, 0.4, 0.5 and 0.6 day^{-1}, corresponding to 30%, 40%, 50% and 60% of volume renewal per day.

2.2. Pretreatment of cane molasses

Cane molasses was obtained from Jiang-men sugar-refinery (Guangdong, PRC). It contained 25% (w/w) water, 35% (w/w) sucrose, 10% (w/w) reduced sugars (glucose and fructose), 2.5% (w/w) other carbohydrates, 4.3% (w/w) crude protein, 0.06% (w/w) crude fat, 9.6% (w/w) ash, 4.6% (w/w) salt, 8.9% (w/w) metal ions such as calcium, potassium, sodium, iron, magnesium, copper, etc. The crude molasses was diluted with distilled water to obtain 10% (w/v) total sugar concentration and centrifuged to remove ash and other undissolved impurities. This molasses solution was used for the pretreatment of cation exchange resin (remove metal ions) as described (Roukas, 1998).

2.3. Determination of sugar concentration and dry cell weight

Cells were centrifuged at 3800 g for 5 min. Sugar (for sucrose, acid hydrolysis is required; adjusted to pH 1.0 with HCl and heated to 100 °C for 30 min) concentration in the supernatant was determined according to Miller (1959). The pellet was washed with distilled water three times and filtered through a pre-dried Whatman GF/C filter paper (1.2 μm pore size). The algal cells on the filter paper discs were dried at 100 °C in a vacuum oven until constant weight and were subsequently cooled down to room temperature in a desiccator before weighting.

2.4. Lipid extraction and analysis

Cells were harvested and lyophilized for lipid extraction and analysis. Total lipids were extracted from 200 mg of lyophilized biomass with a solvent mixture of chloroform, methanol and water (2:1:0.75, by vol.) according to the modified Folch procedure (Christie, 2003). The extract was dried in a rotary evaporator, and then weighed, re-suspended in chloroform, and finally stored at −20 °C under nitrogen gas to prevent lipid oxidation.

Lipids were separated into neutral lipids (NL), glycolipids (GL) and phospholipids (PL) using solid-phase extraction (Christie, 2003). A 500 mg Sep-PakTM cartridge of silica gel (Waters, Milford, MA, USA) was first conditioned by elution with 5 mL of chloroform, and about 50 mg of lipids were then applied to the column. Elution with 10 mL of solvent in the order of chloroform, acetone and methanol yielded NL, GL and PL, respectively. Each fraction was dried under a stream of nitrogen gas, weighted and then re-suspended in 0.1 mL of chloroform.

The NL fraction was subjected to one-dimensional thin-layer chromatography (TLC) for triacylglyerol (TAG) separation and identification, using TLC plates (20 cm × 20 cm) coated with silica gel 60 (Merck, Whitehouse Station, NJ, USA) (Liu et al., 2010a). Plates were activated in an oven at 100 °C for 2 h prior to use. The solvent mixture hexane/diethyl ether/acetic acid (70:30:1, v/v) was used. After co-chromatography with pure standards (Sigma, St. Louis, MO, USA), bands of lipid classes were stained with 2,7-dichlorofluorescein (Sigma) and visualized under UV light. The TAG band was scratched off, re-extracted with hexane, dried under nitrogen gas and weight.

2.5. Fatty acid analysis

Fatty acid methyl esters (FAMEs) were prepared by acid transesterification (Christie, 2003). Briefly, lyophilized cells were incubated with a solvent mixture of toluene and 1% sulfuric acid in methanol (1:2, v/v) overnight at 85 °C for producing FAMEs which were then extracted with hexane. The FAMEs were analyzed by using an HP 6890 capillary gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector (FID) and a HP-INNOwax capillary column (30 m × 0.32 mm) (Agilent Technologies, Inc., Wilmington, DE). Nitrogen was used as carrier gas. Initial column temperature was set at 170 °C, which was progressively raised to 230 °C at 1 °C/min. The injector was kept as 250 °C with an injection volume of 2 μL under splitless mode. The FID temperature was set at 270 °C. FAMEs were identified by chromatographic comparison with authentic standards (Sigma, St Louis, MO, USA). The quantities of individual FAMEs were estimated from the peak areas on the chromatogram using heptadecanoic acid (Sigma) as the internal standard.

