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Culture medium optimization and lipid profiling of *Cylindrotheca*, a lipid- and polyunsaturated fatty acid-rich pennate diatom and potential source of eicosapentaenoic acid

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Abstract

Cylindrotheca, an epipelic benthic pennate diatom, holds promise as a nutraceutical source and may be useful for aquaculture. Experiments were done on two *Cylindrotheca* species, *Cylindrotheca fusiformis* (UTEX 2084) and *C. closterium*, which was isolated from seawater collected offshore from Visakhapatnam, India. *C. closterium* was identified through microscopy and rDNA typing. Type and concentration of nutrient components in the culture medium that promoted best growth and highest lipid accumulation were identified. Lipid content was gravimetrically estimated. For relative comparison of the effects of different culture media on lipid content, we made estimations through rapid *in situ* screening method using Nile red staining and spectrofluorimetry. The fatty acid profile of lipid was obtained through gas chromatography-mass spectroscopy. Nualgi, a commercially available micronutrient ready-mix with elements adsorbed as nanoparticles on a modified silica sol, was found to significantly boost growth in both *Cylindrotheca* species when used *in lieu* of a conventional micronutrient mix prepared from eight compounds. Among the three nitrogen sources tested – sodium nitrate ($NaNO_3$), urea, and ammonium chloride (NH_4Cl) – best growth of *C. fusiformis* occurred on nitrate and urea, while NH_4Cl was best for *C. closterium*. Lipid productivity was much higher in cultures supplied with NH_4Cl for both *Cylindrotheca* species and compensated for lower biomass in *C. fusiformis*. Mixotrophy with glycerol or sodium acetate resulted in no significant increase in growth over photoautotrophy. Both *Cylindrotheca* species were lipid rich; lipid constituted 18–27% of dry biomass in the medium with $NaNO_3$. Among total fatty acids, polyunsaturated fatty acids constituted <40%, eicosapentaenoic acid 25%, and arachidonic acid ~8% and ~4% in *C. fusiformis* and *C. closterium*, respectively. NH_4Cl, phosphate, and Nualgi micronutrient ready-mix in concentrations optimal for each strain contribute to a good culture medium for *Cylindrotheca*.

Keywords: ammonium chloride; aquaculture; *Cylindrotheca*; highly unsaturated fatty acids (HUFAs); Nualgi; nutraceutical; nutrient medium.

Introduction

Commercial production of microalgal biomass as a source of nutritional supplements (nutraceuticals), pigments, antioxidants and polyunsaturated fatty acids (PUFAs), and feed in aquaculture is an increasingly popular industrial enterprise. The most successful taxa for microalgal biotechnology are the green algae *Chlorella*, *Dunaliella salina* (Dunal) Teodoresco, and *Haematococcus pluvialis* Flotow and the blue green alga (cynobacterium) *Arthospira* (*Spirulina*) spp. Diatoms hold great promise for nutraceutical production as they are a source of omega fatty acids, which have proven human health benefits. While the centric diatoms are used as live feed in aquaculture because of their suitable cell size and shape, pennate diatoms hold promise as source of valuable long chain polyunsaturated fatty acids (LC PUFAs) or highly unsaturated fatty acids (HUFAs), the main species of which are eicosapentaenoic acid (EPA), arachidonic acid (ARA), and docosapentaenoic acid (DHA). Due to the nutritional benefit of HUFAs, markets are increasing for microalgae rich in these compounds as health supplements and food enrichment, and for use in animal feeds to modify the fats of poultry, beef, and pork to a healthier profile for human consumption (Barclay et al. 1994). In addition to *Phaeodactylum tricornutum* Bohlin, which is a well-studied species of benthic diatom, *Cylindrotheca* holds promise as a source of EPA in aquaculture (Ying et al. 2002, Liang et al. 2005, Moura Junior et al. 2007) including abalone farming (Liang et al. 2005). *Cylindrotheca* is also a lipid-rich diatom (Elsey et al. 2007). We report here on studies made to identify a suitable nutrient medium for culture and to determine the fatty acid profile of *Cylindrotheca fusiformis* Reimann *et* J.C. Lewin (UTEX 2084) and *C. closterium* (Ehrenberg) Reimann *et* J.C. Lewin from the waters off Visakhapatnam, a coastal city in southeastern India.

Materials and methods

Cylindrotheca fusiformis (UTEX 2084) – a highly studied species – was obtained from the culture collection at the

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University of Texas at Austin. For mass culture, local strains are preferable. Therefore, a pure culture of an available species of *Cylindrotheca* in the waters (Bay of Bengal) off Visakhapatnam, India (i.e., *C. Closterium*) was developed. The species was identified based on its cell structure under the microscope and by rDNA typing.

Study of cell morphology of local isolate of *Cylindrotheca* species under light and electron microscopy

Cells from culture in late exponential phase were used. For fluorescence microscopy, Nile red (NR) staining was done as described by Cooksey et al. (1987). For scanning electron microscopy (SEM), the cells were treated with 10% HCl, followed by 25% hydrogen peroxide, and finally suspended in 80% acetone. The cell suspension (20 µl) was coated on a coverslip and allowed to dry. The dried cells on the coverslip were glued to a stub and sputter coated with gold, then observed by SEM.

rDNA typing of local isolate of *Cylindrotheca*

DNA was extracted with cetyltrimethylammonium bromide as described by Iwatani et al. (2005). The polymerase chain reaction with primers targeted to 18S rRNA was set up as described by Iwatani et al. (2005). The amplified product was sequenced at the sequencing facility at the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. The sequence was deposited in GenBank. Its similarity to the sequences in GenBank was assessed by Basic Local Alignment Search Tool (BLASTx).

Growth studies

Cells growing in cultures in f/2 medium prepared with filtered seawater (Guillard and Ryther 1962) at exponential phase were used as inoculum for the experiments. A volume from such cultures that would give a final concentration of 1×10^5 cells ml^{-1} was inoculated into 100 ml of medium in a 250 ml Erlenmeyer flask. In experiments where addition of carbon source on growth was assessed, a higher concentration of cells (2×10^6 cells ml^{-1}) was inoculated into the medium. The cultures were kept in a culture room at 26±2°C under a 12:12 h light/dark cycle with a light intensity of 100 µmol photons m^{-2} s^{-1}. The cultures were shaken by hand thrice a day.