2.6. Pigments extraction and analysis

Cell samples from different stages were harvested and freeze-dried on a DW3 freeze-drier (Heto Dry Winner, Denmark). Extraction was carried out with acetone and liquid nitrogen until the cell debris was almost colorless. The extracts were filtered through a 0.22 μm Millipore organic membrane. Twenty microliters of each extract was separated by HPLC on a Waters Spherisorb® 5 μm ODS2 4.6 250 mm analytical column with a Waters HPLC system (Waters) using previously described method by Liu et al. (2010b). Pigments were eluted at a flow rate of 1.2 mL min^{-1} with a linear gradient from 100% solvent A [acetonitrile/methanol/0.1 M Tris–HCl (pH 8.0), 84:2:14, v/v/v] to 100% solvent B (methanol/ethyl acetate, 68:32, v/v) over a 15 min period, followed by 10 min of solvent B. Individual carotenoids were identified by their absorption spectra and their typical retention times compared to standard samples of pure carotenoids (Sigma).

2.7. RNA isolation and real time RT-PCR assay

RNA was isolated from aliquots of about 10^8 cells using the TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and treated with DNase I (Invitrogen, Carlsbad, CA, USA) to remove the potentially contaminated genomic DNA. The concentration of total RNA was determined spectrophotometrically at 260 nm. Total RNA (1 μg) extracted from different samples was reverse transcribed to cDNA by using a SuperScript III First-Strand Synthesis System (Invitrogen). Real time RT-PCR analysis was performed from 1 μL

Table 1
Primers used for real time RT-PCR analysis of fatty acid biosynthetic genes and carotenogenic genes in *C. zofingiensis*.

Primer	Sequence (5′–3′)	GenBank accession no.
Bc_qF	GTGCGATTGGGTATGTGGGGGTG	GQ996717
Bc_qR	CGACCAGGACCAGGGCGGAAAT	
Sad_qF	TCCAGGAACGTGCCACCAAG	GQ996719
Sad_qR	GCGCCCTGTCTTGCCCTCATG	
Psy_qF	CGCAGGCGAAAAATTCCTGTCA	FN563998
Psy_qR	TAAGGAATGTCACACCGCTGGC	
Pds_qF	GATTGGGCGGAGTGATGAGG	EF621406
Pds_qR	CTGTCGCTAATGCGGGTTTC	
Chyb_qF	GCCAGCCATGAAACGTGTG	EU016205
Chyb_qR	GTTCCTTCCAGTTATGTACACA	
Bkt_qF	GTGCTCAAAGTGGGGTGGTATG	AY772713
Bkt_qR	CCATTTCCCACATATTGCACCT	

of the RT reaction mixture in a total volume of 20 μL with specific primers and the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). The primers for fatty acid biosynthetic genes (*Bc* and *Sad*) and carotenogenic genes (*Psy*, *Pds*, *Chyb*, and *Bkt*) are listed in Table 1. PCR was run in a BIO-RAD iCycler IQ Multi-Color Real Time PCR Detection System (Bio-Rad, Hercules, CA). The relative levels of the amplified mRNA were evaluated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using actin gene for normalization.

3. Results and discussion

3.1. Growth of C. zofingiensis based on various sugars

Previously *C. zofingiensis* was found to be able to efficiently utilize not only glucose, but also sucrose and fructose for cell growth (Liu et al., 2010a). Thus cane molasses, which contains mainly the three sugars, may serve as a favorite carbon source for the heterotrophic culture of *C. zofingiensis*. The algal growth fed with cane molasses was compared with those fed with glucose or sugar mixture and the results are listed in Table 2. Clearly molasses without any treatment is suboptimal for the cell growth indicated by the much lower cell dry weight, specific growth rate and sugar utilization than that based on glucose or sugar mixture. In contrast, treated molasses with metal ion removal supported higher cell dry weight and growth yield coefficient than the other sugar sources.

Molasses not only contains essential nutrients for microorganisms, but also contains metal ions and suspended colloids. The substantial amounts of impurities in molasses may lower the biomass production of *C. zofingiensis*. This problem can be solved by treating cane molasses with cation exchange resin as proposed by Jiang et al. (2009). Molasses with this treatment was therefore used to investigate if the industrial waste can replace glucose for biodiesel and astaxanthin production by *C. zofingiensis*.

Table 2
Growth of *C. zofingiensis* based on various carbon sources in batch cultures.[a]

Carbon sources	Dry weight ($g\ L^{-1}$)	Specific growth rate (day^{-1})	Sugar utilization[b]	Growth yield coefficient[c]
Glucose	9.9 ± 0.26	0.80 ± 0.03	0.98 ± 0.05	0.48 ± 0.02
Sugar mixture[d]	9.8 ± 0.22	0.78 ± 0.02	0.97 ± 0.04	0.48 ± 0.02
Untreated molasses	6.2 ± 0.19	0.56 ± 0.02	0.64 ± 0.03	0.45 ± 0.02
Treated molasses[e]	11.3 ± 0.24	0.81 ± 0.05	0.98 ± 0.03	0.54 ± 0.03

[a] Cells were grown heterotrophically in flasks with an initial sugar concentration of 20 $g\ L^{-1}$ for 5 days. Data are expressed as mean ± standard deviation of triplicates.
[b] Sugar utilization = consumed sugar ($g\ L^{-1}$)/initial sugar ($g\ L^{-1}$).
[c] Growth yield coefficient = net dry weight obtained ($g\ L^{-1}$)/consumed sugar ($g\ L^{-1}$).
[d] Sugar mixture: 15.4 $g\ L^{-1}$ sucrose, 2.6 $g\ L^{-1}$ glucose, and 2 $g\ L^{-1}$ fructose.
[e] Treated with cation exchange resin.

3.2. Growth, lipids and astaxanthin production of C. zofingiensis fed with pretreated molasses

As initial sugar concentration could influence the growth and metabolite production by *C. zofingiensis* (Ip and Chen, 2005), here we investigated the effect of various concentration of pretreated molasses on growth, lipids and astaxanthin production of the algal cells. As illustrated in Fig. 1, *C. zofingiensis* could grow well in the medium containing up to 50 $g\ L^{-1}$ of total sugar. The dry weight, lipid and astaxanthin contents of the cultures were associated with initial sugar concentrations in medium (Fig. 1B). When over 30 $g\ L^{-1}$, the sugar utilization decreased greatly, leading to lower specific growth rates and no more increase in dry weight, lipid content, and astaxanthin levels (Fig. 1A and B). This result suggests