All experiments were set up in triplicate and repeated twice. The statistical significance of the differences observed between treatments was assessed through one-way analysis of variance (ANOVA). The normality and homoscedasticity of the data was checked through Kolmogorov-Smirnoff and Cochran's tests, respectively, prior to conducting ANOVA. When ANOVA showed a significant F ratio, a Tukey honestly significant difference (HSD) test was performed. The statistical tests were done with applications of Lowry (2005) and Silva and Azevedo (2009).

Growth was assessed by making cell counts in a 1-mm depth hemocytometer on alternate days after setting up the experiment over a period of 10 days, by which time the cultures reached stationary phase. In some of the media tested, the cells were clumped and could not be counted. In such cases, growth was assessed by measurement of chlorophylls a+c as described by Jeffrey and Humphrey (1975). Specific growth rate was estimated using an equation given by Furnas (2002). In some experiments, the biomass was estimated at the end of the experiment. The cultures were centrifuged at ~9500×*g* for 10 min, the supernatant medium was discarded, and the pelleted algal biomass was dried to a constant weight in an oven at 80°C. The nitrate and silica contents in the culture medium during growth were estimated as described by Collos et al. (1999).

Growth in different culture media To identify the best culture medium among different media available for diatom culture, growth of the two *Cylindrotheca* species was studied in f/2, enriched seawater (ESW), Algal-1, Walne's, and complete nutrient medium (Andersen and Kawachi 2005, Subba Rao 2009). The compositions of these media are given in Table 1.

Effect of Nualgi – a micronutrient mix Micronutrients, which are an essential component of culture medium, were added as a mix prepared from 8 to 12 different compounds (Table 1). Some micronutrients, especially iron, precipitate in the medium prepared from seawater and thus become unavailable. A patented (PCT/IN05/00195 US patent application no. 0070275856) micronutrient ready-mix is available in the market with the trade name Nualgi at ~6.25 US$ kg^{-1}. The mix has been used successfully in shrimp ponds in southern India to promote diatom blooms. It is water dispersible and consists of an alumina-modified silica sol with micronutrients adsorbed on it as nanoparticles. As it is not expensive and is in a ready-to-use form, its effect on growth of *Cylindrotheca* was tested.

To test the effect of Nualgi, silica and trace elements in the f/2 Si medium were replaced with Nualgi. The optimal concentration of Nualgi was determined by setting up cultures in media with different concentrations ranging from 0.5 to 3 g l^{-1}. Cultures in f/2 Si medium were used as the reference controls.

Effect of different nitrogen sources Nitrogen is the major micronutrient required for culture of microalgae. It can be supplied as a nitrate, ammonium, or urea. All culture media tested above have sodium nitrate ($NaNO_3$) as nitrogen source. Experiments were conducted to identify the form of nitrogen that promotes maximum growth in *Cylindrotheca*. Different forms of nitrogen [$NaNO_3$, ammonium chloride (NH_4Cl), and urea] at concentrations of 0.41–7.06, 0.58–9.99, and 0.65–11.2 mM, respectively, were tested.

Mixotrophy with different carbon sources Mixotrophy has been found to tremendously boost biomass production of the pennate diatom *Phaeodactylum tricornutum* (Cerón García et al. 2000). We measured growth of *Cylindrotheca* species in mixotrophic culture with f/2 Nualgi medium substituted with

Table 1 Composition of different culture media and growth of two species of *Cylindrotheca* measured as maximum cell number reached at the end of exponential phase (8th day of culture).

Nutrient	Compound	Medium[a]				
		Guillard's f/2 Si	ESW	Complete nutrient medium	Algal-1	Walne's medium
N	Sodium nitrate	75	47	100	34.375	100
P	Sodium dihydrogen orthophosphate	5.65	7	10	4.2	20
P	Potassium phosphate	–		10	–	–
Fe	Ferric chloride hexahydrate	3.15	1.255	3	1.785	1.3
Zn	Zinc sulfate	0.022	0.55	0.044	0.0927	0.021
Mn	Manganese chloride	0.18	4.1	0.36	0.077	0.36
Mo	Sodium molybdate	0.06	–	0.176	0.098	0.009
Co	Cobaltous chloride	0.01	0.12	0.02	0.008	0.021
Cu	Copper sulfate	0.01	–	0.0184	0.008	0.02
Si	Sodium metasilicate	3.50	3.50	3.50	3.50	3.50
EDTA	Ethylenediaminetetraacetic acid	4.36	40	10	–	45
Fe	Ammonium ferrous sulfate hexahydrate	–	17.55	–	–	–
Bo	Boric acid	–	28.5	–	–	33.6
Mo	Ammonium molybdate	–	–	0.0907	–	–
		Maximum cell number ($\times 10^6$ cells ml^{-1}) attained on the 8th day of culture[b]				
C. fusiformis		2.00 (±0.10)	1.92 (±0.05)	1.66 (±0.05)	1.77 (±0.04)	2.09 (±0.06)
C. closterium		1.88 (±0.03)	1.77 (±0.09)	1.50 (±0.06)	1.68 (±0.06)	1.92 (±0.03)

[a]Composition is given in mg l^{-1} (Andersen and Kawachi 2005, Subba Rao 2009).
[b]The difference in growth between f/2 Si and all other media except complete nutrient medium was insignificant as calculated by Tukey HSD test (ANOVA df=4, F=7.35, p<0.01 for *C. fusiformis* and df=4, F=8.16, p<0.01 for *C. closterium*). The difference between f/2 Si and complete nutrient medium was significant for both *C. fusiformis* and *C. closterium* [Tukey HSD p<0.01, HSD 0.01=0.44 and 0.39 for *C. fusiformis* and *C. closterium* respectively; HSD is the absolute (unsigned) difference between any two sample means required for significance at the designated probability].

glycerol (at 0.1, 0.2, and 0.4 M concentrations) or sodium acetate (at 0.2, 0.4, and 0.8 M concentrations).

Lipid studies

Cylindrotheca is reported to be lipid rich (Cooksey et al. 1987, Priscu et al. 1990, Elsey et al. 2007). We studied the lipid content and fatty acid profile of the two *Cylindrotheca* species.