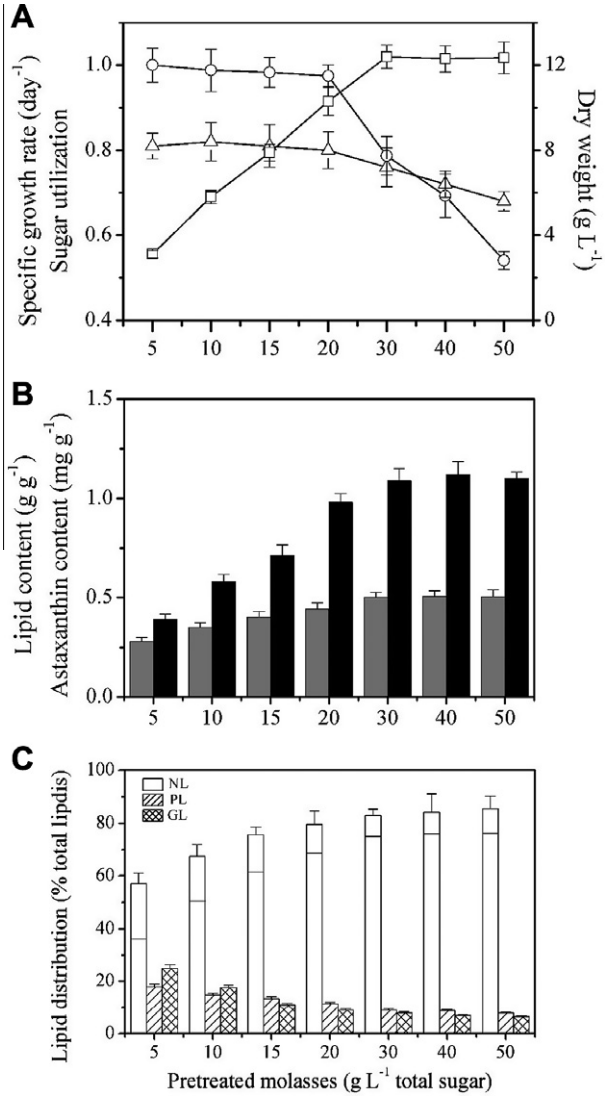

Fig. 1. Growth (A), lipid and astaxanthin contents (B), and lipid composition (C) of *C. zofingiensis* at different concentrations of pretreated molasses. (△) Specific growth rate; (□) dry weight; (○) sugar utilization; (gray column) lipid content; (black column) astaxanthin content. The horizontal line inside the NL column (C) marks the portion of TAG in this fraction. Cells were harvested after 8 days of heterotrophic cultivation. Data are expressed as mean ± standard deviation of triplicates. NL, neutral lipids; PL, phospholipids; GL, glycolipids; TAG, triacylglyerol.

Table 3
Fatty acid profile of *C. zofingiensis* cultured with different concentrations of pretreated molasses in heterotrophic batch cultures.[a]

Fatty acids	Pretreated molasses (g L^{-1} total sugar)						
	5	10	15	20	30	40	50
C16:0	25.20 ± 1.12	23.75 ± 1.01	23.47 ± 1.24	22.30 ± 0.98	22.31 ± 1.18	22.55 ± 0.92	22.82 ± 1.25
C16:1	2.39 ± 0.12	2.02 ± 0.09	1.92 ± 0.08	1.96 ± 0.11	2.09 ± 0.09	2.40 ± 0.12	2.52 ± 0.14
C16:2	8.30 ± 0.51	8.22 ± 0.39	8.75 ± 0.36	7.99 ± 0.43	7.91 ± 0.41	7.64 ± 0.34	7.54 ± 0.44
C16:3	3.89 ± 0.21	3.07 ± 0.16	2.37 ± 0.12	1.60 ± 0.10	1.75 ± 0.08	1.86 ± 0.10	1.79 ± 0.07
C16:4	0.47 ± 0.02	0.42 ± 0.01	0.31 ± 0.01	0.12 0.01	0.06 ± 0	0.07 ± 0	0.08 ± 0.01
C18:0	2.29 ± 0.05	2.50 ± 0.09	2.33 ± 0.13	2.49 ± 0.11	2.51 ± 0.12	2.71 ± 0.15	2.66 ± 0.11
C18:1	20.40 ± 1.11	24.87 ± 1.21	29.92 ± 1.63	33.76 ± 1.75	33.84 ± 1.51	33.88 ± 1.42	34.21 ± 1.79
C18:2	23.19 ± 1.26	22.29 ± 1.13	20.02 ± 0.96	20.03 ± 0.85	20.04 ± 1.05	19.27 ± 1.09	19.68 ± 1.17
C18:3 (*n*-6)	1.24 ± 0.05	1.50 ± 0.08	1.36 ± 0.08	1.10 ± 0.06	0.90 ± 0.03	1.05 ± 0.06	0.46 ± 0.03
C18:3 (*n*-3)	11.26 ± 0.61	9.75 ± 0.55	7.63 ± 0.42	6.80 ± 0.39	6.90 ± 0.42	6.68 ± 0.26	6.87 ± 0.29
C18:4	0.81 ± 0.03	1.08 ± 0.04	0.64 ± 0.02	0.42 ± 0.02	0.43 ± 0.01	0.48 ± 0.02	0.48 ± 0.03
Others	0.56 ± 0.02	0.53 ± 0.03	1.27 ± 0.07	1.43 ± 0.06	1.26 ± 0.05	1.41 ± 0.08	0.89 ± 0.05
Mounsatd[b]	22.80 ± 1.33	26.90 ± 1.38	31.85 ± 1.21	35.72 ± 1.99	35.93 ± 1.26	36.28 ± 1.482	36.73 ± 1.79
Pounsatd[c]	49.16 ± 2.78	46.33 ± 2.55	41.07 ± 2.07	38.06 ± 1.59	37.99 ± 1.86	37.05 ± 1.96	36.89 ± 2.02
Unsatd[d]	71.96 ± 4.12	73.23 ± 3.28	72.92 ± 3.11	73.78 ± 3.52	73.92 ± 3.87	73.33 ± 3.29	73.62 ± 3.65
DUS (▽/mol)[e]	1.40 ± 0.06	1.37 ± 0.05	1.27 ± 0.04	1.22 ± 0.08	1.22 ± 0.05	1.21 ± 0.07	1.21 ± 0.04
TFA[f]	15.92 ± 1.04	21.21 ± 1.29	26.78 ± 1.55	35.34 ± 2.48	41.80 ± 2.06	42.19 ± 2.34	41.95 ± 1.89