Estimation of lipid content The cells at early stationary phase were harvested for lipid estimation and fatty acid analysis. Lipid content was estimated by a rapid screening method, viz., *in situ* measurement of fluorescence after staining with NR, a lipid-soluble fluorescent probe. In diatoms, there is a significant relationship (r=0.95) between *in vivo* fluorescence of cells stained with NR and lipid content determined gravimetrically (Cooksey et al. 1987). The staining protocol was devised by combining methods adopted by Cooksey et al. (1987), Priscu et al. (1990), and Elsey et al. (2007). Briefly, 5 ml aliquot of cultures at early stationary phase was drawn and centrifuged at ~500×*g* for 5 min. The supernatant was discarded and the cell pellet was resuspended in filtered seawater (FSW). Cell concentration in this suspension was estimated from cell counts made using a hemocytometer. The suspension was used for lipid estimation through fluorescence measurement with a spectrofluorometer (FluoroMax; HORIBA Jobin Yvon Ltd., Edison, NJ, USA). First, autofluorescence of cells was measured at excitation and emission wavelengths of 475 and 580 nm, respectively, with a slit width of 2 nm and an integration time of 0.5 s (Priscu et al. 1990). Then, 5 µl of 1% NR (in acetone) (Sigma-Aldrich, St. Louis, MO, USA) was added to the 5 ml cell suspension and vortexed (Cooksey et al. 1987). The time course of fluorescence development in the cell suspension was recorded at 1 min intervals (Elsey et al. 2007). The cell suspension was well mixed before measurement. The maximum emission intensity occurring at a specific time point was recorded. NR fluorescence of FSW was also measured. Lipid content was expressed as triolein equivalents. For this purpose, a calibration curve was drawn from the fluorescence emission values of triolein (Sigma-Aldrich) at concentrations of 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, and 2000 ng ml^{-1}. Lipid content was calculated using the formula:

$$\text{ng equivalent triolein } ml^{-1} = [F_L - (F_A + F_{DW})]/b$$

where F_L=maximum fluorescence of cell suspension in FSW after addition of NR, F_A=autofluorescence of cell suspension in FSW before NR addition, F_{DW}=fluorescence of FSW after NR addition, and b=slope of the standard curve drawn for fluorescence against triolein concentration (ng ml^{-1}) (Priscu et al. 1990). Triolein+NR and algal lipid+NR may have different fluorescence responses owing to differences in hydrophobicity

and size between triolein micelles and cellular lipid droplets. Consequently, the lipid estimates through this method can only be interpreted in relative terms (Priscu et al. 1990).

Gravimetric estimation of lipid was performed in some experiments through extraction of lipids. Total lipid was extracted following the Bligh and Dyer method (1959) modified for algal lipids. Briefly, 1.5 ml of methanol and 1 ml of chloroform were added to the dried biomass and the mixture was vortexed thoroughly for 2 min. To this mixture, 1 ml of water and 2 ml of chloroform were added and vortexed for 1 min. The mixture was centrifuged for 5 min at 1100×*g*, after which the chloroform layer was collected and dried in a stream of nitrogen. The mass of the lipid was measured. Similarly, extracted lipid was used for fatty acid profiling.

Fatty acid profiling

Preparation of fatty acid methyl esters (FAMES) For saponification and esterification (Carreau and Dubacq 1978, Carvalho and Xavier Malcata 2005), the sample (the extracted lipid) was mixed with 2 ml of 0.5 N NaOH solution, heated on a heat block at 90°C for 15 min, cooled to room temperature, mixed with 2 ml of 0.7 N HCl in methanol and 1 ml of 14% BF3-methanol, heated on a heat block at 90°C for 10 min, and cooled. Next, 3 ml of saturated aqueous NaCl solution and 2 ml hexane were added and vortexed for 2 min. The upper liquid layer was collected and dried in a gentle stream of nitrogen. The resulting FAMEs were reconstituted in 100 µl of hexane and used for lipid profiling through gas chromatography-mass spectroscopy (GC-MS).

GC-MS An Agilent 6890 GC coupled with MS (Agilent 7673; Agilent Technologies, Palo Alto, CA, USA) equipped with an HP-5MS fused silica capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness) was used with helium as carrier gas at 0.6 ml min^{-1}. A sample (2 µl) was injected into the column under the following conditions: column temperature at 120°C for initial 0.5 min with a thermal gradient from 250°C at 5°C min^{-1} to 305°C at 5°C min^{-1}; the final temperature was maintained for 5 min. Injector and mass selective detector temperatures were 250°C and 280°C, respectively. Peaks were identified by comparing their retention time with authentic references and by comparison of mass spectra with the spectra available in the Wiley National Institute of Science and Technology combined library.

For quantification of the fatty acids, the standards, which came with a MIDI identification system called Sherlock Microbial Identification System (MIS) (MIDI Part no. 1300-AA) were used. The Sherlock MIS uses an external calibration standard developed and manufactured by Microbial ID, Inc., Newark, DE. The standard is a mixture of straight-chained saturated fatty acids of 9–20 carbons (9:0–20:0) in length and five hydroxy acids. FAME standards of known composition were chromatographed to ensure accurate quantification. Fatty acid identifications were confirmed by GC-MS using the mass fragmentation pattern in a comparison with the Wiley database. Peak areas in the chromatogram were quantified using the integrated software provided with the GC-MS ChemStation (Agilent Technologies). The total peak areas of all fatty acids identified in the chromatogram were computed. The relative quantities of individual fatty acids were estimated from the peak area of each fatty acid as a percentage of the total peak area.

Results

Identification of the local isolate of *Cylindrotheca*

The cells were ellipsoidal with two long tapering arms on opposite ends (Figure 1A). Each cell had two chloroplasts (Figure 1B). In the NR-stained cells, lipid globules stained