[a] Data are expressed as mean ± standard deviation of triplicates.
[b] Mounsatd: percentage of monounsaturated fatty acids (% of total fatty acids).
[c] Pounsatd: percentage of polyunsaturated fatty acids (% of total fatty acids).
[d] Unsatd: percentage of unsaturated fatty acids (% of total fatty acids).
[e] DUS (▽/mol): degree of fatty acid unsaturation = [1.0 (% monoenes) + 2.0 (% dienes) + 3.0 (% trienes) + 4.0 (% tetraenes)]/100.
[f] FA: total fatty acids (g)/cell dry weight (g) × 100%.

that the growth of *C. zofingiensis* was inhibited by the high sugar concentration due to substrate inhibition, a common issue confronted in batch culture.

The lipid distribution was found to be associated with the initial concentrations of molasses (Fig. 1C). Neutral lipid (NL) is the major lipid class, whose proportion increased with higher molasses concentrations and could account for up to 85.5% of total lipids. Similar to NL, TAG levels were promoted by higher concentrations of molasses. At the concentration of 30 g L^{-1} sugar, TAG represented 75.0% and 90.4% of total lipids and NL, which are respectively 1.45 and 2.1 times of those obtained at 5 g L^{-1} sugar (Fig. 1C). In contrast, the membrane lipids phospholipid (PL) and glycolipid (GL) decreased in response to the increased molasses (Fig. 1C). NL in particular TAG is superior to PL or GL as biodiesel precursor. The high content of TAG (0.38 g g^{-1} dry weigh, Fig. 1B and C) obtained here suggested the suitability of using *C. zofingiensis* lipids for biodiesel production.

The fatty acid profiles of oils by heterotrophic *C. zofingiensis* are summarized in Table 3. C16:0, C16:2, C18:1, C18:2 and C18:3 are the major fatty acids and represented more than 85% of total fatty acids. The levels of C16:0, C16:2 and C18:2 remained nearly unchanged under all molasses concentrations. In contrast, C18:1 and C18:3 levels were significantly affected: the former was enhanced by higher molasses concentrations while the latter by lower molasses concentrations. In addition, the content of total fatty acids based on dry weight rose as the molasses concentration increased and could reach as high as 42.2%, which was obtained at 40 g L^{-1} sugar.

Table 4
Production of lipid and astaxanthin based on pretreated molasses or glucose by *C. zofingiensis* in heterotrophic batch cultures.[a]

	Sugar	
	Glucose	Pretreated molasses
Biomass	11.3	12.9
Biomass productivity (g L^{-1} day^{-1})	1.35 ± 0.05	1.55 ± 0.07
Lipid productivity (g L^{-1} day^{-1})	0.66 ± 0.04	0.71 ± 0.03
TAG productivity (g L^{-1} day^{-1})	0.51 ± 0.02	0.53 ± 0.03
Astaxanthin productivity (mg L^{-1} day^{-1})	1.6 ± 0.02	1.7 ± 0.08

[a] Data are expressed as mean ± standard deviation of triplicates. The initial sugar of both cultures was 30 g/L. Cells were harvested after 8 days of cultivation.

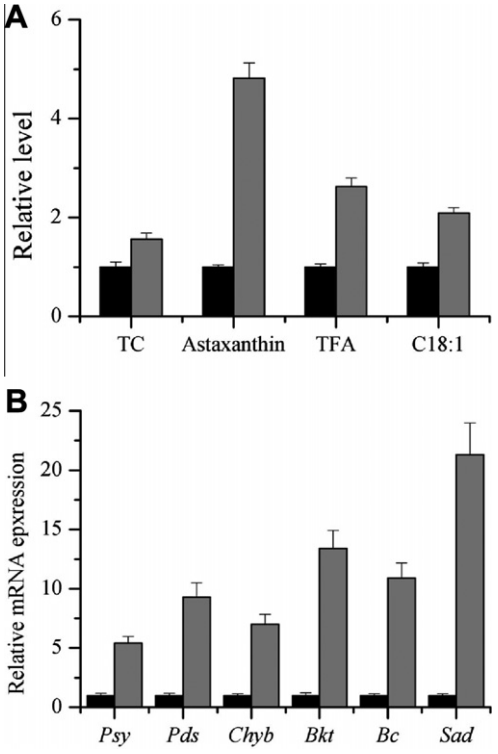

Fig. 2. Relative accumulation of fatty acids, carotenoids and the transcript levels of carotenogenic genes (*Psy*, *Pds*, *Chyb*, and *Bkt*) and fatty acid biosynthetic genes (*Bc* and *Sad*) of *C. zofingiensis*. The contents of fatty acids and carotenoids (A) and the levels of gene transcripts (B) from cultures without molasses were set to 1 (black column) to normalize those from cultures with pretreated molasses (10 g L^{-1} total sugar) (gray column). Data are expressed as mean ± standard deviation of triplicates. TC, total carotenoids; TFA, total fatty acids; Psy, phytoene synthase; Pds, phytoene desaturase; Chyb, carotenoid hydroxylase; Bkt, carotenoid ketolase; Bc, biotin carboxylase; Sad, stearoyl ACP desaturase.

Table 5
Growth of *C. zofingiensis* based on untreated molasses in batch cultures.[a]

Molasses (g L^{-1} total sugar)	Dry weight (g L^{-1})	Specific growth rate (day^{-1})	Sugar unitization	Growth yield coefficient
2.5	1.8 ± 0.09	0.76 ± 0.04	1.00 ± 0.00	0.52 ± 0.02
5	3.1 ± 0.14	0.78 ± 0.03	0.98 ± 0.038	0.52 ± 0.03
10	4.8 ± 0.19	0.71 ± 0.02	0.89 ± 0.04	0.48 ± 0.02
20	6.2 ± 0.19	0.56 ± 0.02	0.64 ± 0.03	0.45 ± 0.02

[a] Cells were grown heterotrophically with different initial total sugar concentrations for 5 days. Data are expressed as mean ± standard deviation of triplicates.