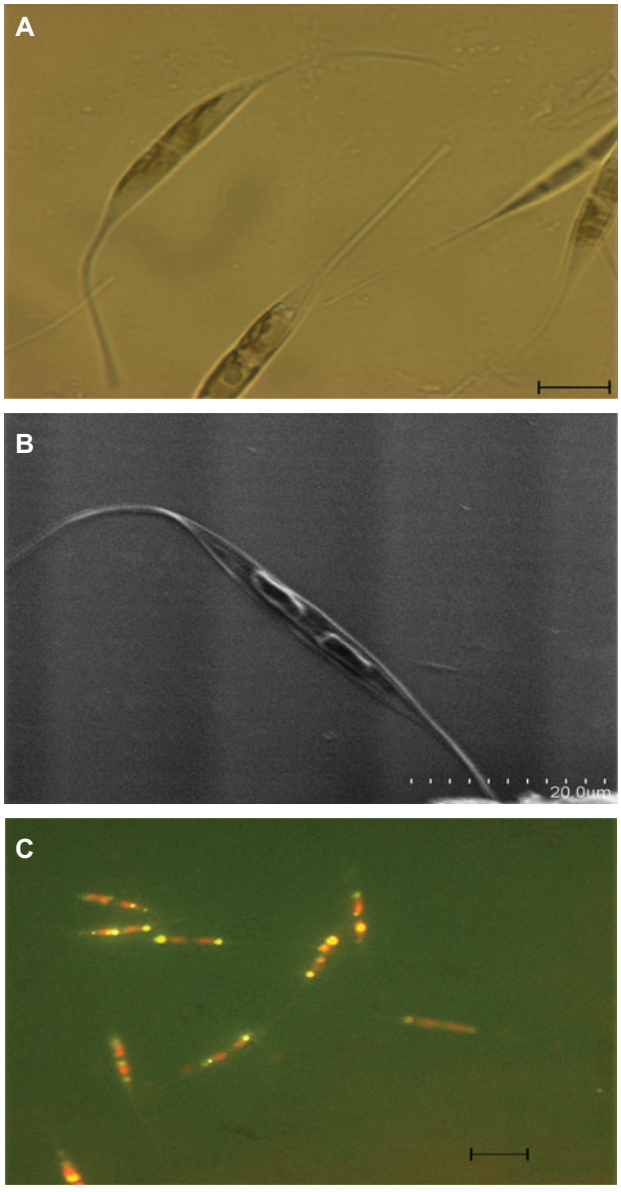

Figure 1 Identification of *C. closterium* by light microscopy, SEM, and fluorescence microscopy.
(A) Light microscopy. (B) SEM showing two chloroplasts per cell. (C) Fluorescence microscopy of NR-stained cells with chloroplasts and lipid goblets showing red and yellow fluorescence, respectively. Bar in A represents 10 µm; bars in B and C represent 20 µm.

yellow, while chloroplasts displayed red fluorescence (Figure 1C). The images matched the description of *Cylindrotheca closterium*. The amplification of a partial region of 18S rRNA resulted in a ~480 bp product from which a 422 bp sequence was obtained. The sequence was deposited in GenBank with accession number JN232979. BLASTx analysis showed 98% similarity with partial sequences of 18S rRNA from *C. closterium*. Thus, the *Cylindrotheca* isolate from local bay waters was identified as *C. closterium*.

Growth studies

Effect of different culture media on growth In both *Cylindrotheca* species, no significant difference in growth was observed among different media tested except complete nutrient medium, in which it was low (Table 1). The f/2 Si medium has lower levels of nitrate, phosphate compounds, and ethylenediaminetetraacetic acid (EDTA) than other media and has no boric acid unlike Walne's medium. For mass culture, lower concentration of chemicals can have a favorable influence on input costs. Therefore, we chose to standardize type and concentration of various components in the medium.

Effect of Nualgi Substitution of Nualgi for micronutrients and silicon in f/2 Si medium significantly improved growth (Figure 2A,B; Table 2). A concentration of 0.5 g l^{-1} of Nualgi proved optimal for *C. fusiformis*, but for *C. closterium*,

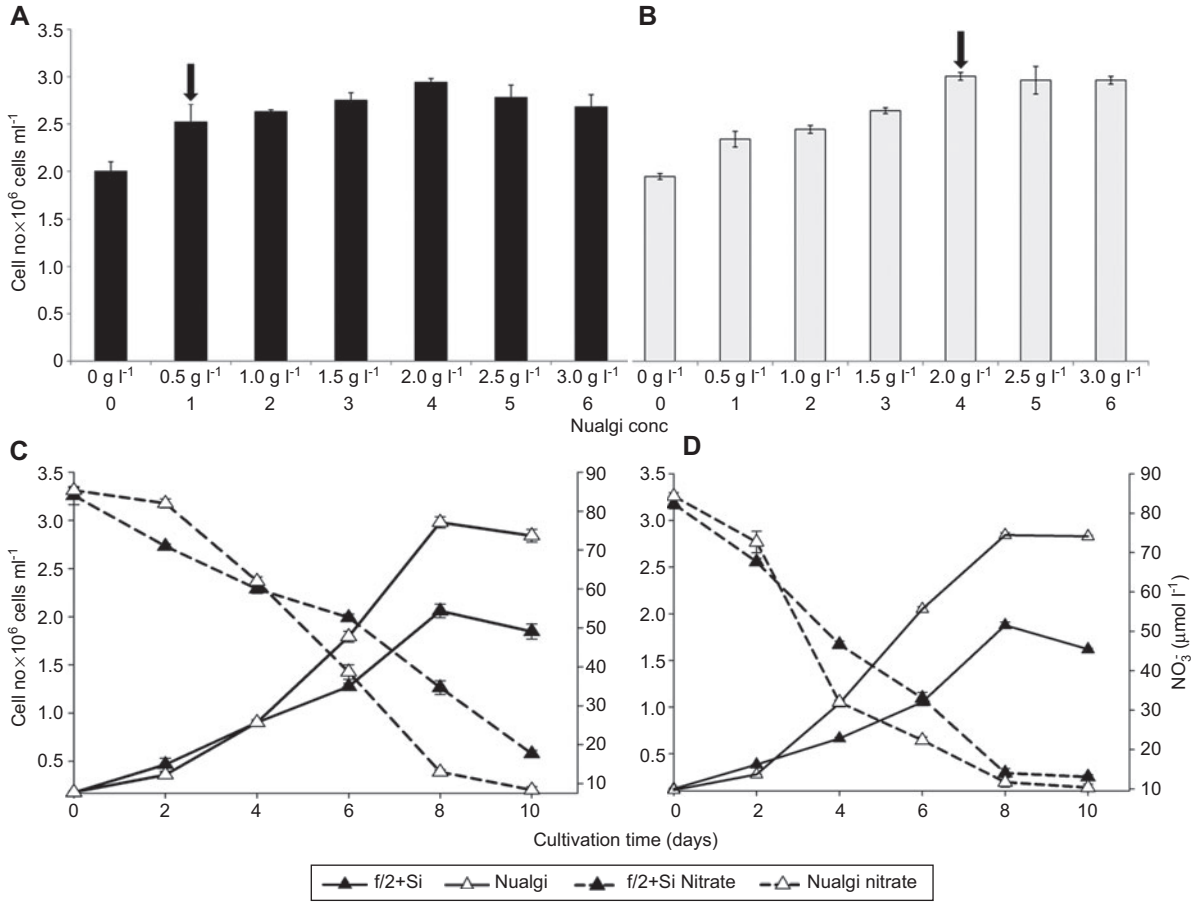

Figure 2 Growth measured by cell counts for *C. fusiformis* and *C. closterium* in f/2 Si medium in which the micronutrients and Si were replaced by different concentrations of Nualgi, a commercially available micronutrient ready-mix.
Initial cell concentration was 1×10^5 cells ml^{-1}. (A) In *C. fusiformis*, the differences in growth between the control [f/2 Si(1)] and f/2 with Nualgi at all concentrations [2 (0.5 g l^{-1}), 3 (1.0 g l^{-1}), 4 (1.5 g l^{-1}), 5 (2.0 g l^{-1}), 6 (2.5 g l^{-1}), and 7 (3.0 g l^{-1})] were statistically significant (ANOVA df=6, F=9.83, p<0.01; Tukey HSD 1 vs. 2/3 p<0.05, 1 vs. 4/5/6/7 p<0.01; HSD 0.05=0.52, 0.01=0.71). The differences in growth between f/2 medium substituted with 0.5 g Nualgi and other concentrations were not statistically significant (Tukey HSD test). (B) In *C. closterium*, the differences in growth between the control [f/2 Si(1)] and f/2 with Nualgi at all concentrations [2 (0.5 g l^{-1}), 3 (1.0 g l^{-1}), 4 (1.5 g l^{-1}), 5 (2.0 g l^{-1}), 6 (2.5 g l^{-1}), and 7 (3.0 g l^{-1})] were statistically significant (ANOVA df=6, F=31.42, p<0.01; Tukey HSD p<0.01, HSD 0.01=0.28). The difference between 2 and 3 was not statistically significant; the differences between 2 and 4, and 2 and 5 were statistically significant (Tukey HSD p<0.01, HSD 0.01=0.28); the differences between 4 and 5, and 4 and 6 were not statistically significant. Arrow indicates the optimum concentration of Nualgi for each species. (C and D) Growth and nitrogen utilization curves [as the amount of unutilized nitrogen (nitrate) in the culture medium] for *C. fusiformis* and *C. closterium* in f/2 Si medium compared to f/2 medium supplemented with the optimal concentration of Nualgi. The values are means of nine replicates (three in space and time); bars represent standard error (SE).

Table 2 Growth and lipid parameters in cultures of *Cylindrotheca* species grown in f/2 Si medium substituted with optimal concentration of Nualgi in comparison with the control (f/2 Si).

Parameter	*C. fusiformis*		*C. closterium*		f/2 Si vs. f/2 Nualgi for *C. fusiformis* and *C. closterium*	
	f/2 Si	f/2+Nualgi	f/2 Si	f/2+Nualgi	ANOVA results	Tukey HSD test
Cell count ($\times 10^6$ cells ml^{-1})	2.00 (±0.10)	2.98 (±0.06)	1.88 (±0.03)	2.84 (±0.02)	df=3, F=90.41, p<0.001	p<0.01, HSD 0.01=0.37
Dry weight (g l^{-1})	0.48 (±0.01)	0.80 (±0.01)	0.53 (±0.01)	0.89 (±0.04)	df=3, F=100.65, p<0.0001	p<0.01, HSD 0.01=0.14
Total lipid yield (g^{-1})	0.13 (±0.00)	0.19 (±0.001)	0.11 (±0.00)	0.16 (±0.001)	df=3, F=16.48, p<0.003	p<0.01, HSD 0.01=5.84
Lipid as percentage of dry weight of biomass	27.08	23.75	20.75	17.98		

2.0 g l^{-1} was required for maximum growth (Figure 2A,B). In f/2 medium with micronutrients and Si replaced with optimal concentration of Nualgi, nitrate in the culture medium was more quickly assimilated than in the control (f/2 Si) (Figure 2C,D). In f/2 medium substituted with an optimal concentration of Nualgi, both biomass and lipid of both species of *Cylindrotheca* were significantly higher than those of the control (f/2 Si) (Table 2). Lipid constituted ~18–27% of dry weight in the biomass (Table 2). Though overall content of lipid in algal biomass harvested from medium with Nualgi was elevated, the lipid content of cells was reduced in this medium, as evident when lipid values were represented as percentage of dry weight of biomass (Table 2).

Effect of different nitrogen sources on growth In *C. fusiformis* cultures, concentrations of 1.77 mM $NaNO_3$, 0.59 mM of urea, and 0.65 mM of NH_4Cl proved optimal (Figure 3A). Growths at optimal concentration of $NaNO_3$ and urea were not significantly different (Tukey HSD test). The growth at optimal concentration of NH_4Cl was, however, significantly lower (ANOVA df=2, F=8.45, p=0.037; HSD 0.05=0.44, 0.01=0.71) than growth in $NaNO_3$ at optimal concentration. In *C. closterium*, the optimal concentrations of $NaNO_3$ and NH_4Cl were 150 mg l^{-1}, while it was 35 g l^{-1} for urea (Figure 3B). Growth in NH_4Cl at optimal concentration was significantly higher than growth in $NaNO_3$ (ANOVA df=2, F=20.71, p<0.001; Tukey HSD p<0.01) or urea (p<0.05) (HSD 0.05=0.44, 0.01=0.71) at optimal concentrations. Thus, urea and $NaNO_3$ proved to be good nitrogen sources for growth of *C. fusiformis*, while NH_4Cl was best for *C. closterium*.

Effect of different carbon sources on growth in mixotrophic culture

Growth performance in f/2 Nualgi medium supplemented with different carbon sources (glycerol and sodium acetate) is depicted in Figure 4. The cells of *C. closterium* were clumped when grown in medium with a carbon source. Thus, we measured growth from the concentration of chlorophyll a+c. Both species of *Cylindrotheca* grew in mixotrophy mode with glycerol or sodium acetate. A 0.2 M concentration of sodium acetate was optimal for both *C. fusiformis* and *C. closterium* (Figure 4). Glycerol at 0.1 M was optimal for *C. fusiformis* (Figure 4). The growth rates of *C. fusiformis* at optimal concentrations of sodium acetate or glycerol were not different from growth in control mineral (f/2 Nualgi) medium (ANOVA df=2, F=1.54, p=0.319). In *C. closterium*, poor growth in glycerol even at a concentration of 0.4 M (Figure 4) was significantly lower than at optimal concentration of sodium acetate or the control (ANOVA df=2, F=146.18, p<0.0002; Tukey HSD p<0.01, HSD 0.01=0.61). Growth in 0.2 M (optimal concentration) sodium acetate was not significantly different from growth in mineral medium (Tukey HSD, HSD 0.01=0.61). Thus, addition of carbon sources did not improve growth in the two tested strains of either *Cylindrotheca* species.