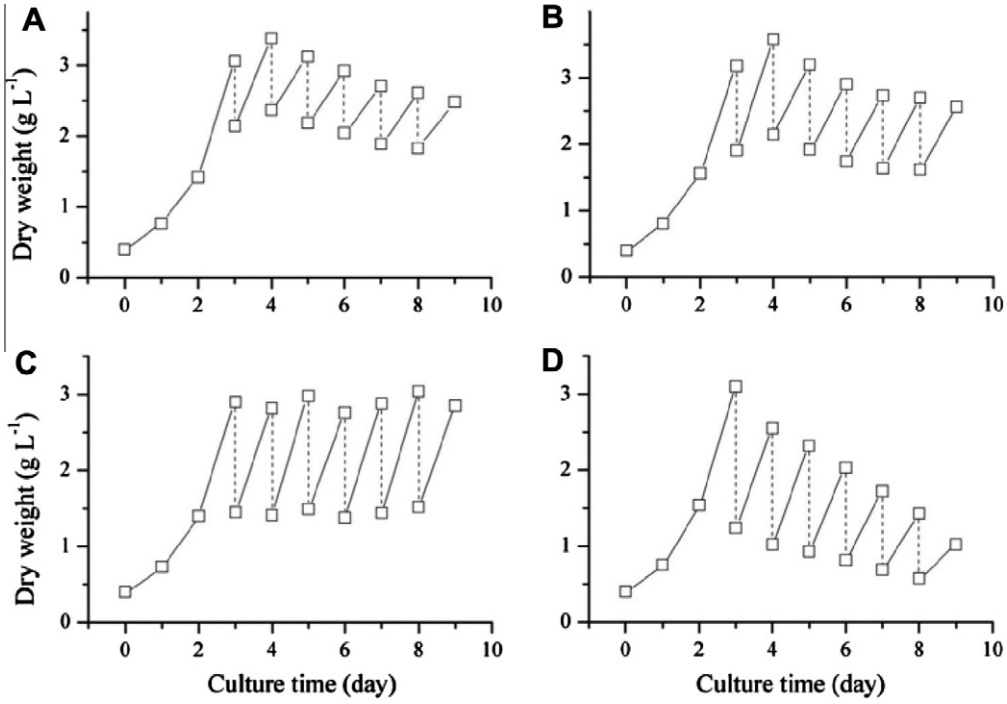

Fig. 3. The growth of *C. zofingiensis* in semicontinuous cultures at various daily dilution rates of 0.3 (A), 0.4 (B), 0.5 (C), and 0.6 (D) under heterotrophic conditions.

The production of *C. zofingiensis* fed with pretreated molasses and glucose was compared with regard to the productivities of biomass, lipid and TAG as well as astaxanthin (Table 4). The sugar concentration of both cultures was 30 g/L. *C. zofingiensis* fed with molasses achieved a biomass productivity of 1.55 g L^{-1} day^{-1}, slightly higher than that fed with glucose. The higher biomass productivity might result from other essential nutrients besides sugars in molasses which can exert positive effect on the growth of *C. zofingiensis*. Accordingly, molasses gave the alga somewhat higher production of lipid, TAG and astaxanthin.

3.3. Molasses up-regulates genes involved in fatty acid and astaxanthin biosynthesis

The accumulations of fatty acids and astaxanthin are associated with the expression of specific genes. The transcription of several of these genes was detected in order to identify the limiting steps for the metabolites. Carotenogenic genes include phytoene synthase (Psy), phytoene desaturase (Pds), carotenoid hydroxylase (Chyb) and carotenoid ketolase (Bkt), and fatty acid biosynthetic genes are biotin carboxylase (Bc, a subunit of ACCase) and stearoyl ACP desaturase (Sad). As illustrated by Fig. 2A, the presence of molasses greatly enhanced the production of fatty acids and in particular astaxanthin whose content was about five times of control. In accordance with the significant enhancement in levels of astaxanthin and fatty acids, molasses triggered remarkable up-regulation of carotenogenic genes *Psy*, *Pds*, *Chyb* and *Bkt*, and fatty acid biosynthetic genes *Bc* and *Sad* (Fig. 2B). This result provides essential information in genetic engineering of microalgae for enhancing oil and astaxanthin production.

3.4. Growth of C. zofingiensis based on untreated molasses

The requirement of molasses pretreatment for optimal growth of *C. zofingiensis* greatly limits its application in heterotrophic culture of the algal cell for biodiesel production on huge scales. To overcome this problem, an effective procedure was developed by using semi-continuous cultures coupled with feeding molasses at a low concentration. A range of molasses concentrations giving 2.5, 5, 10, 20 g L^{-1} sugar were first tested for the heterotrophic growth of *C. zofingiensis* and the result is listed in Table 5. With 5 g L^{-1} sugar, *C. zofingiensis* grew well and obtained relatively higher biomass yield (3.1 g L^{-1}), specific growth rate (0.78 day^{-1}) and sugar utilization (98%). Lower molasses concentrations (e.g., 2.5 g L^{-1} sugar) gave comparable specific growth rates and sugar utilization efficiency, but much lower biomass yields; whereas higher molasses concentrations obtained lower specific growth rate, sugar utilization efficiency and growth yield coefficient (Table 5). This can be caused by the various contents of essential nutrients and also metal ions in molasses-based media. Thus it is important to balance the two compositions in media so that untreated molasses can support optimal growth of the alga for target metabolite production. To enhance biomass production of *C. zofingiensis* fed with low concentration of untreated molasses, semi-continuous

cultivation was introduced. Fig. 3 showed the semi-continuous *C. zofingiensis* with four dilution rates (0.3, 0.4, 0.5 and 0.6 day^{-1}, see Section 2.1). Among them, 0.5 day^{-1} was the best dilution rate that gave a biomass productivity of 1.44 g L^{-1} day^{-1}, which is 81.6%, 30.1% and 48.7% higher than that obtained with dilution rates of 0.3, 0.4 and 0.6 day^{-1}, respectively. With lower dilution rates (e.g., 0.3 and 0.4), the biomass increased after the first cycle and then started to decrease possibly resulted from carbon limitation coupled with excess of other nutrients.

Among a substantial amounts of algal species selected to investigate their potential production for biofuels, several *Chlorella* species have the highest potential as feedstocks for biodiesel due to their ease of mass culture, fast growth and high lipid content as well as multiple cultivation modes. In batch cultures fed with molasses, *C. zofingiensis* could achieve 1.55 and 0.71 g L^{-1} day^{-1} for biomass and lipid productivities, respectively, top among other oleaginous microalgae. *Chlorella protothecoides* fed with molasses pretreated by invertase to release reducing sugars from sucrose showed similar growth yield coefficient to that of *C. zofingiensis* fed with untreated molasses (Table 5, Yan et al., 2011). No reducing treatment was required for *C. zofingiensis* because it could utilize sucrose directly for biomass production (Liu et al., 2010a). Interestingly, molasses could be efficiently utilized by *C. zofingiensis* when it was used at a low concentration, e.g., 5 g L^{-1} (Table 5). Coupled with a semi-continuous culturing approach fed with crude molasses, *C. zofingiensis* achieved high biomass productivity (1.44 g L^{-1} day^{-1}). In addition, *C. zofingiensis* is able to produce the high value astaxanthin. Integrating the production of oil with high value co-products will enable *C. zofingiensis* more cost-affordable as biodiesel feedstocks, though some technical challenges remain to be addressed. The procedures described in this study can serve as a technology platform to transform industrial wastes, e.g., cane molasses to oils and other high-value metabolites by specific microalgae.

4. Conclusions

The utilization of cane molasses by *C. zofingiensis* for growth and production of oil and astaxanthin was investigated. Through simple pretreatment, molasses could support *C. zofingiensis* for high productivities of biomass, lipid, TAG and astaxanthin. *C. zofingiensis* was also able to directly utilize molasses for rapid growth and high biomass by using semi-continuous cultures fed with low concentrations of crude molasses. This study highlights the possibility of using *C. zofingiensis* to deal with industrial wastes and to produce profitable biodiesel as well as the high-value astaxanthin.

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