In both the species, ~60–70% of nitrate in the medium remained unused when sodium acetate was added (Figure 4).

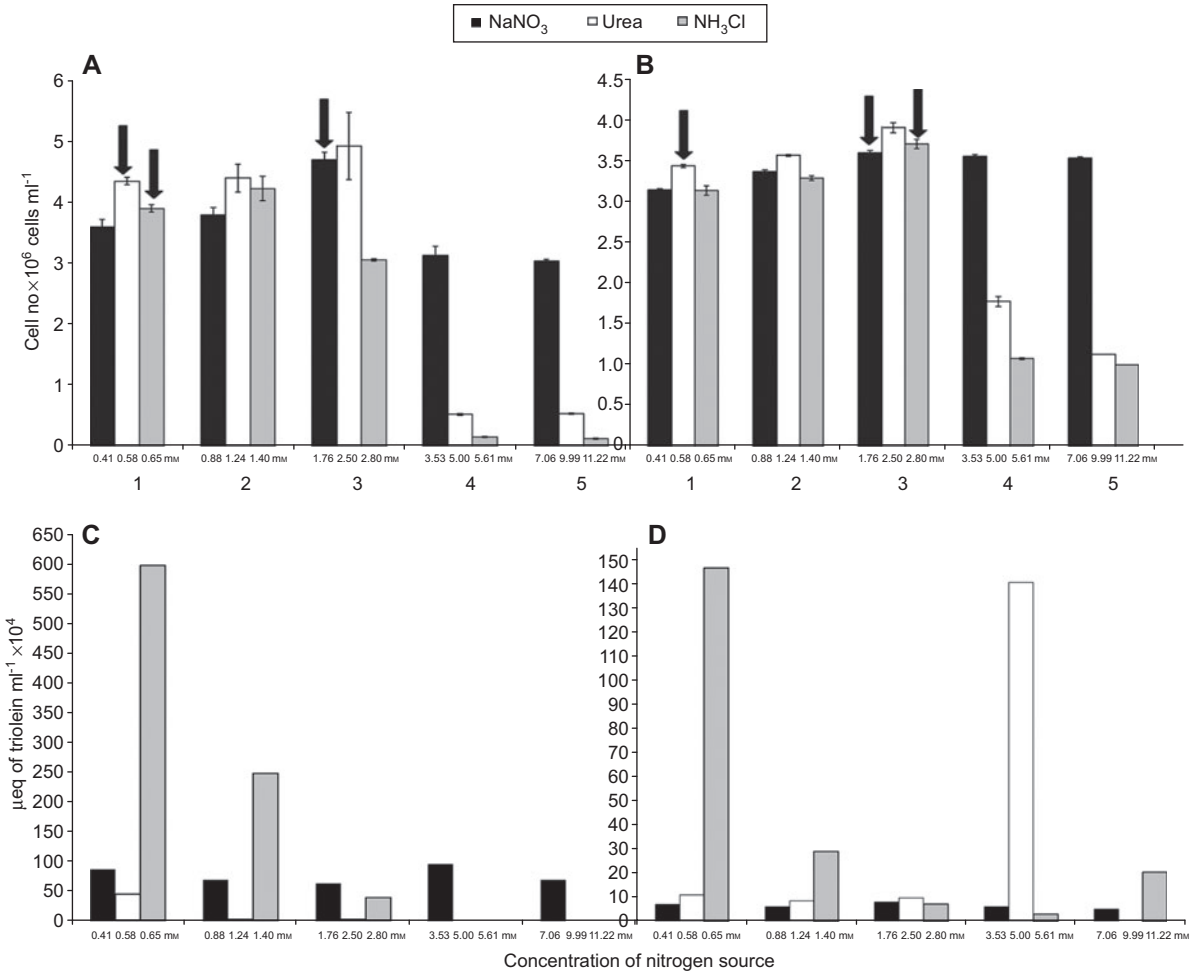

Figure 3 Growth measured by cell counts at the end of exponential phase (8th day of culture) for *C. fusiformis* and *C. closterium* in culture medium with different sources of nitrogen ($NaNO_3$, urea, and NH_4Cl) given in mM values at different concentrations (35, 75, 150, 300, and 600 mg l^{-1}) for each compound.

The medium also contained phosphate and Nualgi (a micronutrient ready-mix) at a concentration of 0.5 g l^{-1} for (A) *C. fusiformis* and 2.0 g l^{-1} for (B) *C. closterium*. Initial cell concentration was 1×10^5 cells ml^{-1}. Arrows indicate the concentrations for optimal growth. For $NaNO_3$, the growth at optimal concentration was significantly higher than at other concentrations [$p<0.01$ (Tukey HSD test) for both *C. fusiformis* (ANOVA df=4, F=35.76, $p<0.0001$; HSD 0.01=0.68) and *C. closterium* (ANOVA df=4, F=80.31, $p<0.0001$; HSD 0.01=0.14). For urea, growth at optimal concentration was not significantly different from growth at higher concentrations (*C. fusiformis*: ANOVA df=4, F=57.02, $p<0.0001$; Tukey HSD $p<0.01$, HSD 0.01=0.68 and *C. closterium*: ANOVA df=4, F=186.13, $p<0.0001$; Tukey HSD $p<0.01$, HSD 0.01=0.14). For NH_4Cl, growth at optimal concentration was not significantly different from growth at the next highest concentration in *C. fusiformis* (ANOVA df=4, F=412.77, $p<0.0001$; HSD not significant), while growth decreased significantly ($p<0.01$, HSD 0.01=0.66) at still higher concentrations. In *C. closterium*, growth at the optimal concentration of NH_4Cl was significantly higher than at lower concentrations of NH_4Cl (ANOVA df=4, F=2404.74, $p<0.0001$; Tukey HSD $p<0.01$, HSD 0.01=0.18). (C and D) Lipid contents of the cultures in stationary phase of A and B expressed as ng triolein equivalents. Note that the nitrogen sources ideal for growth ($NaNO_3$ and urea) were not ideal for lipid accumulation in *C. fusiformis*. The values are means of nine replicates (three in space and time); bars represent SE.

Thus, a very low concentration of nitrogen is utilized when mixotrophy with sodium acetate is operating.

Lipid content

Lipid content was highest in both *Cylindrotheca* species in cultures grown in medium with NH_4Cl as the nitrogen source (Figure 3C,D). However, growth rate of *C. fusiformis* in medium with NH_4Cl was significantly lower than in medium with $NaNO_3$ or urea (Figure 3A,B).When cultured for lipid, lower biomass in NH_4Cl may be compensated for by the higher lipid content. At high concentrations of NH_4Cl, growth was very poor and lipid content could not be estimated in *C. fusiformis*. In medium with $NaNO_3$, there were low levels of lipid. In medium with urea, through growth was good in both species, while lipid accumulation was very poor in *C. fusiformis*; high lipid concentrations accumulated in *C. closterium* in medium with a urea concentration of 300 mg l^{-1} (Figure 3C,D).

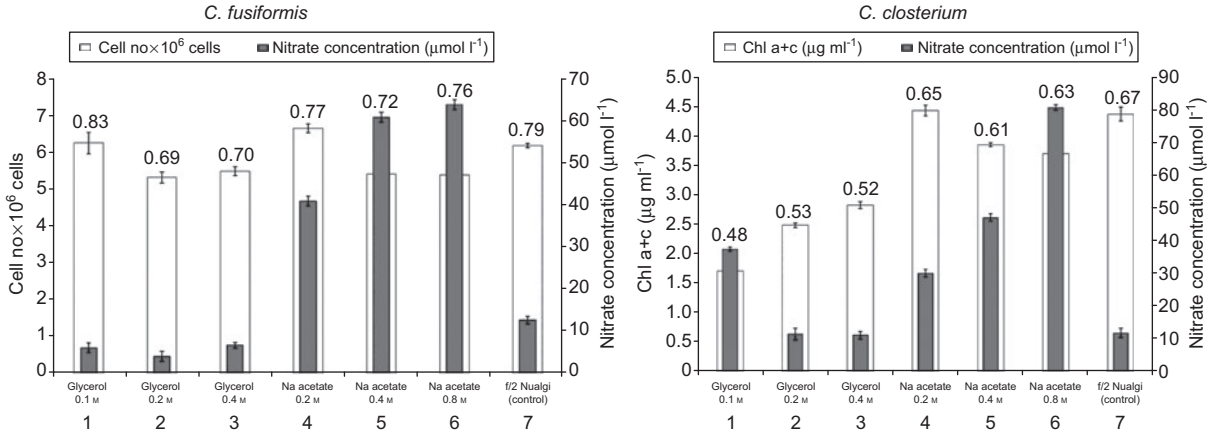

Figure 4 Growth of *C. fusiformis* and *C. closterium* in f/2 medium with micronutrients and Si replaced with an optimal concentration of Nualgi, a commercial formulation of micronutrient ready-mix, and organic carbon sources (glycerol and sodium acetate) at different concentrations. The white bars represent growth measured by cell counts at the end of exponential phase (8th day of culture) for *C. fusiformis* and growth by chlorophyll a+c concentrations (in mg l^{-1}) for *C. closterium*. The dark bars represent nitrate remaining unutilized in the culture medium at the end of the exponential phase. The values over each bar are specific growth rate $\mu = K/0.693$ in each culture medium. These experiments, unlike all other experiments in this study, were set up under continuous illumination. The values are means of nine replicates (three in space and time); bars represent SE.

The fatty acid profiles were not affected by substitution of micronutrients in f/2 Si medium with Nualgi (Table 3). The saturated fatty acid profile was similar in both *Cylindrotheca* species except for the presence of small amounts of 24:0 in *C. closterium* that was absent in *C. fusiformis* (Table 3). Among the monoenoic fatty acids, palmitoleic acid was the major fatty acid in both species, but was present in a higher proportion in *C. fusiformis* than in *C. closterium* (Table 3). *cis*-Vaccenic acid 18:1(n-7) was present in much higher quantities (~9% of total fatty acids) in *C. closterium* than in *C. fusiformis* in which this fatty acid represented <1% of the total fatty acids. In both species, EPA 20:5(n-3) constituted the major LC HUFA (~25%) (Table 3). ARA (20:4) was another LC HUFA that was present, constituting ~8% and

Table 3 Relative percentages of fatty acids in *C. fusiform* and *C. closterium* grown in Nualgi (f/2 Nualgi, 0.5 and 2.0 g l^{-1}, respectively) substituted (instead of micronutrients and Si) f/2 medium compared to the control (f/2 Si) medium.

	C. fusiformis		*C. closterium*	
	f/2 Si	f/2 Nualgi	f/2 Si	f/2 Nualgi
Saturated fatty acids				
14:0	8.46	8.21	9.36	9.51
15:0	1.93	1.94	1.06	1.02
16:0	18.11	19.08	17.12	17.76
18:0	2.87	2.73	3.03	2.89
24:0	–	–	1.41	1.23
Monoenoic fatty acids				
16:1(n-7) Palmitoleic acid	17.89	17.71	12.52	12.44
16:1(n-5)	–	–	2.19	1.68
18:1(n-9)	0.78	0.91	1.56	1.41
18:1(n-7)	0.80	0.82	9.10	8.73
PUFAs				
16:2(n-4)	–	–	6.03	5.99
16:3(n-4)	11.80	12.09	8.02	8.39
18:2(n-6) Linoleic acid omega 6	1.40	1.29	–	–
18:3(n-6) γ-Linolenic acid omega 6	1.77	1.91	1.17	1.02
LC HUFAs				
20:3(n-6) Dihomo-γ-linolenic acid omega 6	1.37	1.24	–	–
20:4(n-6) ARA	8.19	7.98	3.78	3.91
20:5(n-3) EPA	24.63	24.09	23.65	24.02
Total PUFAs (PUFA+LC HUFA)	49.16	48.60	42.65	43.33

~4% of the total fatty acids in *C. fusiformis* and *C. closterium*, respectively (Table 3). Linoleic acid and α/γ-linolenic acid were present though in small quantities (~1% of total fatty acids) in *C. fusiformis* but not in *C. closterium*. The PUFA profile differed between the two species (Table 3). A rare polyenic PUFA 16:2(n-4) constituted ~9% of total fatty acids in *C. closterium* but was absent in *C. fusiformis* (Table 3). The PUFA 16:3(n-4) occurred at a higher proportion in *C. fusiformis* (~12% of total fatty acids) than in *C. closterium* (~8% of total fatty acids) (Table 3). The PUFAs constituted ~48–49% in *C. fusiformis* and ~42–43% in *C. closterium*.

Discussion

Morphological and rDNA typing enabled identification of the pennate diatom isolated from the local bay waters as *Cylindrotheca closterium*. Based on ribosomal small subunit gene (18S rRNA) typing (Li et al. 2007), *C. closterium* is recognized as a species complex.

The ready-made micronutrient mix adsorbed on metallate silica available under the trade name Nualgi promoted good growth in both *Cylindrotheca* species. This could be due to the relatively high quantities of iron in the formulation and the minor elements being in a readily available (nano) form in the product (claimed by the manufacturers). The product is not expensive and can be used in mass production of these nutraceutically important *Cylindrotheca* species. The cumbersome procedure of making micronutrient mix from 8 to 12 different compounds can be avoided by using Nualgi. The optimal concentration of Nualgi differed between the two species, being 0.5 and 2.0 g l^{-1} for *C. fusiformis* and *C. closterium*, respectively.

Both $NaNO_3$ and urea were equally good in promoting growth in *C. fusiformis*. Nitrate transporter genes in *C. fusiformis* are actively expressed in the presence of nitrate and urea but repressed in the presence of ammonium ions (Hildebrand and Dahlin 2000). Nitrate and urea are better nitrogen sources than ammonium salts in another pennate diatom, *Phaeodactylum tricornutum* (Yongmanitchai and Ward 1991). The optimal concentrations of nitrate and urea for *P. tricornutum* are much higher (~1.5 g l^{-1}) (Yongmanitchai and Ward 1991). The medium used by Yongmanitchai and Ward (1991) was Mann and Myers medium. Growth rate when nitrogen was supplied as ammonium was lower in *C. fusiformis*, while it was significantly higher in *C. closterium* compared to nitrate and urea. Grant et al. (1967) reported that when ammonium, urea, and nitrate are supplied in combination to *C. closterium*, ammonium is utilized first, followed by urea, and finally nitrate. Ammonium transport genes in *C. fusiformis* have also been cloned and characterized (Hildebrand 2005); these genes are expressed more in nitrogen-starved and nitrate-supplied cells than in cells supplied with ammonium salts (Hildebrand 2005). However, in both the *Cylindrotheca* species we studied, maximum lipid accumulation occurred in cultures that were fed NH_4Cl. Though maximum cell number attained by *C. fusiformis* in the medium with ammonium as the nitrogen source was lower than in the medium with nitrate or urea, lipid productivity was higher and more than compensated for decreased biomass production. Lipid constituted 18–23% of dry weight (estimated from gravimetric analysis) in the two *Cylindrotheca* species studied when cultured in medium with $NaNO_3$. Lipid contents estimated by NR staining in cultures grown in NH_4Cl were several folds higher than in medium with $NaNO_3$. Thus, lipid might account for much more than 23% of dry weight in *Cylindrotheca* biomass cultivated in medium with ammonium.

A culture medium with NH_4Cl, phosphate, and Nualgi at concentrations optimal for each strain is probably ideal for mass culture of *Cylindrotheca*. A medium developed with these components and used in outdoor mass culture in a 1500 l volume yielded a dry biomass of ~1.4 g l^{-1} in *C. fusiformis* compared to 0.8 g l^{-1} in medium without Nualgi; in *C. closterium*, the yields were ~1.9 and ~1.1 g l^{-1} in medium with and without Nualgi, respectively (authors' unpublished results).

Mixotrophic culture with glycerol or sodium acetate had no advantage in either of the tested strains of *Cylindrotheca* species for growth compared to photoautotrophy in mineral medium. Saks et al. (1976) demonstrated that *C. closterium* is a facultative heterotroph. Mixotrophy with glycerol as a carbon source and ammonium as a nitrogen source greatly boosted growth in a related pennate EPA-rich diatom, *P. tricornutum*, with a biomass production of ~16 g l^{-1} (Cerón Garćia et al. 2000). A very fast growth rate has been reported for *P. tricornutum* in mixotrophic culture with glycerol (Liu et al. 2009).

Cylindrotheca species are reported to have maximum accumulation of HUFAs in exponential phase (Ying et al. 2002). Therefore, we studied the fatty acid profile in the late exponential phase. The fatty acid profiles in the two *Cylindrotheca* species were largely similar to that reported in *C. fusiformis* by Liang et al. (2005). Both *Cylindrotheca* species had a high EPA content, ~25% of the total fatty acids, similar to the levels reported in *P. tricornutum* (Yongmanitchai and Ward 1991) but higher than earlier reports of ~17–18% of EPA in *C. fusiformis* (Liang et al. 2000, 2005). As *P. tricornutum*, these *Cylindrotheca* species have high EPA content and no DHA; hence, downstream processing for isolation of EPA is easy (Yongmanitchai and Ward 1991). The purification of EPA from these diatom species is reported to be inexpensive in comparison to fish liver oil, the other natural source, without the disadvantage of peculiar taste, instability, and high purification costs (Lebeau and Robert 2003). EPA has potential as an antibacterial agent and has been recommended for topical application on human infections (Desbois et al. 2009). It is antibacterial for aquaculture pathogens (Benkendorff et al. 2005). Thus, *Cylindrotheca* species can be used in aquaculture both as a nutritious feed (Moura Junior et al. 2007) and also as an antibacterial agent (Desbois et al. 2009). Use of antibiotics against aquaculture pathogens is not permitted.

ARA 20:4(n-6), another essential fatty acid, was also present in moderate amounts in both *Cylindrotheca* species we studied; concentrations were much higher than those reported by Liang et al. (2005) (maximum of 1.4% among 60 clones of *C. fusiformis*). Chu et al. (1994) reported ARA production by *Nitzschia inconspicua* Grunow in the range of 0.6–4.7% in

total fatty acids. ARA is a biogenetic precursor of the biologically active prostaglandins and leukotrienes, and is a component of mature human milk (Koletzko et al. 1996). The usual source of this essential fatty acid is animal viscera and fungi (Lebeau and Robert 2003).

Cylindrotheca species have many good characteristics, such as rapid growth and multiplication rate; they are easy to culture and harvest, and can endure contamination (Ying et al. 2002). Thus, they can be used in aquaculture and can also be cultured for use in poultry and animal feed to improve the nutritional status of meat and eggs (Barclay et al. 1994). Under the present circumstances of climate change, a boost in microalgal biotechnology is relevant for biosequestration of carbon dioxide, and *Cylindrotheca* is a good candidate genus for cultivation. *C. closterium* isolated from the Bay of Bengal and investigated in this study is an ideal candidate for mass culture in tropical climates of southern India.

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